Electroconvulsive therapy for depression: optimising treatment and exploring molecular mechanisms

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I. Declaration

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II. Summary

The objective of this project was to explore clinical and molecular aspects of electroconvulsive therapy (ECT) in the treatment of depression, with a particular focus on the role of microRNAs (small, non-coding molecules that can regulate gene expression).

The patients receiving ECT in this study were participants in a randomised controlled trial (the EFFECT-Dep Trial) of high-dose (6x seizure threshold) right unilateral and standard dose (1.5x seizure threshold) bitemporal brief-pulse ECT for depression. The objective of the EFFECT-Dep Trial was to compare the effectiveness and cognitive side-effects of these two forms of ECT. In this thesis, a systematic review and meta-analysis was carried out with a similar objective. PubMed, PsycInfo, Web of Science, Cochrane Library, Embase and the International Clinical Trials Registry Platform databases were searched for with the terms "electroconvulsive" or "electroshock" and "trial". Seven randomised controlled trials (n=792) met inclusion criteria, including the EFFECT-Dep Trial.

Bitemporal ECT did not differ from high-dose unilateral ECT on depression rating change scores (Hedges's g = -0.03; 95% confidence interval (CI) -0.17 to 0.11), remission (RR 1.06; 95% CI 0.93 to 1.20), or relapse at 12-months (RR 1.42; 95% CI 0.90 to 2.23). There was an advantage for unilateral ECT on reorientation time after individual ECT sessions (mean difference in minutes = -8.28; 95% CI -12.86 to -3.70) and retrograde autobiographical memory (Hedges's g =-0.46, 95% CI=-0.87 to -0.04) after completing an ECT course. There were no differences for general cognition, category fluency and delayed visual and verbal memory. The immediate clinical implication of this is that high-dose right unilateral ECT should be considered as a first-line option when prescribing ECT.

Peripheral blood samples from patients participating in the EFFECT-Dep Trial and healthy controls were collected in PaxGene© bottles. MicroRNAs and mRNAs were extracted using PaxGene© blood miRNA/RNA kits. An initial discovery phase deep sequencing study (n=16) was carried

out on the SOLiD™ platform. This yielded over 23 million reads mapping on to known microRNAs in the human genome. However, in the group as a whole there were no significant differences in microRNA levels from baseline to ECT following strict correction for multiple testing. However, post-hoc analysis indicated that there were significant changes in microRNA levels in patients with psychotic depression. These changes were confirmed in the same sample using quantitative real-time polymerase chain reaction (qRT-PCR). In a separate cohort (37 depressed, 34 controls), candidate microRNAs from deep sequencing were analysed in a validation study. Two microRNAs (miR-126-3p and miR-106a-5p) were significantly elevated in patients with psychotic depression (n=7) at baseline when compared to healthy controls. After ECT treatment, the levels of these microRNAs normalised.

Bioinformatic analysis revealed a high number of genes, molecular and biological processes as well as signalling pathways targeted by miR-126-3p and miR-106a-5p. *VEGFA, SIRT1* and *E2F1* were shared gene targets of both microRNAs. Molecular and biological processes involving genes targeted by miR-126-3p and miR-106a-5p revealed that cell cycle regulation, regulation of transferase activity and response to abiotic stimuli were particularly over-represented. A number of over-represented pathways relevant to depression and the potential mechanism of ECT were identified. These included several growth factor pathways (VEGF, IGF, TGF-Beta, PDGF, EGF) and cell cycle regulatory pathways implicated in depression and neuroplasticity.

Downstream *VEGFA* mRNA levels were subsequently quantified using qRT-PCR (97 depressed, 53 controls). *VEGFA* mRNA levels were raised in depressed (psychotic and non-psychotic) subjects compared to healthy controls. Following ECT however, there was a significant decrease in *VEGFA* levels in the psychotic depression group (n=21) only. *VEGFA* levels correlated with psychotic symptomatology, but no correlations with cognitive outcomes were observed. There was no effect of electrode placement on *VEGFA* levels.

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Abbreviations

°C	degrees Celsius
µ	micro
5-HT	serotonin
5HTT/SERT	serotonin transporter
7-CTKA	7-chloro-kynurenic acid
A	absorbance
ACE-R	Addenbrooke's Cognitive Examination - Revised
ACTH	adrenocorticotropic hormone
ANCOVA	analysis of covariance
ANK3	ankyrin 3
ANOVA	analysis of variance
APA	American Psychiatric Association
APP	amyloid precursor protein
ATHF	Antidepressant Treatment History Form
AUC	area under the curve
BA	Brodmann's area
BAS	baseline
BCL-2	B-Cell CLL/lymphoma 2
BDNF	brain-derived neurotrophic factor
BH	Benjamini-Hochberg
bp	base pair
BPAD	bipolar affective disorder
BPRS	Brief Psychiatric Rating Scale
BT	bitemporal
BZD	benzodiazepine
C	coulombs
Ca^{2+}	calcium
CUAMI	Columbia Autobiographical Memory Inventory
cAMP	cyclic adenosine monophosphate
CACNA1C	calcium channel, voltage dependent, L type, alpha 1c subunit
CBT	cognitive behavioural therapy
CCKR	cholecystokinin receptor
cDNA	complementary DNA

CDK	cyclin-dependent kinase
CDKN	cyclin-dependent kinase inhibitor
Chr	Cytogenetic location
CI	confidence interval
CLOCK	circadian locomotor output cycles kaput
CMS	chronic mild stress
CNS	central nervous system
COMT	catechol-o-methyltransferase
CORT	exogenous corticosterone
CpG	5'—C—phosphate—G—3'
CREB	cAMP response-element binding protein
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
C_T	cycle threshold
CUPS	chronic unpredictable stress
DA	dopamine
DAVID	Database for Annotation, Visualisation and Integrated Discovery
DG	dentate gyrus
DGCR8	DiGeorge syndrome critical region 8 protein
DLPFC	dorsolateral prefrontal cortex
DMNT3B	DNA (cytosine-5-)-methyltransferase 3 beta
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribose nucleoside triphosphate
DR	dopamine receptor
DRN	dorsal raphe nucleus
DSM	diagnostic and statistical manual
DST	dexamethasone suppression test
DTI	diffusion tensor imaging
E2F1	E2 transcription factor 1
EASE	Expression Analysis Systematic Explorer
ECS	electroconvulsive shock
ECT	electroconvulsive therapy
EDTA	ethylenediamine tetraacetic acid

EEG	electroencephalogram
EGF	epidermal growth factor
EGFL	EGF-like domain
EGR	early growth response
ePCR	emulsion PCR
EOT	end of treatment
ERK	extracellular signal related kinase
FEAST	Focal Electrically Administered Seizure Therapy
FCSRT	Free and Cued Selective Reminding Test
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FKBP5	FK 506 binding protein
Flk	fetal liver kinase
Flt	fms-like tyrosine kinase
FOXO	forkhead box, class O transcription factor
FST	forced swim test
g	gram
GABA	gamma-Aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDNF	glial cell line-derived neurotrophic factor
GLM	general linear model
GO	gene ontology
GR	glucocorticoid receptor
GWAS	genome-wide association study
HDRS	Hamilton depression rating scale
HIF	hypoxia inducible factor
HIPK	homeodomain interacting protein kinase
HPA	hypothalamic-pituitary-adrenal
HTRA2	HtrA serine peptidase 2
ICD	International Classification of Diseases
IL	interleukin
IPT	interpersonal psychotherapy
IV	inverse variance
k	kilo
KEGG	Kyoto Encyclopedia of Genes and Genomes

L	litre
LH	learned helplessness
LIMK	LIM domain kinase
LTP	long-term potentiation
m	milli
mm	millimetre
M	molar
MAOI	monoamine oxidase inhibitor
MAPK	mitogen-activated protein kinase
MD	maternal deprivation
MDD	major depressive disorder
miR	microRNA
MMSE	mini-mental state examination
MPC	medical prefrontal cortex
MR	mineralocorticoid
MRE	microRNA response elements
MRI	magnetic resonance imaging
mRNA	messenger RNA
MRS	magnetic resonance spectroscopy
MS	maternal separation
NA	noradrenaline
NAA	N-acetylaspartate
NART	National Adult Reading Test
NaSSA	noradrenergic and specific serotonergic antidepressant
NF-κB	nuclear factor-kappa beta
NGF	nerve growth factor
NICE	National Institute for Health and Care Excellence
NMDA	N-methyl D-aspartate
NRG	neuregulin
OFT	open field test
OR	odds ratio
PANTHER	Protein Analysis Through Evolutionary Relationships
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDAS	Psychotic Depression Assessment Scale

PDGF	platelet-derived growth factor
PG	prostaglandin
PGF	placental growth factor
PFC	prefrontal cortex
PI3K	phosphatidylinositol 3-kinase
PIK3R2	phosphoinositide-3-kinase, regulatory subunit 2
PLC	phospholipase C
PM	post-mortem
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus
qRT-PCR	real-time polymerase chain reaction
RB1	retinoblastoma 1
RCT	randomised controlled trial
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
ROC	receiver operating curve
rpm	revolutions per minute
RR	risk ratio
RS	restraint stress
rs	reference SNP cluster ID
RT	reverse transcription
rTMS	repetitive transcranial magnetic stimulation
RUL	right unilateral
s	second
SAT	spermidine/spermine N1-acetyltransferase
SCID	Structure Clinical Interview for DSM-IV Disorders
Scz	schizophrenia
SD	standard deviation
SEM	standard error of mean
SIRT1	sirtuin 1
SMD	standardised mean difference
SMOX	spermine oxidase
SNP	single nucleotide polymorphism
SNRI	serotonin-noradrenaline reuptake inhibitor

SPUH	St. Patrick's University Hospital
SPRED1	sprouty related, EVH1 domain containing 1
SSRI	selective serotonin reuptake inhibitor
ST	seizure threshold
STAR*D	Sequenced Treatment Alternatives to Relieve Depression
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TCA	tricyclic antidepressant
TGF	transforming growth factor
TMT	trail-making test
TNF	tumour necrosis factor
TPH2	tryptophan hydroxylase 2
TRBP	TAR RNA-binding protein
TrkB	tropomyosin-related kinase B
U	units of activity
UK	United Kingdom
UNG	uracil N-glycosylase
US	United States
UV	ultra-violet
VEGF	vascular endothelial growth factor
VTA	ventral tegmental area
WBC	white blood cells
WHO	World Health Organisation
x g	times gravity

Chapter 1
Introduction

1 Introduction

1.1 Major Depressive Disorder

1.1.1 General overview

Depression is one of the most common psychiatric disorders with a huge individual, economic and social burden. It is the second leading cause of years of life lived in less than ideal health (years lived with disability) (Vos et al., 2012). The World Health Organisation (WHO) predicts that unipolar depression will be the leading contributor to the global burden of disease by 2030 (World Health Organisation, 2008). It is the costliest brain disorder in Europe, accounting for 1% of the European economy (Gustavsson et al., 2011). Electroconvulsive therapy (ECT) is the most acutely effective treatment for ECT (UK ECT Review Group, 2003, Eranti et al., 2007, Spaans et al., 2015). However, patients and psychiatrists have concerns about ECT's cognitive side effects. Many patients fail to respond to standard treatments such as pharmacotherapy and psychotherapy (Kolshus et al., 2011). Although there have been some advancements in the understanding of the molecular basis of depression, there is a lack of new effective treatments and biomarkers to clarify diagnosis, prognosis and treatment choice.

1.1.2 Diagnosis

The two main diagnostic classification systems in use today are the Diagnostic and Statistical Manual for Mental Disorders V (DSM-V) (American Psychiatric Association, 2013) and the International Classification of Diseases, 10th revision (ICD-10) (World Health Organisation, 1992). There are only minor differences between these classification systems, and the main system used in research, including this work, is the DSM criteria, outlined in Table 1.1 below. Depressive episodes can occur in both unipolar and bipolar depression. In addition to a diagnosis of a major depressive episode, the severity of the episode can be classified as mild, moderate or severe depending on the number of symptoms and functional impairment.

The use of diagnostic criteria helps to maintain reliability across various research settings. However, the criteria, though empirically based, are not based on aetiological or biological evidence, which may limit their validity (Jones, 2012). The lack of a unifying pathophysiology and the heterogeneity of clinical presentations of depression indicates there may be discrete depressive disorders beyond unipolar and bipolar depression (Hasler, 2010). Sub-classification categories identified in DSM-V that may represent such discrete entities include psychotic, melancholic, catatonic, atypical, post-partum and seasonal depression.

In psychotic depression a patient suffers from the symptoms of depression as well as psychosis, including delusions or hallucinations. Delusional themes are often mood-congruent, such as nihilistic delusions (Rothschild, 2013). Some have argued that psychotic depression is distinct from other forms of depression and should be treated as such in upcoming diagnostic classifications (Ostergaard et al., 2012). This is based on differences in clinical presentation, prognosis, response to therapies and biological differences. For example, ECT is recommended as a first line treatment in psychotic depression by international guidelines produced by organisations such as the American Psychiatric Association (APA) and the World Federation of Societies of Biological Psychiatry (Rothschild, 2013). Dysregulation of the hypothalamic-pituitary-adrenal axis (HPA-axis) is also more pronounced in those with psychotic depression when compared to non-psychotic depression (Brown et al., 1988). Melancholic depression is another severe form of the disorder characterised by anhedonia, lack of reactivity, diurnal variation and psychomotor disturbance amongst other symptoms (American Psychiatric Association, 2013). Historically, melancholic depression was also thought to show better response to ECT, although more modern trials have failed to consistently validate this (Rasmussen, 2011).

A. Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure	
	1. Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad or empty) or observation made by others)
	2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation made by others)
	3. Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day.
	4. Insomnia or hypersomnia nearly every day
	5. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down)
	6. Fatigue or loss of energy nearly every day
	7. Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick)
	8. Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others)
	9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide
B. The symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning	
C. The symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition (e.g., hypothyroidism).	

Table 1.1 List of DSM-V criteria for major depressive disorder

1.1.3 Epidemiology

Lifetime prevalence rates of unipolar depression are in the region of 10-20% (Kessler and Bromet, 2013, Kessler et al., 2003). It is almost twice as common in women when compared to men (Kessler et al., 1994). Depression is frequently recurrent, difficult to treat and is associated with

increased suicide rates (Bostwick and Pankratz, 2000). Rates of depression in Europe tend to be higher in urban areas, whereas this appears to be reversed in the United States (Ayuso-Mateos et al., 2001, Probst et al., 2006). In contrast to many other psychiatric disorders there is a wide range in the median age of onset of depression, which lies in the region of 20-45 years. In high-income countries, depression is more common in lower socio-economic groups, but this pattern is not consistently observed in low-medium income countries. Other risk factors include adverse childhood events, traumatic life events and being divorced or separated (Kessler and Bromet, 2013). Importantly, patients with depression have an increased risk of death, which is not fully accounted for by the risk of suicide. Depression is associated with an increased risk of various other conditions, such as cardiovascular disease, diabetes, cancer and respiratory disorders (De Hert et al., 2011).

The estimated heritability of unipolar depression is in the region of 39-42%, indicating a genetic component to depression. Candidate genes include brain-derived neurotrophic factor *(BDNF)*, serotonin-transporter protein *(5HTT)*, FK506 binding protein *(FKBP5)*, tryptophan hydroxylase 2 *(TPH2)* and HTRA serine peptidase 2 *(HTRA2)* (Flint and Kendler, 2014). However, the high discordance rate of 50% between monozygotic twins suggests factors other than genetics contribute to disease genesis (Haque et al., 2009). Bipolar affective disorder (BPAD), which shares genetic risk factors with schizophrenia, has an even higher heritability estimate in the region of 70-80% (Craddock and Sklar, 2013). Bipolar depression is less common than unipolar depression, with a lifetime prevalence rate around 2.5% with an equal gender distribution. Although environmental factors are likely to play an important role, there is still a lack of clarity as to what specific risk factors come into play. Although no single gene with a large effect has been found, genes such as calcium channel, voltage dependent, L type, alpha 1C subunit *(CACNA1C)*, ankyrin 3, node of Ranvier *(ANK3)* and genes involved in circadian rhythm have been associated with BPAD (McCarthy et al., 2012).

1.1.4 Models of depression

1.1.4.1 Monoamine theory of depression

The accidental discovery of the antidepressant effects of some anti-tubercular medications led to the development of the tricyclic antidepressants (TCAs) in the 1950s (Berton and Nestler, 2006). This laid the premise for early models of major depression, which postulated a 'chemical imbalance' in monoamine neurotransmitters.

The majority of current antidepressants are thought to work by increasing the availability of serotonin or catecholamines at the synapse. This includes monoamine oxidase inhibitors (MAOIs), TCAs, selective serotonin reuptake inhibitors (SSRIs), serotonin-noradrenaline reuptake inhibitors (SNRIs) and others. The monoamine hypothesis of depression states that depression is caused by a deficiency of these monoamines in the brain and that antidepressant treatment will normalize these levels (Berton and Nestler, 2006). However, although catecholamine levels at the synapse increase acutely in response to treatment, a clinical response can take weeks or months. Also, tryptophan depletion, which lowers the amount of serotonin available to the brain, has no effect on individuals without a history of depression (Berton and Nestler, 2006).

A number of neurotransmitters have been implicated in BPAD, chiefly serotonin (5-HT) and the catecholamines dopamine (DA) and noradrenaline (NA). Drugs that cause an increase in serotonin or catecholamines (e.g. antidepressants, amphetamine, cocaine) can induce hypomanic or manic episodes (Manji et al., 2003).

Although monoamine neurotransmitters and their pathways play a part in the pathophysiology of mood disorders, it has become clear they are only part of a more complex system.

1.1.4.2 HPA-Axis/Stress theory of depression

Hyperactivity of the HPA-axis has long been linked to depression. In response to stress, the hypothalamus secretes corticotropin-releasing hormone (CRH), which acts on the anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH in turn stimulates cortisol

release from the adrenal glands (Pariante and Lightman, 2008). Excess cortisol levels are thought to have neurotoxic effects on the brain and are associated with smaller hippocampal volumes (Frodl and O'Keane, 2013, Cotter et al., 2001). Circulating cortisol normally triggers a negative feedback loop inhibiting further release of CRH and ACTH. However, in many depressed patients this negative feedback system appears to be dysregulated, leading to chronically high levels of CRH, ACTH and cortisol. CRH interacts with other neurotransmitter systems, including the catecholamines. There is also evidence for elevated levels of CRH in the hypothalamus and cerebrospinal fluid (CSF) (Berton and Nestler, 2006). It is postulated that in patients vulnerable to developing depression, the glucocorticoid receptors lose their inhibitory effect leading to the loss of the negative feedback loop but the exact mechanisms remain unclear. Possible mechanisms include genetic variation, with some evidence implicating the *FKBP5* gene (Binder et al., 2004, Tozzi et al., 2016), and the impact of early adverse life events (the stress-diathesis model) (Heim and Nemeroff, 1999). A number of drugs targeting the HPA-axis have been studied, with further clinical trials under way, but the latest Cochrane review of this area found clear evidence for their use to be lacking (Gallagher et al., 2008).

1.1.4.3 Inflammatory model of depression

Some of the first evidence linking the immune system to mood disorders came from the observation that patients with immune system disorders had increased rates of psychiatric disorders and many of the key symptoms of depression are also seen in immune-related illnesses. In later years, patients undergoing interferon (a form of cytokine) therapy for cancers or viral infections were observed to have high rates of depression (Hoyo-Becerra et al., 2014). Elevated levels of some cytokines, other inflammatory markers and activity of transcription factors like nuclear factor-kappa beta (NF-KB) have been found in depressed patients (Dowlati et al., 2010, Pace et al., 2006). It is still not known, however, how these cytokines and other inflammatory markers may be involved in psychiatric symptomatology, but they appear to interact with other neural pathways, neurotransmitters and the HPA-axis.

1.1.4.4 Neurotrophic theory of depression

In recent years, there has been much interest in the neurotrophic model of depression. In this model, neural circuits and connections can undergo both detrimental changes in response to stress or negative life events as well as beneficial adaptations in response to environmental stimuli. The neurotrophic model suggests that antidepressants act by inducing transcriptional and translational processes, culminating in neuroplastic changes, e.g. hippocampal neurogenesis, synaptogenesis, increased dendritic spines and dendrites (Krishnan and Nestler, 2008). In animals, Electroconvulsive Shock (ECS) - an animal model of ECT, induces such neuroplastic changes more robustly than antidepressants, possibly accounting for its more powerful therapeutic effect. The timing of these changes mirror the delayed clinical response observed with antidepressants (Malberg et al., 2000).

The development of the neurotrophic theory of depression arose from initial observations in animal studies where stress led to reduced levels of BNDF, neurons and glia in the hippocampus, amygdala and pre-frontal cortex, key areas in mood regulation and depression (Duman, 2004). BDNF is widely expressed throughout the brain, promoting neuronal survival and maturation, synaptic plasticity and synaptic function. The BDNF receptor, tropomyosin-related kinase B (TrkB), when activated, leads to downstream signalling changes in pathways including phospholipase C γ (PLC γ), mitogen-activated protein kinase (MAPK) / extracellular signal related kinase (ERK), and the phosphatidylinositol 3-kinase (PI3K) pathways. These pathways are involved in neuronal survival, growth and plasticity as well as acting on the transcription factor cAMP response-element binding protein (CREB), a potent regulator of gene expression (Autry and Monteggia, 2012). BDNF is also involved in the modulation and maintenance of various neurotransmitter pathways, including serotonergic, dopaminergic and gamma-Aminobutyric acid (GABA) pathways (Pillai, 2008).

These findings have since been mirrored using neuroimaging or post-mortem investigations of brains of depressed patients, including reduced BDNF and TrkB levels, reduced hippocampal volume, reduced hippocampal

neurogenesis, and reduced size and number of neurons and glia in the prefrontal cortex (Wainwright and Galea, 2013, Bremner et al., 2000, Castren and Rantamaki, 2010).

A key support for the neurotrophic hypothesis comes from animal models where various antidepressant therapies have been shown to reverse and protect the brain from the neurotoxic effects of stress (Duman, 2004). Direct injection of BDNF into the rodent hippocampus leads to antidepressant-like effects in animal models of depression (Shirayama et al., 2002). Also, in knockout or knockdown mouse models where BDNF has been deleted, administration of antidepressants fails to elicit behavioural antidepressant response, implicating BDNF as a key mediator in these processes, possibly acting through the cyclic adenosine monophosphate (cAMP) pathway (Monteggia et al., 2007, Duman, 2004).

Peripheral serum levels of BDNF are lower in depressed patients compared to controls and anti-depressant treated patients (Molendijk et al., 2014). However, it is not known which of the several pathways outlined above is important for antidepressant function.

Other potential therapeutic targets include: vascular endothelial growth factor (VEGF-A); the transcription factor CREB, involved in synaptic plasticity and memory formation; and LIM domain kinase-1 (LIMK-1), an enzyme regulating dendrite size (Duman and Aghajanian, 2012). Neuropeptides, like the non-acronymic VGF, have also been implicated (Thakker-Varia and Alder, 2009). VEGF-A is one member of the VEGF family, which in humans currently includes five members; VEGF-A, VEGF-B, VEGF-C, VEGF-D and Placental Growth Factor (PGF) (Holmes and Zachary, 2005). The VEGF-A protein was the first of these to be discovered, and was initially known simply as VEGF (Li and Eriksson, 2001). VEGF-A influences both vascular and neuronal function.

In the brain, VEGF-A binds to two main tyrosine kinase receptors; fetal liver kinase (Flk-1 / VEGFR-2) or fms-like tyrosine kinase 1 (Flt-1 / VEGFR-1), whereas VEGF-C and VEGF-D bind to fms-like tyrosine kinase 4 (Flt-4 / VEGFR-3) (Newton et al., 2013), see Figure 1.1 below. Flk-1 in particular is involved in the maturation of neuronal progenitors and plays a key role in neurogenesis. Like BDNF, VEGF has been shown to play a role in the action of several antidepressants and ECS (Segi-Nishida et al., 2008). It

also induces antidepressant-like effects in animal models of depression (Warner-Schmidt and Duman, 2007).

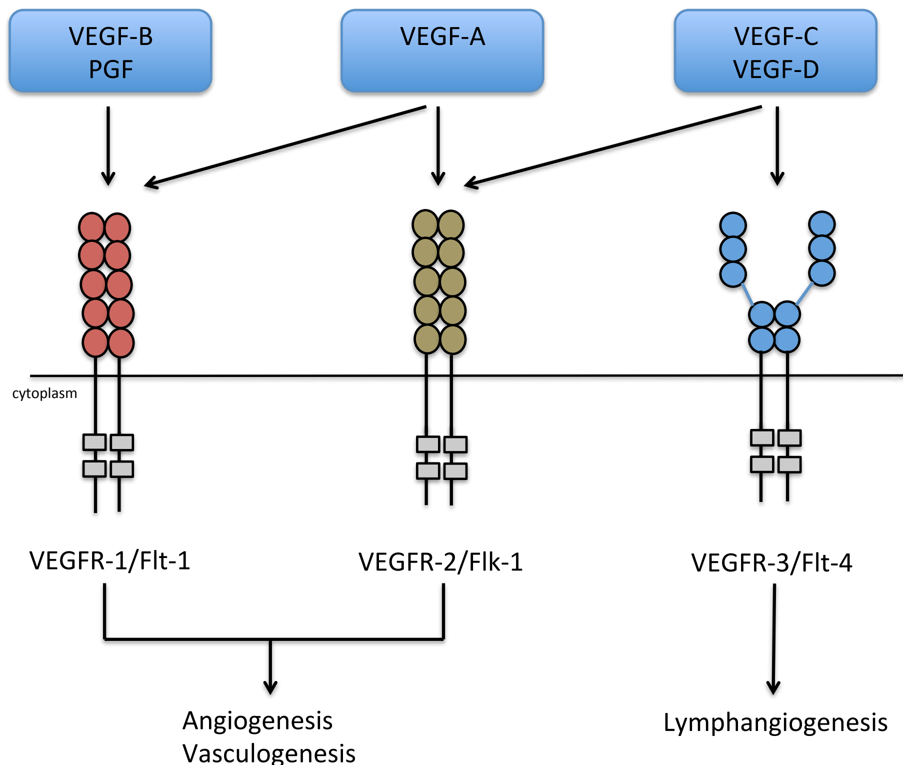


Figure 1.1 VEGF family subtypes and their receptors

There is less evidence for the role of VGF in depression, but it has been shown to be upregulated by antidepressants and downregulated in animal models of depression (Cattaneo et al., 2010, Thakker-Varia and Alder, 2009). VGF, exclusively expressed in neurons, plays a role in hippocampal neuroplasticity and neurogenesis (Thakker-Varia and Alder, 2009).

Lending further support to the role of neuroplasticity in depression is the promise shown by the N-methyl D-aspartate (NMDA) glutamate receptor antagonist ketamine as a fast-acting antidepressant (Coyle and Laws, 2015). This rapid clinical response is matched by a rapid induction of new synapses and dendrite spines (Duman and Aghajanian, 2012).

The various theoretical models are likely to have some overlap and may represent various entrance points or stages in complex pathways

involved in mood regulation and depression. For example, the hippocampus is densely populated by mineralocorticoid (MR) and glucocorticoid receptors (GR), which in response to chronic stress may lead to reductions in hippocampal volume and reduced neuroplasticity (Wainwright and Galea, 2013). Integrating pathophysiological mechanisms such as reduced monoamine neurotransmission, dysregulation of the HPA-axis, dysregulation of the inflammatory cytokines and reduction in neuroplasticity and neurogenesis may be more helpful than looking at these models in isolation (see Figure 1.2).

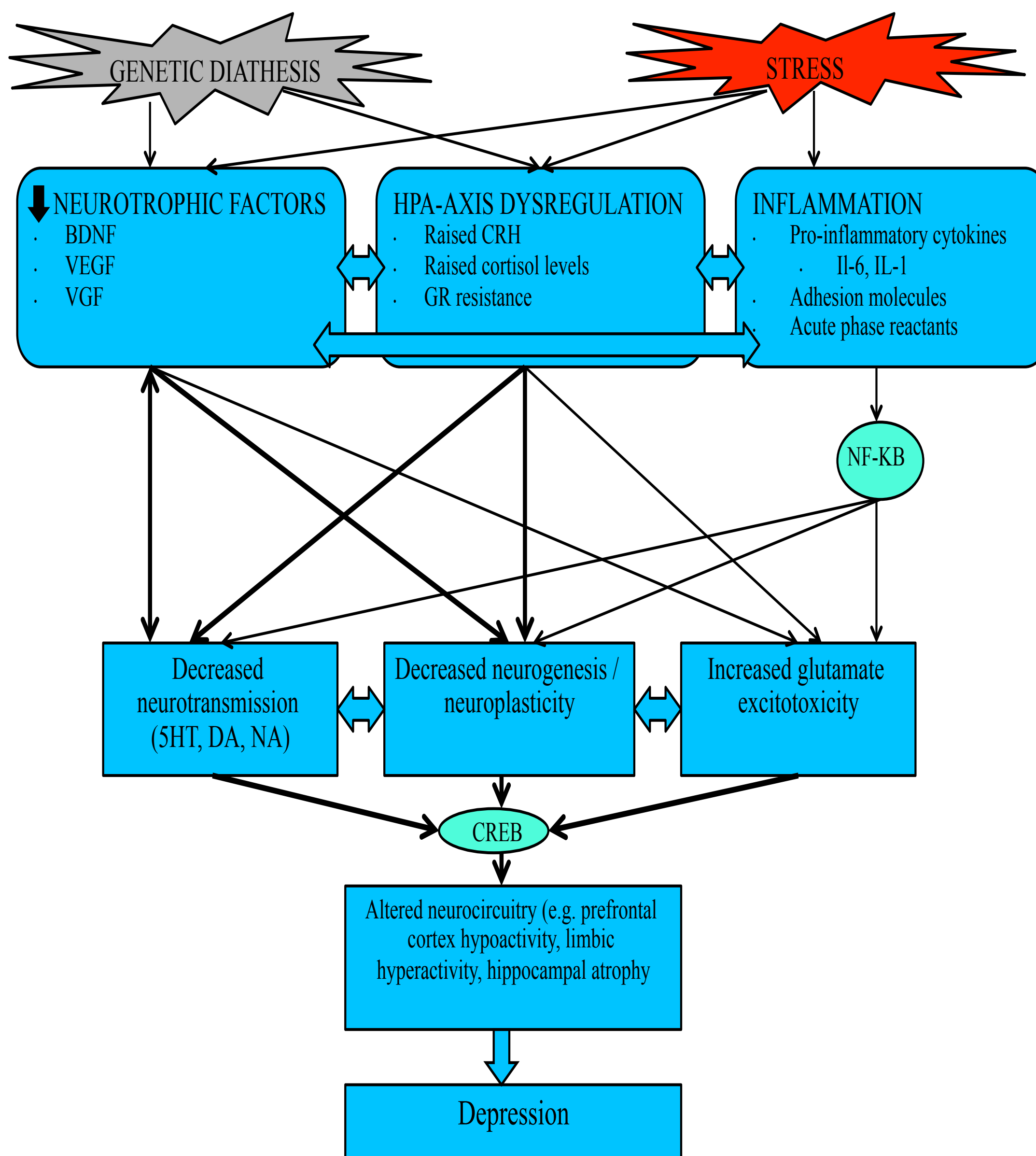


Figure 1.2 An integrated model of the neurobiology of depression

1.1.5 Available treatments and treatment resistance

The 1950's and 1960's heralded a new era in the treatment of depression with the development of TCAs and MAOIs. A major advance at the time, there has been disappointingly few new genuine breakthroughs in treatments for depression in latter decades (Berton and Nestler, 2006). In fact, the most acutely effective treatment for depression remains ECT, which has been used since the 1930s (UK ECT Review Group, 2003). Newer reuptake inhibitors such as SSRIs, SNRIs, bupropion (a noradrenaline and dopamine reuptake inhibitor), as well as noradrenergic and specific serotonergic antidepressants (NaSSAs) such as mirtazapine, have an improved side effect profile but similar efficacy. These drugs all aim to increase the amount of monoamines, such as serotonin or noradrenaline, at the synapse. Drug treatments with a truly novel mechanism of action such as agomelatine have been disappointing to date (Gahr, 2014). Newer physical therapies such as transcranial magnetic stimulation (Martin et al., 2003) and transcranial direct current stimulation (Loo et al., 2012a) have also been disappointing.

There is good evidence that psychological therapies, including interpersonal psychotherapy (IPT) or cognitive behavioural therapy (CBT) can be helpful in depression (Barth et al., 2013, Linde et al., 2015). However, they are not effective for all patients and in the case of severe depression may not be suitable (Kolshus et al., 2011). Clinical guidelines, such as those provided by the UK National Institute for Health and Care Excellence (NICE), suggest that initial treatment for moderate to severe depression should normally consist of a single SSRI for at least six to eight weeks at an adequate dose, ideally in combination with psychotherapy (NICE, 2010). The treatment of BPAD can involve the use of antidepressant medications during a depressive episode, but typically involves the use of mood stabilisers such as lithium and certain anticonvulsants for continuation therapy, and mood stabilisers and antipsychotics or benzodiazepines in the acute phase (NICE, 2010).

Although some patients respond to this approach, there are many cases where there is either an incomplete or no response. Some researchers have suggested that failure of a single antidepressant is sufficient for establishing treatment resistance (Sackeim et al., 1990). This is likely to grossly overestimate the prevalence of treatment resistance. There is a growing consensus that a more practical and reasonable definition involves the failure of at least two antidepressants, at an adequate dose, for an adequate duration and with proper adherence (Berlim and Turecki, 2007). The reported prevalence of treatment resistance varies from 20% to 50% (Fava, 2003). In the large publicly funded Sequenced Treatment Alternatives to Relieve Depression (STAR*D) trial, about one third of those treated for depression failed to achieve remission with progressively higher rates of treatment resistance in those who required multiple treatment steps (Rush et al., 2006). There is little evidence yet to guide clinicians as to which antidepressant is most likely to have the best chance of response in a given patient, and combination and augmentation treatment for depression is common in clinical practice (Kolshus et al., 2011).

Despite recent setbacks, work continues to develop novel treatments for depression. New treatments under investigation include deep brain stimulation, ketamine / NMDA antagonists, and drugs that target dopamine pathways (Smith, 2014, Krishnan and Nestler, 2008, Mathew et al., 2012). Further down the line, developments in the understanding of the role of micro-Ribonucleic Acid (microRNAs) (Kolshus et al., 2014), neurotrophic factors (Castren and Rantamaki, 2010) and epigenetics (Dalton et al., 2014) in depression may lead to novel antidepressant treatments.

1.1.6 Biomarkers in depression

In current clinical practice there is no objective 'blood test' or other biomarker to guide diagnostic assessment, prognosis and treatment choice or response. We are limited to patient history, mental state examination and collateral or self-report. By the time a diagnosis is made a patient will already have suffered significant morbidity, and a biomarker that could identify patients at a prodromal stage has been described as the "holy grail"

of psychiatry (Macaluso et al., 2012). Treatment choice is typically based on general guidelines tailored to the patient's clinical presentation and the treatment's side effects. A biomarker that could predict a patient's likelihood of clinical response to a given treatment would alleviate suffering, and reduce time and costs spent in treating depression. It would be particularly useful for treatments with a significant side effect burden like ECT. However, the heterogeneity of depressive disorders makes research in this area difficult, as does the high levels of comorbidity with other disorders (Lopresti et al., 2014).

A biomarker (biological marker) was defined by the US National Institutes of Health (Biomarkers Definitions Working Group, 2001) as:

> *"a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."*

For practical purposes, a biomarker should ideally be easily accessible or non-invasive (such as peripheral blood), inexpensive, reliable, reproducible and should have acceptable (>80%) sensitivity and specificity (Schneider and Prvulovic, 2013). The *sensitivity* of a test represents its ability to correctly identify those who have a disorder (e.g. the proportion of those with depression who are identified as having depression). The *specificity* of a test reflects its ability to correctly identify those who do not have a disorder (e.g. the proportion of those without depression who are identified as not having depression).

Several biomarkers are under investigation, including biochemical/molecular markers, genetic or epigenetic markers and neuroimaging markers.

1.1.6.1 Biochemical/molecular markers

The HPA-axis was one of the earliest targets of biomarker research in depression with the dexamethasone suppression test (DST) being briefly in

vogue in the clinical setting in the 1980s (The APA Task Force on Laboratory Tests in Psychiatry, 1987). It was thought particularly to differentiate melancholic and psychotic as distinct from other forms of depression. However, meta-analytic evidence failed to find an association between DST non-suppression and melancholic depression, and in psychotic depression this association was only moderate (Nelson and Davis, 1997). Plasma levels of cortisol and other steroid hormones have also been put forward as potential biomarkers. Cortisol has been found to be higher in depressed patients compared to controls. However, studies to date have found limited evidence that steroid levels correlate with clinical response to treatment and cortisol levels are raised in response to a number of stressors apart from depression (Markopoulou et al., 2009, Strickland et al., 2002).

BDNF and other neurotrophic factors are also potential biomarkers. Several studies have identified lower serum levels of BDNF protein in depressed patients compared to controls (Molendijk et al., 2014). Some studies show that these levels are normalised by antidepressant treatment but this is not a universal finding (Sen et al., 2008, Bus et al., 2012). Indeed, studies using CSF have failed to replicate these findings (Martinez et al., 2012). Other trophic factors such as VEGF, Fibroblast Growth Factor-2 (FGF2), VGF and glial cell line-derived neurotrophic factor (GDNF) have been explored in small studies but require validation in other larger cohorts (Clark-Raymond et al., 2014, Evans et al., 2004, Cattaneo et al., 2010, Diniz et al., 2012).

Given the potential role of cytokines and other inflammatory markers in the pathophysiology of depression these have also been evaluated as potential biomarkers. Elevated levels of some cytokines, other inflammatory markers and activity of transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) have been found in depressed patients (Dowlati et al., 2010, Pace et al., 2006). In particular, plasma levels of interleukin-6 (IL-6) and tumour necrosis factor–alpha (TNF-α) are higher in depressed patients compared to controls, and patients with treatment resistant depression have higher levels of IL-6 than those who respond to antidepressant treatment (Schmidt et al., 2011).

1.1.6.2 Genetic and epigenetic markers

Although there is significant heritability in both unipolar and bipolar depression no single gene of large effect has been found to date. Rather, it is thought that numerous genes of small effect underlie the heritability (Flint and Kendler, 2014). Polymorphisms in the serotonin transporter, *BDNF* and tryptophan hydroxylase encoding genes have all been associated with depression, but only have a modest effect and are not suitable as biomarkers (Schmidt et al., 2011, Gyekis et al., 2013). New research is starting to show that epigenetic mechanisms such as histone modification, deoxyribonucleic acid (DNA) methylation and microRNA expression are important mediators between genes and the environment. These epigenetic mechanisms may therefore prove to not only help improve the molecular mechanisms of depression but also act as potential biomarkers in the future (Kolshus et al., 2014, O'Connor et al., 2012).

In summary, although numerous molecular markers have been explored in depression, none has met the criteria for actual clinical use, particularly due to limited sensitivity and specificity. One solution may be the use of panels of biomarkers to improve on these criteria (Schmidt et al., 2011). The use of machine learning and other bioinformatic approaches to handle the large datasets that this would entail has made this more of a possibility in recent years (Phillips, 2012).

1.2 Electroconvulsive therapy

1.2.1 Brief history of electroconvulsive therapy

ECT has been used to treat mental illness for over 75 years, replacing earlier forms of ‘shock’ treatments such as insulin coma therapy and metrazol therapy (Payne and Prudic, 2009, Shorter, 2009). In 1938, Cerletti & Bini administered the first electroconvulsive treatment to a Milanese mechanic (Enrico X.) suffering from schizophrenia. The results were positive and newspapers heralded a "Brilliant New Method of Treating Psychosis” (Shorter and Healy, 2007). In the following years, before the dawn of effective pharmacological agents for mental illness, ECT spread throughout

the globe offering hope for previously 'incurable' patients. Early ECT was limited by the risk of fractures from excessive seizures. This was essentially ended by the development of modified ECT through the use of muscle relaxants, such as succinylcholine in 1951, as well as short-acting anaesthetics, such as methohexital. The use of unmodified ECT however, is still used in many parts of the developing world.

Figure 1.3 Early electroconvulsive therapy (ECT) apparatus
This apparatus was previously used in the 1970s and 1980s for treating patients at the Eg Psychiatric Hospital, in Kristiansand, Norway. Exhibit at the Technical Museum of Norway, Oslo. Photo: Erlend Bjørtvedt (licensed under CC-BY-SA).

ECT was initially used for a wide range of psychiatric disorders, with 35% of total admissions to the Maudsley and Bethlem Royal hospitals in South London receiving ECT in 1956 (Lambe et al., 2014). Gradually, a growing evidence base arising from a series of randomised trials in the 1960's through to the 1980's clarified ECT's superior efficacy in depression when compared to other newly available antidepressant treatments such as MAOIs and TCAs (Payne and Prudic, 2009). Meta-analysis of trials using

sham ECT also clarified that real ECT was significantly superior to its placebo equivalent (UK ECT Review Group, 2003).

The use of ECT dramatically dwindled in the late 1960s and early 1970s and appears to have largely plateaued in the developed world, although variations in its use exist both within and between countries (Lambe et al., 2014, Thompson et al., 1994, Leiknes et al., 2012). Growing concerns over cognitive side effects, its overuse in conditions unlikely to benefit from ECT (and sometimes clear misuse), the growing role of psychotropic agents, and political changes such as the 'anti-psychiatry' movement of the 1970s, are all likely contributors to its reduced availability and use in contemporary practice (Payne and Prudic, 2009, Lambe et al., 2014).

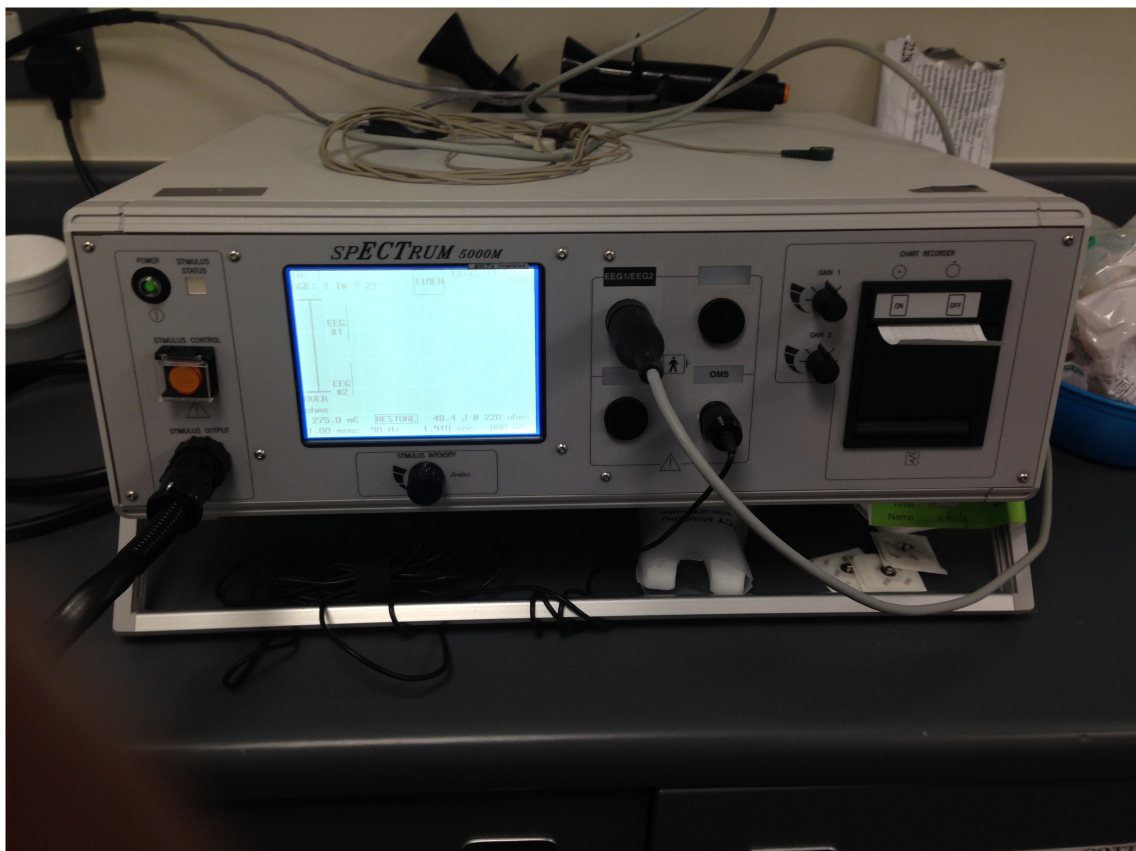


Figure 1.4 Modern day brief-pulse ECT apparatus

The machine shown is the Spectrum 5000Q (MECTA corporation, USA), as used in the EFFECT-Dep trial.

1.2.2 Mechanism of action of electroconvulsive therapy

One of the common criticisms aimed at ECT is that its mechanism of action is not understood. Although it is true that there are large gaps in the understanding of how ECT works, it is also true that similar gaps exist for many psychiatric treatments, including antidepressant drugs, as well as the pathophysiology of depression itself. It is also important to note that empirical data clearly shows the efficacy of ECT. In the history of medicine, numerous treatments (e.g. Vitamin C for scurvy, willow bark (containing salicylate) for pain, penicillin as an antibiotic) have been used to great effect prior to their actual mechanism of action being established (Payne and Prudic, 2009, Scott, 2011, Hacker et al., 2009).

Although large gaps exist, there is some knowledge that can help us understand ECT's possible mechanism of action and guide future research. One of the problems is the wide range of actions of ECT on the brain, which makes it hard to identify which are involved in its antidepressant effect. Various early hypotheses that have since been discredited include psychological mechanisms such as a pure placebo effect, self-punishment, forced regression and induced amnesia of painful or depressing memories (McCall et al., 2014). Current theories of ECT's antidepressant effects focus on the role of the seizure itself as well as changes in neurotransmission, neuroplasticity and HPA-axis regulation amongst others.

1.2.2.1 The role of the seizure in ECT

Modified ECT involves passing a small electrical current to a patient's scalp after administering anaesthesia and a muscle relaxant (Dunne and McLoughlin, 2013). The electrical current excites neurons in the brain inducing a seizure. Early studies indicated that the induction of a seizure was an essential component of the therapeutic effects of ECT with ensuing changes in regional blood-flow and metabolism.

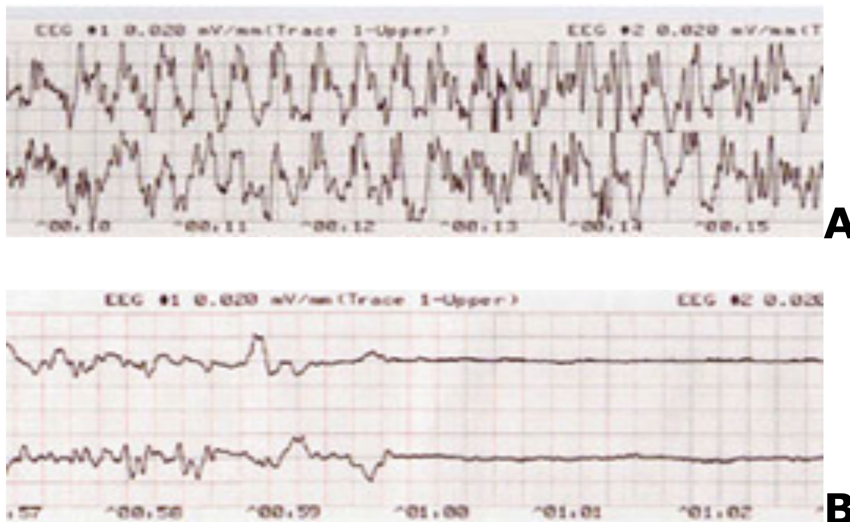


Figure 1.5 The EEG in ECT

Two EEG strips showing A) Bilateral 3 Hertz spike and wave complexes; B) Termination of seizure with post-ictal suppression.

Subsequent studies, however, indicated that the induction of a seizure is necessary, but not sufficient, to have a therapeutic effect. Electrode placement and charge above seizure threshold (the electrical charge just sufficient to induce a seizure) modulates the therapeutic effects of ECT, especially for unilateral ECT (Scott, 2011).

1.2.2.2 Effects on neurotransmission

Whereas pharmacological antidepressants typically mainly act on one or possibly two monoamine systems, ECT has actions on serotonergic, noradrenergic and dopaminergic pathways. Direct evidence for this comes chiefly from animal studies using ECS (Merkl et al., 2009, Scott, 2011). In humans, CSF levels of dopamine metabolites were increased following a course of ECT (Nikisch and Mathe, 2008). Neuroimaging studies have also found a reduction in dopamine receptor binding in the anterior cingulate cortex following successful ECT, although this was not found in brain serotonin (5HTA1) receptors (Saijo et al., 2010a, Saijo et al., 2010b). The major dopamine-metabolising enzyme in the prefrontal cortex is catechol-o-methyltransferase (*COMT*). A study examining polymorphisms in the COMT gene found that those with high enzyme activity (Val/Val genotype)

responded better to ECT (Huuhka et al., 2008). This reinforces the role that dopamine may have in the therapeutic effect of ECT.

ECT also affects other neurotransmitters, but their role in depression is even less clear. ECT increases GABAergic functioning, which may have an antidepressant as well as anticonvulsant effect (Merkl et al., 2009). Glutamate, the main excitatory neurotransmitter in the brain has been identified as a potential target for new antidepressant treatments (Sanacora et al., 2008). ECT can modulate or normalize glutamate levels in areas of the brain such as the dorsolateral prefrontal cortex (DLPFC) and the anterior cingulate cortex (Michael et al., 2003a, Merkl et al., 2011).

1.2.2.3 Effects on neuroplasticity and brain structure

Animal experiments have shown that ECS leads to changes in neurogenesis and neuroplasticity in line with the neurotrophic model of depression (Duman et al., 1997, Duman and Vaidya, 1998). These changes occur rapidly with ECS, mirroring the rapid antidepressant effects of ECT. Rodent models have shown that ECS causes increased neurogenesis (Madsen et al., 2000), increased mossy fibre sprouting in the dentate gyrus (Lamont et al., 2001), and increases in hippocampal cytoskeletal proteins involved in neuroplasticity (O'Donovan et al., 2014b). Increased neurogenesis has also been shown in a non-human primate model of ECS (Perera et al., 2007). Some of these neuroplastic changes are likely brought about through changes in BDNF signalling. ECS has been shown to block downregulation of *BDNF* messenger ribonucleic acid (mRNA) in rat hippocampus following restraint stress (Nibuya et al., 1995), increase mRNA levels of the *BDNF* activating transcription factor *CREB* (Nibuya et al., 1996), increase BDNF protein levels following glucocorticoid administration (O'Donovan et al., 2014a), and increase *BDNF*-regulating microRNAs (Ryan et al., 2013). Animal studies have also shown increases in *VEGF* mRNA expression following ECS and their role in guiding neurogenesis (Segi-Nishida et al., 2008, Elfving and Wegener, 2012, Warner-Schmidt and Duman, 2007). Other neurotrophic factors induced by ECS include neuritin, nerve growth factor (*NGF*) and *FGF2* (Rotheneichner et al., 2014).

Whether these changes are also seen in humans is yet not known. Directly measuring molecular brain changes in living human subjects is not possible, and findings are based on peripheral sources or neuroimaging studies. Meta-analytic evidence has shown that ECT causes increases in serum protein levels of BDNF, although these increases do not correlate with clinical response (Brunoni et al., 2014). Only one study to date has looked at VEGF protein levels in blood following ECT. Significant increases were found one month after the end of ECT in a group of treatment resistant patients. In this group there was a correlation between the change in VEGF levels and score on a depression rating scale (Minelli et al., 2011).

Neuroimaging studies have also given some insights into the possible neuroplastic mechanism of action of ECT. Proton magnetic resonance spectroscopy (^{1}H MRS) is a non-invasive method of observing brain metabolism in-vivo. In a cohort of treatment-resistant patients, ECT responders were found to have increased levels of N-acetylaspartate (NAA), an indirect measure of neuron viability and functionality (Michael et al., 2003b). Functional magnetic resonance imaging (fMRI) has shown that ECT appears to decrease functional connectivity in the DLPFC, and this decrease was correlated with clinical improvement (Perrin et al., 2012). In contrast, a structural connectivity study using diffusion tensor imaging (DTI) to measure white matter microstructures in fronto-limbic pathways including DLPFC, found increased activity. Structural imaging studies implicate the amygdala and hippocampus as key areas in depression and the response to ECT. In one study, pre-treatment amygdala volume significantly predicted treatment response to ECT (Ten Doesschate et al., 2014). Another study found patients to have smaller hippocampal volumes than healthy controls at baseline (Joshi et al., 2015). Following ECT, structural imaging studies indicate an increase in both hippocampal and amygdala volumes (Joshi et al., 2015, Jorgensen et al., 2015).

Together, these findings indicate that ECT results in both structural and functional changes in brain areas and pathways thought to be involved in depression.

1.2.2.4 Effects on the HPA-axis

Following the administration of ECT there is typically a surge in various hormones, including cortisol, which has been linked with the acute cognitive side effects of ECT (Payne and Prudic, 2009). Following a course of ECT however, several studies have found a normalisation of HPA-axis dysregulation as measured by the dexamethasone suppression test (Yuuki et al., 2005, Kunugi et al., 2006). Ultimately, ECT's effects on the HPA-axis may lead to increased neurogenesis and neuroplasticity as these are inversely correlated to glucocorticoid activity in neural cells with GR or MR receptors (Rotheneichner et al., 2014).

1.2.3 Indications for electroconvulsive therapy

Over 1.4 million patients are treated with ECT worldwide on an annual basis (Leiknes et al., 2012). Historically, ECT has been predominantly used for affective disorders and schizophrenia, although there is significant variance across global regions, with ECT being predominantly a treatment for depression in the developed world and a treatment for psychotic disorders across most of Africa, Asia and South America (Leiknes et al., 2012). ECT is also used in rare occasions in Neuroleptic Malignant Syndrome, as well as a treatment of 'last resort' in conditions such as Parkinson's Disease, Obsessive-Compulsive Disorder and treatment-resistant epilepsy (Baghai and Moller, 2008). The current UK NICE guidelines recommend ECT for the acute treatment of severe depression, catatonia or severe mania where a rapid response is required and other treatments have been ineffective or when the condition is life-threatening (NICE, 2010).

1.2.4 Efficacy of electroconvulsive therapy for depression

ECT remains the most acutely effective treatment for depression (UK ECT Review Group, 2003). Early trials established that real ECT was superior to a sham ECT, although a high placebo effect was noted in some trials (Rasmussen, 2009, Barton, 1977). However, these trials did not have the same level of methodological rigour that would be expected of

contemporary trials. These trials included patients with various diagnoses, small numbers, and often were not carried out under blinded or randomised conditions (Read and Bentall, 2010). Subsequent trials under randomised controlled conditions (RCTs) were therefore carried out. One of the first of these trials failed to find an advantage for ECT over simulated ECT in a sample of 32 patients, however low-dose right unilateral ECT, subsequently identified as an ineffective treatment, was used as the active arm (Lambourn and Gill, 1978). A number of other RCTs carried out around this time did find significant differences with separate meta-analytic evidence clearly showing the advantage of real over simulated ECT (UK ECT Review Group, 2003, Pagnin et al., 2004). As simulated ECT is likely to have a larger placebo effect than a placebo pill, meta-analyses have also been carried out on the effects of ECT vs. pharmacological placebo. This has found that patients undergoing ECT were significantly more likely to achieve a treatment response (OR 11.1; CI 95% 3.1 to 39.7) than those receiving a placebo (Pagnin et al., 2004, Janicak et al., 1985).

The efficacy of ECT has been compared to antidepressant pharmacotherapy. The UK ECT Review Group included 18 RCTs totalling 1144 patients and found ECT to be significantly more efficacious. The difference as measured in points on the Hamilton Depression Rating Scale (HDRS) was estimated to be equivalent to 5.2 (95% CI 1.4-8.9) (UK ECT Review Group, 2003). The majority of these trials used either TCAs or MAOIs. ECT also performs favourably in the short-term against more recent brain stimulation treatments such as repetitive transcranial magnetic stimulation (rTMS) (Ren et al., 2014, Berlim et al., 2013).

ECT has been found to be particularly effective in psychotic depression, with remission rates superior to non-psychotic depression (Petrides et al., 2001). This offers support to the hypothesis that psychotic depression may represent a distinct disorder separate from the current unified concept of depression. In contrast, treatment resistant depression is associated with poorer ECT response, although it is the most common indication for ECT in the developed world (Sackeim et al., 2009, Heijnen et al., 2010).

Although its short-term efficacy is well established, 12-month relapse rates are in the region of 51%, with the majority of relapses occurring in

the first six months (see Figure 1.6 below) (Jelovac et al., 2013). Continuation pharmacotherapy following a course of ECT halves the risk of relapse (Jelovac et al., 2013).

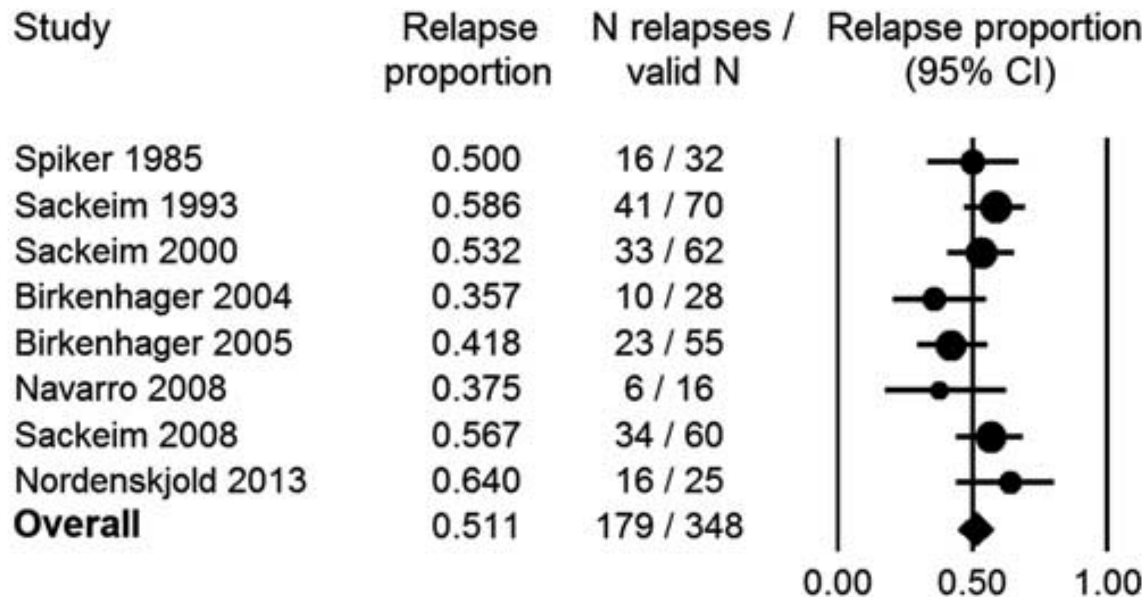


Figure 1.6 Forest plot of relapse rates at 12 months after ECT
Adapted from Jelovac et al. (2013).

1.2.5 Side effects of electroconvulsive therapy

ECT, like most medical interventions, has the potential to cause both minor and major side effects, but carries a disproportionate level of fear and stigma considering efficacy and safety data (Wilkinson and Daoud, 1998). Side effects can be divided into cognitive and physical side effects.

1.2.5.1 Cognitive side effects of ECT

The cognitive side effects of ECT continue to be the single most controversial side effect of ECT, and remain an area of concern to psychiatrists and patients alike. The majority of recent ECT research has focused on maintaining efficacy whilst minimising cognitive side effects (Semkovska and McLoughlin, 2013, UK ECT Review Group, 2003, Tirmizi et al., 2012).

The cognitive side effects of ECT can be divided into immediate, short-term and long-term side effects. The immediate cognitive side effects of ECT involve a temporary disruption to orientation, the ability of a person to place themselves in relation to their environment. This is typically measured by establishing their orientation in terms of person, place and time (Food and Drug Administration, 2011). Although a brief confusional period lasting minutes is the norm, some patients experience prolonged disorientation lasting more than an hour. Some researchers have found a correlation between the speed of reorientation and later performance on retrograde memory tests at the end of an ECT course (Sobin et al., 1995, Martin et al., 2015). Rarely, some patients may experience an episode of delirium with agitation and severe confusion (Sackeim et al., 1983). There is no evidence suggesting any long term or persistent disorientation following ECT (Food and Drug Administration, 2011).

Meta-analytic evidence indicates that ECT, when used for depression, does have a significant short-term impact on cognitive function. However, these impairments return to baseline (prior to ECT) function within 2-3 weeks and continue to improve in the ensuing period (Semkovska and McLoughlin, 2010). Different cognitive domains have been shown to show various degrees of impairment in the days and weeks following ECT. Domains that appear most vulnerable to ECT in the short-term include executive functioning and verbal and visual episodic memory. Global cognition, processing speed, attention and spatial problem solving abilities appear to only show small deficits in the days and weeks after ECT. There is no apparent impairment in overall intellectual ability (Semkovska and McLoughlin, 2010).

The longer-term cognitive effects of ECT have not been well described. Retrograde amnesia, particularly for autobiographical memory remains a concern for patients and clinicians alike, but its measurement is fraught with difficulty due to multiple confounders (passage of time, depression) and limitations of current assessment instruments (Semkovska and McLoughlin, 2013, Soderlund et al., 2014). There are also discrepancies between objective measures of retrograde memory and subjective reports of long-term memory loss (Rose et al., 2003).

1.2.5.2 Physical side effects of ECT

The physical side effects of modern modified ECT are well established (Lisanby, 2007). Some may be due to the seizure and its effects whilst others may be due to the anaesthetic and muscle relaxant. Typical side effects include headache, nausea and muscle pain and transient alterations in blood pressure. Headache may occur in up to 45% of patients but typically responds to mild analgesia, although some may require anti-migraine treatment. Nausea is the second most common (up to 23% of patients) physical side effect, and is usually attributed to anaesthetic medication. Anti-emetics may be used in some cases. Muscle soreness can also occur, typically after the initial treatments. This is attributed to the fasciculations induced by the muscle relaxant (Payne and Prudic, 2009). Beyond these common side effects, physical side effects include arrhythmias, dental trauma, prolonged seizures and pulmonary complications. The reported risk of death is in the region of one in 10,000, i.e. similar to minor surgical procedures (Food and Drug Administration, 2011).

1.2.6 Refining electroconvulsive therapy

1.2.6.1 Electrode placement

Although bitemporal (BT) electrode placement remains the most commonly used worldwide, right unilateral (D'Elia) and bifrontal placements are preferred in some countries (Leiknes et al., 2012). In 1949 Douglas Goodman first reported the use of right unilateral (RUL) ECT in an attempt to spare the speech areas of the brain (Goldman, 1949). He found it not only equally efficacious to bitemporal ECT, but also be associated with less confusion. The first controlled trial of right unilateral ECT was carried out in 1958, again finding no significant clinical advantage for bitemporal ECT (Abrams, 2002). Over subsequent years, numerous studies with varying techniques and procedures produced conflicting results, which failed to settle the unilateral vs. bitemporal 'controversy' (Janicak et al., 1985, Pettinati et al., 1986).

Figure 1.7 Three common electrode placements in use today

From top; Bitemporal, Right unilateral (d'Elia) and bifrontal electrode placements.

Clarification on this issue eventually arrived through a series of studies by Harold Sackeim's group in Columbia University, New York. These studies made it clear that the effectiveness of right unilateral ECT depends on the strength of the electrical dose above the seizure threshold (ST), being relatively ineffective at the lower doses (i.e. 1.5 x ST) used in bitemporal ECT (Sackeim et al., 1987, Sackeim et al., 1993, McCall et al., 2000). This also indicated that a generalised seizure was necessary, but was not in itself sufficient for clinical response from unilateral ECT. The UK ECT Review Group concluded in 2003 that high-dose ECT was more effective than low-dose ECT but at this stage there were not enough studies to ascertain whether high-dose unilateral ECT was as effective as bitemporal ECT, nor whether it was associated with less cognitive side effects (UK ECT Review Group, 2003).

Several RCTs have now been carried out to test this hypothesis with varying results (Ranjkesh et al., 2005, McCall et al., 2002, Kellner et al., 2010, Sackeim et al., 2000, Sackeim et al., 2008, Sackeim et al., 2009, Semkovska et al., 2016). These trials indicate that both forms of ECT are effective in treating depression, but there appear to be some differences in cognitive performance. Some of the trials show little difference in cognitive side effects following high-dose (5-8 x ST) right unilateral or low to moderate dose (1.0-1.5 x ST) ECT (Ranjkesh et al., 2005, McCall et al., 2002, Kellner et al., 2010), whereas others have found a cognitive advantage when using high-dose (6 x ST) right unilateral ECT over bitemporal ECT (Sackeim et al., 2000, Sackeim et al., 2008, Sackeim et al., 2009, Semkovska et al., 2016).

With regard to bifrontal ECT, meta-analytic evidence suggests it is not more effective than the other placements and requires further characterisation before it can be recommended (Dunne and McLoughlin, 2012).

1.2.6.2 Pulse-width

Aside from electrode placement, further refinement of ECT has come from modifications in the waveform of the electrical current applied. One of the major changes was the move from the original sine-wave stimulus with its

long pulse-width (8.3ms) to current square-wave brief-pulse (0.5-1.5ms) stimulus. This development led to a marked reduction in cognitive side effects but maintained efficacy (Loo et al., 2012b). Although this was an improvement, it is known that the chronaxie of neurons (a measure of the length of stimulus required for a neuron to discharge) is in the region of 0.1-0.2ms (Sackeim et al., 2008). Pulsewidths longer than the chronaxie could be deemed inefficient as stimulus is applied during the refractory period of a neuron (see Figure 1.8). This has led to the development of ultra-brief ECT with a pulsewidth <0.5ms. A recent meta-analysis supported the advantage of ultra-brief ECT in terms of cognitive side effects, however, this was at a significant cost in antidepressant efficacy (Tor et al., 2015). Brief pulse ECT is therefore likely to continue to remain a widely used form of ECT in the near future (Spaans et al., 2013).

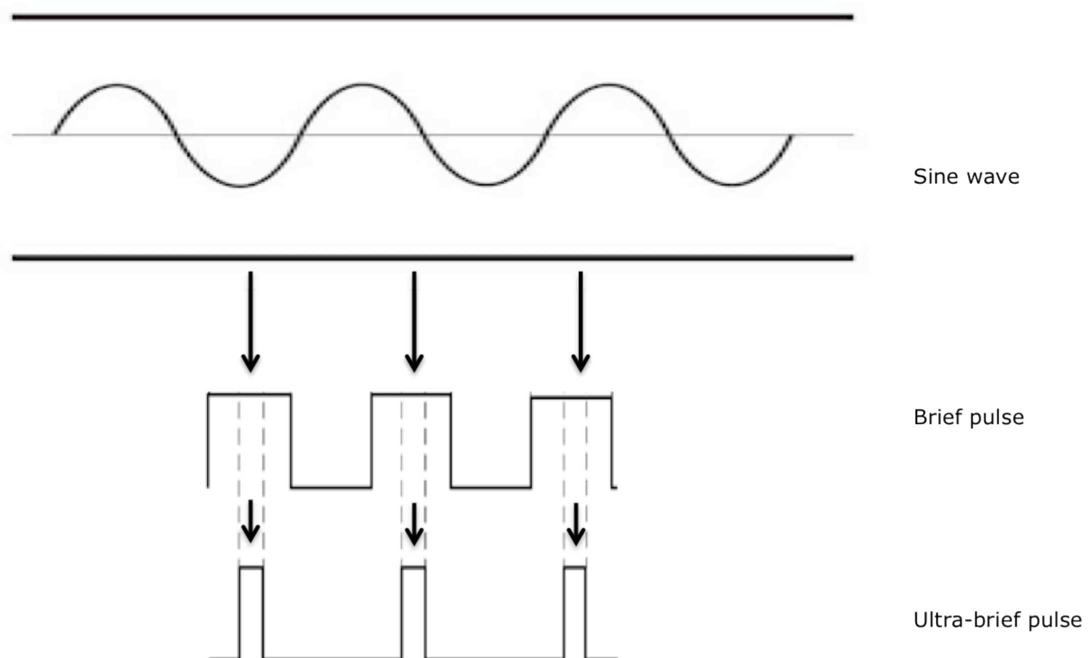


Figure 1.8 Different electrical stimuli used in ECT
From top; Sine wave ECT (8.3 ms), brief-pulse ECT (0.5-1.5 ms), ultra-brief pulse (<0.5 ms). Dotted line represents chronaxie.

1.2.7 Current use of ECT in Ireland

ECT in Ireland is regulated by the Mental Health Commission, which provides annual reports on national use. Since 2008 there has been an annual average of 300-400 ECT treatments on a national basis, with 35% carried out in St. Patrick's University Hospital (Mental Health Commission, 2015, Semkovska et al., 2016). The rates of use of ECT in Ireland, which lie in the range of 5-10/100,000 are similar to other Western countries (Dunne and McLoughlin, 2011). In 2013, the latest year for which there is available data, the mean age of patients having ECT was 60 years, and there was a predominance of female patients (63.4%). 14.5% of programmes were on an involuntary basis (Mental Health Commission, 2015). In Ireland, a programme of ECT can be up to 12 treatments, and some patients will have more than one programme per year. As is the case with the majority of the developed world, the most common indication for ECT in Ireland is depression, particularly for those that are refractory to medication (Mental Health Commission, 2015).

1.2.8 Summary

In summary, ECT, which is still in regular use in contemporary psychiatric practice, has wide-ranging mechanisms of action but we do not yet know which are necessary for antidepressant function, which may contribute to side effects, and which are merely incidental. ECT creates much "noise" and disentangling this will likely need various approaches, including pre-clinical and clinical studies. As ECT is the most acutely effective treatment for depression, understanding its mechanisms of action could help the development of novel treatments. One new area of research that may help shed light on the pathophysiology of depression as well as the mechanism of action of its treatments is epigenetics, which includes microRNAs (Kolshus et al., 2014, Dalton et al., 2014).

1.3 MicroRNAs and depression

1.3.1 MicroRNA background and function

The first microRNA, lin-4, was discovered in 1993 but it took another seven years until a second microRNA was identified (Lee et al., 1993). Since then microRNAs have rapidly emerged as important regulators of post-transcriptional gene expression (see Figure 1.9). They are endogenous non-protein coding RNA molecules of approximately 21-23 bases. Over 2500 mature human microRNA sequences have been reported to date (Griffith-Jones, 2015) and over 50% of mammalian mRNA species are potential targets for microRNAs (Friedman et al., 2009). A single mRNA may be regulated by multiple microRNAs and, conversely, some microRNAs have the potential to target hundreds of mRNAs.

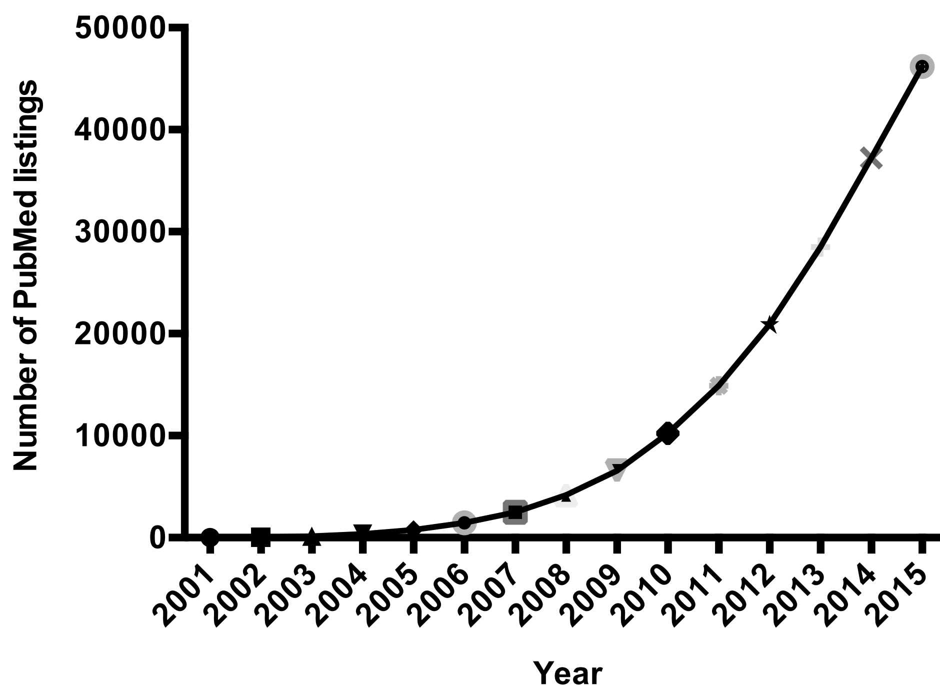


Figure 1.9 Growth of microRNA research from 2001-2015.

A search for published papers on PubMed using the term "microRNA" has increased from five in 2001 to 46205 by the end of 2015.

Typically, microRNAs suppress target gene expression; however, reciprocal relationships between microRNAs levels and their targets are emerging (Pasquinelli, 2012). They are expressed during development and in adulthood, with the expression of some subsets being organ-specific (Sempere et al., 2004) and region-specific within organs such as the brain (Olsen et al., 2009). Functional studies indicate that microRNAs are involved in the control of neuronal cellular processes including neurogenesis, synaptic plasticity, cell fate decision and apoptosis (Kloosterman and Plasterk, 2006, Magill et al., 2010, Saba and Schratt, 2010, Bredy et al., 2011, Luikart et al., 2012). Furthermore, Dicer, one of the key microRNA processing enzymes has been shown to be necessary for correct embryonic brain development in zebrafish and for maintenance of neuron survival in the mature brain (O'Connor et al., 2012).

Synaptic plasticity refers to the ability of synapses to make adaptive changes in response to activity in their neuronal circuits. These changes include altered neurotransmitter release, altered receptor expression and alteration of dendritic density and size. These adaptive changes are thought to be key parts of the molecular mechanisms underlying higher functions such as learning and memory and, when dysregulated, may be involved in the pathogenesis of psychiatric disorders. Altered dendritic spine density and volume have been found to be associated with specific microRNAs, including miRNA-132 and miRNA-134 (Edbauer et al., 2010, Schratt et al., 2006). Target genes of these microRNAs include those encoding: brain-derived neurotrophic factor (BDNF), widely expressed throughout the brain where it promotes neuronal survival and maturation, synaptic plasticity and synaptic function; CREB, a transcription factor involved in synaptic plasticity and memory formation; and LIM domain kinase-1 (LIMK-1), an enzyme that regulates dendrite size. MicroRNA suppression of LIMK-1 can be reversed by BDNF (Schratt et al., 2006). MicroRNAs are also key regulators of postnatal neurogenesis, and specifically, miR-124 has been shown to dictate adult neurogenesis in mice (Akerblom et al., 2012). Antidepressant therapies stimulate neurogenesis, whereas stress and depression inhibit neurogenesis. Given microRNAs’ role in neurogenesis, there is therefore added impetus to their study in psychiatric disorders (Baudry et al., 2011).

1.3.2 MicroRNA biogenesis and dysregulation

Two pathways from which microRNAs arise have been described to date. In the canonical pathway, primary microRNAs (pri-microRNA) are transcribed by RNA polymerase II or III from independent microRNA genes and typically form a stem and terminal loop structure with flanking segments (see Figure 1.10).

Within the nucleus, a microprocessor complex that is generally made up of Drosha, an RNAse type III endonuclease, and a cofactor for activation, such as DiGeorge syndrome critical region 8 protein (DGCR8), removes the flanking segments and stem region to generate a precursor microRNA (pre-microRNA) (Rose et al., 2003). Pre-microRNAs are between 70-110 nucleotides in length and form a hairpin secondary structure (see Figure 1.11).

In the second Drosha/DGCR8-independent pathway, pre-microRNAs are derived from introns as a result of splicing and debranching by nuclear splicing machinery (Chong et al., 2010). In both cases, pre-microRNAs are then exported from the nucleus to the cytoplasm via Exportin 5, a double stranded RNA-binding protein that mediates nuclear export (Yi et al., 2003). In the cytoplasm, the pre-microRNA hairpin loop is cleaved to form a ~22 base pair duplex by the RNAse III enzyme Dicer in combination with a cofactor such as TAR RNA-binding protein (TRBP) (Chendrimada et al., 2005). One strand from the duplex is then preferentially loaded into the RNA-induced silencing complex (RISC) in a sequence of steps regulated by Dicer, Argonaute homologue proteins, TRBP and other regulatory proteins.

Once loaded into the RISC, the microRNA guides the complex to target sequences known as microRNA response elements (MRE), generally located within the 3' untranslated region of mRNA transcripts. These match on to seed sequences in the microRNA molecule. The mechanism of microRNA-induced regulation of mRNA translation depends on complementarity between the mRNA target and microRNA. Degradation of mRNA usually occurs when the microRNA RISC binds with near exact complementarity. On the other hand, when the microRNA binds with incomplete complementarity, mRNA translation to protein can also be prevented due to steric hindrance (Carthew and Sontheimer, 2009).

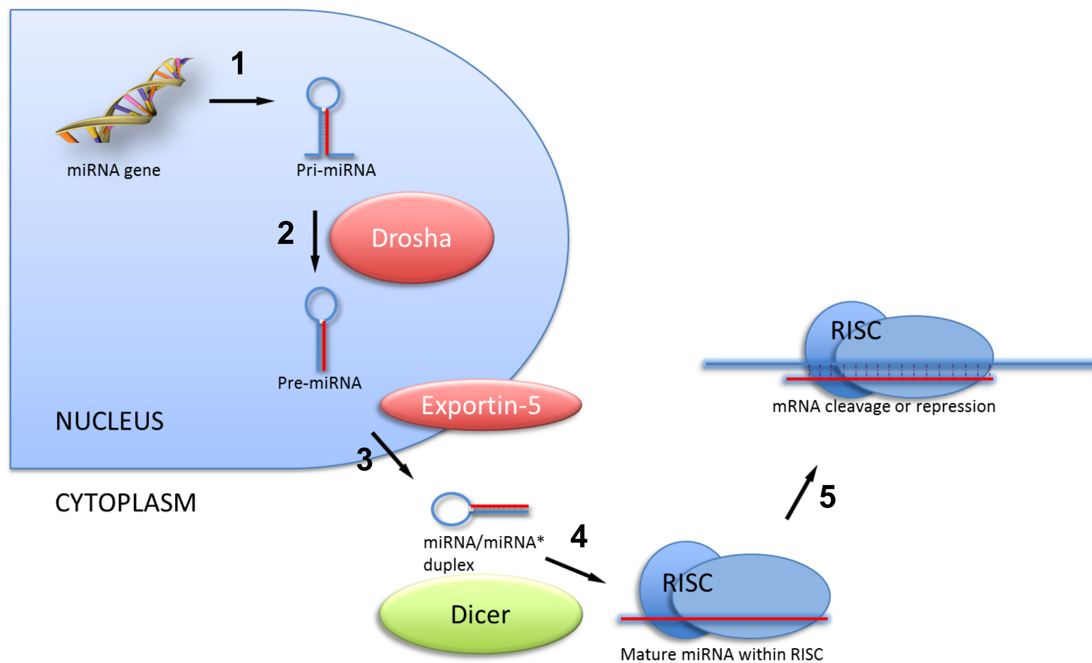


Figure 1.10 MicroRNA Biogenesis.
Mature microRNAs (miRNAs) are formed following a series of steps involving: (1) RNA transcription to generate a primary miRNA (pri-miRNA), followed by (2) RNAse modification by Drosha to form a precursor miRNA (pre-miRNA), which is (3) exported from the nucleus to the cytoplasm by Exportin-5 and cleaved to form a 22 base pair miRNA/miRNA* duplex by Dicer. (4) From the duplex, one miRNA is then preferentially loaded into a RNA-induced silencing complex (RISC) where it (5) binds to a target mRNA transcript, preventing translation.

Dysregulation of microRNAs can be through altered expression or changes to microRNA targets. Deletions, amplifications or single nucleotide polymorphisms (SNPs) in a microRNA can lead to its over- or under-expression with ensuing changes to its targets. Alternatively, alterations in the microRNA processing machinery or SNPs in the target region for a microRNA could lead to inappropriate targeting of mRNAs (Sethupathy and Collins, 2008).

The discovery of microRNAs and their role in regulation of gene expression has led to a revision of the prevailing 'central dogma' of genetics. Recent findings have highlighted the regulatory role of microRNAs and other non-coding genetic material (Djebali et al., 2012). It is therefore no surprise that dysregulation of microRNAs has been linked with human

pathologies such as major depressive disorder, schizophrenia and cancer (Esteller, 2011, Im and Kenny, 2012, Miller and Wahlestedt, 2010). It is hoped that further understanding of the role of microRNAs will elucidate molecular mechanisms of psychiatric illness, act as biomarkers and potentially become novel therapeutic targets (Baudry et al., 2011). In other medical fields, microRNAs have already been identified as biomarkers (Cho, 2010) and therapeutic targets (Janssen et al., 2013).

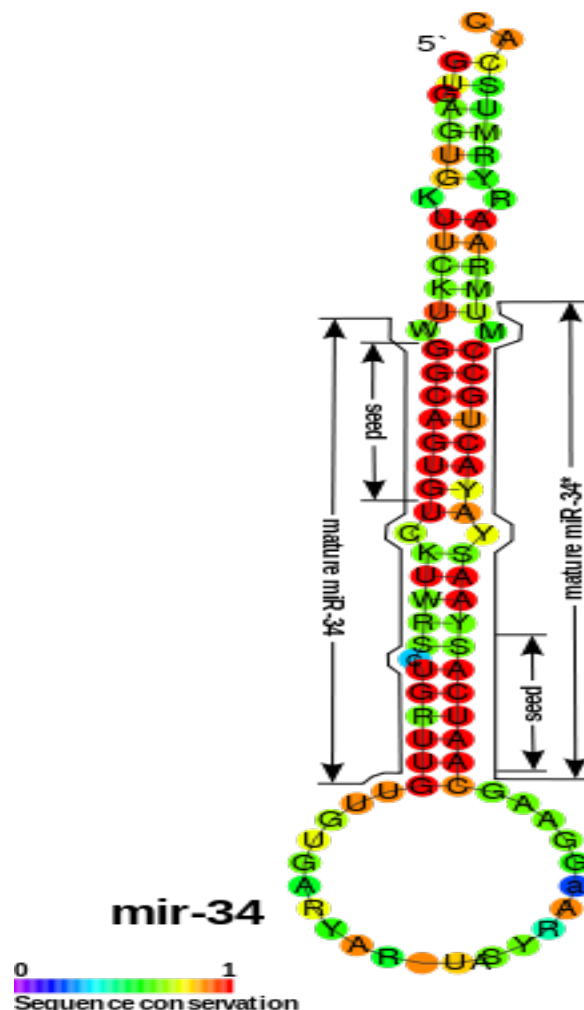


Figure 1.11 Structure of a microRNA (mir-34).

Precursor structure of miR-34 showing hairpin loop as well as the mature microRNA segment and its seed sequence. Colours indicate evolutionary conservation. Source: Paul Gardner (licensed under CC-BY-SA) using Rfam (Burge et al., 2013).

1.3.3 MicroRNA analysis

The techniques used to characterise microRNAs are similar to those used to study RNA or DNA, with some modifications. Studies investigating the role and distribution of microRNAs may be undertaken using cell culture, animal models and human samples, including post-mortem brain specimens, cerebrospinal fluid (CSF) and peripheral blood. The study of post-mortem specimens has the advantage of direct access to human brain, but has limitations in terms of variations in pre- and post-mortem conditions, diagnostic uncertainty and usually small sample sizes (McCullumsmith and Meador-Woodruff, 2011). CSF sampling has the advantage of the ability of tracking dynamic changes but requires an invasive procedure that may not be acceptable to patients, especially for repeated sampling, and may limit ethical approval; therefore, studies using CSF are relatively rare.

In contrast, peripheral blood sampling provides the benefit of easy and acceptable access, and microRNAs are present in a remarkably stable form in human blood (Dwivedi, 2014). There is considerable communication between the central nervous system (CNS) and the immune system, and lymphocytes express several of the same neurotransmitter receptors seen in the CNS (Woelk et al., 2011). Interestingly, microRNAs specific to brain diseases can be detected in peripheral blood, and peripheral blood levels can correlate with brain levels (Gladkevich et al., 2004).

To analyse microRNA levels, researchers may use deep sequencing technology, microarrays or quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) techniques (Pritchard et al., 2012). Deep sequencing (also known as next generation sequencing) is a relatively recent technological advance that allows for quantification of every microRNA in a sample. Such analysis enables robust quantification of known microRNAs as well as potential novel microRNA discovery. However, it is a costly procedure, which limits its widespread use. Microarrays are less costly, but rely on a priori knowledge of sequences of interest. These consist of chips with probes for known microRNAs, usually covering hundreds of microRNAs. MicroRNAs in a sample will then hybridise to the probes, allowing for absolute or relative quantification. Typically, microRNAs identified by sequencing or array approaches are then confirmed using qRT-

PCR. Because microRNAs are too short to accommodate standard primer pairs, they are first lengthened by stem-loop reverse transcription (Kramer, 2011). An essential element of microRNA analysis is the use of bioinformatic approaches and databases to link identified microRNAs with their target genes and biological function. This "in-silico" approach is crucial due to the ability of microRNAs to target hundreds of genes and complex pathways (Pritchard et al., 2012). A separate approach includes genotyping patients and controls to identify structural variants in microRNAs, microRNA targets or microRNA biosynthesis machinery that may be associated with psychiatric phenotypes.

Names are assigned to experimentally confirmed microRNAs using the following nomenclature: the prefix "miR", indicating a mature microRNA, is followed by a dash and a number, usually indicating the order of naming. Pre-microRNAs refer to precursor microRNAs, and use the prefix "mir". MicroRNAs with nearly identical sequences except one or two nucleotides are annotated with an additional lower case letter, e.g. miR-26a and miR-26b would be very closely related. Some of the first microRNAs to be discovered were the let-family of microRNAs. At that time, the existence of the amount of microRNAs now discovered was not known and they therefore do not follow the numerical system. If two microRNAs originate from opposite arms of the same pre-microRNA (which is in a hairpin loop, see Figure 1) they are denoted with a -3p or -5p suffix. If relative expression levels of these opposite strands are known, an asterisk (*) following the microRNA denotes a microRNA expressed at low levels relative to its opposite arm of the hairpin loop. It is proposed that the use of the asterisk denomination be replaced by consistent use of the -3p or -5p suffix (Griffith-Jones, 2015).

1.3.4 MicroRNAs in depression

1.3.4.1 Pre-clinical studies of microRNAs and stress

Animal and other pre-clinical models have been crucial to most medical developments, but have their limitations. Animal models of psychiatric disorders suffer from additional limitations because some of the symptoms experienced such as suicidality, guilt and sad mood are presumably linked to the human condition and cannot be replicated in commonly used small animals (Krishnan and Nestler, 2011). Simple rodent behavioural tests that are commonly used for initial screening of potential new antidepressants include the Forced Swim Test and Tail Suspension Tests (Castagne et al., 2011). Agents used to mimic depressive symptoms include cortisol, retinoic acid and pro-inflammatory cytokines. There are also chronic stress models involving both physical and psychosocial stresses (Krishnan and Nestler, 2011).

Studies of the role of microRNAs in HPA-axis dysregulation and stress have largely focused on Glucocorticoid receptor (GR) regulation, as well as the effects of early-life stress and other psychological stressors on microRNA function and expression. These are summarized in Table 1.2 below.

Author	Species/ Model	Tissue	Analysis	Key Findings
Uchida et al. (2008)	Fischer 344 rats; chronic RS; SH-SY5Y cells	PVN	RT-qPCR; northern blotting	• miR-18a ↓ GR protein in vitro • ↑ pre-miR-18a & ↑ mature miR-18a in F344 rat PVN • ↓ GR protein in F344 rat PVN
Vreugdenhil et al. (2009)	Long-Evans rats; NS1, A549, COS-1 cells	Frontal cortex, hippo-campus	RT-qPCR	• miR-18 & miR-124a ↓ GR-mediated events, ↓ GR protein levels
Meerson et al. (2010)	Male rats; acute/ chronic RS	Amygdala hippo-campal CA1	Micro-array; RT-qPCR	• Acute RS amygdala: ↑ miR-106b, miR-134, miR-183, miR-382; ↓ let-7a-1, miR-202, miR-361, miR-376b, miR-381, miR-9-1 • Acute RS hippocampus: ↑ miR-1-2, miR-376b, miR-182*, miR-424, miR-190, miR-19a, miR-208, miR-216, miR-32; ↓ let-7f-2, miR-124a-1, miR-138-1. miR-15b, miR-202, miR-422a, miR-9-1 • Chronic RS amygdala: ↑ miR-1-2, miR-15a, miR-190, miR-193, miR-208, miR-22, miR-322, miR-361, miR-369, miR-376b, miR-381; ↓ let-7a-1, let-7c, let-7f-1, let-7f-2, miR-103-1, miR-134, miR-138-1, miR-182, miR-216, miR-222, miR-298, miR-323, miR-34a, miR-368, miR-9-1, miR-96 • Chronic RS hippocampus: ↑ miR-132, miR-17-5p, miR-208, miR-23a, miR-369, miR-376b, miR-410; ↓ let-7c, let-7f-1, miR-100, miR-134, miR-148a, miR-16-1, miR-182*, miR-219-1, miR-22, miR-221, miR-30a-3p, miR-330, miR-376a, miR-9-1, miR-96 • miR-134 & miR-183 altered by RS

Kawashima et al. (2010)	Rat primary cortical cultures	-	RT-qPCR	• BDNF ↑ miR-132 in vitro • Exogenous ds-miR-132 ↑ postsynaptic proteins (NR2A, NR2B, GluR1) • Dexamethasone ↓ BDNF-induced miR-132 expression & postsynaptic proteins
Rinaldi et al. (2010)	Male CD1 mice; acute/ chronic RS	Frontal cortex	Micro-array; northern blotting	• Acute RS: ↑ miR-9, miR-9*, miR-26b, miR-29b, miR-29c, miR-30b, miR-30c, miR-30e, miR-125a, miR-126-3p, miR-129-3p, miR-207, miR-212, miR-351, miR-487b, miR-690, miR-691, miR-709, miR-711, let7a-e; ↓ miR-423 & miR-494 • Repeated RS: ↑ miR-29b, miR-29c, miR-129-3p, miR-207, miR-212, miR-351, miR-423, miR-487b, miR-494, miR-690, miR-691, miR-709, miR-711; ↓ miR-9, miR-9*, miR-26b, miR-30b, miR-30c, miR-30e, miR-125a, miR-126-3p, let7 a-e
Uchida et al. (2010)	Sprague-Dawley rats; MD	MPC	RT-qPCR; northern blotting	• MD ↑ pre-miR-132,-124-1, 9-1, -9-3, -212, -29a • MD ↑ RE-1-containing genes - *Glur2*, *Nr1*, *CamKIIa, L1, Adcy5, Kcnc1* • MD ↑ mature miR-132, -124, -9 and -29a • REST4 overexpression in neonatal mice ↑ pre-miR-132,-212 & -9-3 & ↑ *CamKIIa, Glur2, Adcy5, Nr1, Crh, 5htr1a*
Bai et al. (2012)	Male Sprague-Dawley rats; MD	Hippo-campus	RT-qPCR	• MD ↓ BDNF • MD ↑ miR-16 • BDNF positively correlates with depressive-like behaviours in FST & OFT • miR-16 negatively correlates with depressive-like behaviours in FST & OFT
Rodgers et al. (2013)	C57BL/6:129 F1 hybrid mice; CUPS of sires	Sperm	Micro-array	• Paternal stress ↑ miR-29c, miR-30a, miR-30c, miR-32, miR-193-5p, miR-204, miR-375, miR-532-3p, miR-698 • mRNA targets: *DNMT3a, Tnrc6b, Mtdh* • Offspring show ↓ HPA-axis

	during puberty or adulthood			stress response
Cao et al. (2013)	Sprague-Dawley rats; CUPS and MS	Hippo-campus	Micro-array	• ↓miR-298, miR-130b, miR-135a, miR-323, miR-503, miR-15b, miR-532, miR-125a • ↑miR-7a, miR-212, miR-124, miR-139, miR-182 • miR-125a, miR-182 normalised following Chaihu Shugan San treatment
Zhang et al. (2013b)	Sprague-Dawley rats; MD, CUPS	Nucleus accumbens	RT-qPCR	• MD + CUPS ↑ miR-504 • ↑ miR-504 negatively correlates with *DRD2* mRNA expression • DRD2 mRNA and protein expression negatively correlates with immobility in FST
Zucchi et al. (2013)	Long-Evans rats; MS	Dams: frontal cortex; offspring: whole brain	Micro-array; RT-qPCR	• Dams: ↑ 147 miRNAs, ↓147 miRNAs; ↓miR-329, miR-380, miR-20a, miR-500, let-7c, miR-23b, miR-181, miR-186; ↑ miR-24-1 • Offspring: ↑ 205 miRNAs, ↓ 131 miRNAs; • ↓ miR-361, miR-17-5p, miR-425, miR-345-5p, miR-505, miR-103, miR-151, miR-145; ↑ miR-23a, miR-129-2, let-7f, miR-98, miR-9, miR-216-5p, miR-667, miR-219-2-3p, miR-323
Smalheiser et al. (2014a)	Male Sprague-Dawley rats; LH model; enoxacin treatment	Frontal cortex	RT-qPCR	• Enoxacin ↓ LH behaviour following inescapable shock • Enoxacin ↑ let-7a, miR-124, miR-125a-5p & miR-132 in frontal cortex

Issler et al. (2014)	5HT neuronal cultures; male C57BL/6 mice	-	Micro-array; RT-qPCR	• 5HT neurons: ↑ miR-375, miR-376c, miR-7a, miR-137, mghv-miR-M1-2, miR-709, miR-291b-5p, miR-1224, miR-1892, miR-702, miR-139-3p, miR-762, miR-671-5p, miR-483*; ↓ miR-691, miR-466l, miR-17, miR-376b, miR-124, miR-218, miR-128, miR-140*, miR-148a, miR-340-5p, miR-181c, miR-210, miR-135a, miR-27a, miR-452, miR-370, miR-300, miR-376a, miR-127, miR-15b, miR-101a, miR-16, miR-324-5p, miR-434-5p, miR-92a, miR-669i, • miR-135a ↑ by SSRI antidepressants • miR-135a overexpression in 5HT neurons ↓ anxiety- & depression-like behaviours in mice • Knockdown of miR-135a ↑ anxiety- & depression-like behaviours in mice
Zurawek et al. (2016)	Male Wistar rats; CMS	VTA, nucleus accumbens, hippo-campus, MPC, Blood	RT-qPCR	• ↑serum and VTA miR-16 in stress-resilient rats ↓miR-16 in MPC in stress-resilient rats
Dwivedi et al. (2015)	Male Sprague-Dawley rats; CORT model	Pre-frontal cortex	Micro-array, RT-qPCR	• Chronic CORT ↑miR-19b, miR-29c, miR-101a, miR-124, miR-137, miR-153, miR-181a/c, miR-203, miR-218, miR-324-5p, miR-365, miR-409-5p, miR-582-5p, miR-29a, miR-30e, miR-721, miR-699 • Chronic CORT ↓miR-146a, miR-200c, miR-351, miR-155, miR-678, miR-764-5p, miR-135a-3p

Rodgers et al. (2015)	C57/Bl6: 129S6/ SvEvTac hybrid mouse zygotes; RS	PVN	RT-qPCR	• miR-29c, miR-30a, miR-30c, miR-32, miR-193-5p, miR-204, miR-375, miR-532-3p, miR-698 microinjected into zygotes led to same phenotype as paternal-stress model
Chen et al. (2015a)	Male Sprague-Dawley rats; Chronic social defeat stress	MPC, blood	RT-qPCR	• ↓Circulating miR-24-2-5p, miR-27a-3p, miR-30e-5p, miR-3590-3p, miR-362-3p, miR-532-5p in stress-vulnerable rats • ↓Circulating miR-139-5p, miR-28-3p, miR-326-3p, miR-99b-5p in stress-resilient rats • ↑miR-126a-3p, miR-708-5p in MPC of vulnerable rats
Li et al. (2016)	Male Wistar rats; CUPS	Hippo-campus	RT-qPCR	• CUMS ↓BDNF and ↑miR-182 • Injection of BDNF and miR-182 silencer led to anti-depressant like effect • miR-182 ↓ BDNF protein in hippocampus • *BDNF* a functional target of miR-182

Table 1.2 Pre-clinical studies of microRNAs in stress.

Abbreviations: PVN= paraventricular nucleus; GR= glucocorticoid receptor; RT-qPCR= real-time quantitative polymerase chain reaction; BDNF= brain derived neurotrophic factor; MPC= Medial prefrontal cortex; RS= restraint stress; MD= maternal deprivation; LH= learned helplessness; FST= forced swim test; OFT= open field test; HPA= hypothalamic-pituitary-adrenal axis; MS= maternal separation; CUPS= chronic unpredictable stress; CMS Chronic mild stress; VTA= ventral tegmental area; CORT= exogenous corticosterone.

Two studies have identified miR-18 as potential regulator of GR function. In the first, miR-18 was found to inhibit GR mRNA translation. Following repeated restraint stress, miR-18 was also increased in the paraventricular nucleus (PVN) in a strain of rats hyper-responsive to stress (Uchida et al., 2008). MiR-18 and miR-124a were subsequently shown to reduce GR protein levels and reduce GR mediated events in a different strain of rat (Vreugdenhil et al., 2009).

MicroRNAs have been identified as a potential mediator of glucocorticoid induced neuronal atrophy. BDNF upregulates levels of miR-132 in cortical cultures, and miR-132 in turn can increase levels of glutamate receptor proteins such as NR2A, NR2B and GluR1 in postsynaptic neurons (Vo et al., 2005). This process is inhibited by the administration of dexamethasone (Kawashima et al., 2010).

The molecular changes brought about by stress on the microRNA system appear to vary temporally and spatially. Using similar models of restraint stress two different studies showed very little overlap in terms of microRNA expression change. One study was in the amygdala and hippocampus in rats (Meerson et al., 2010), while the other analysed changes in the frontal cortex of mice (Rinaldi et al., 2010). MicroRNA levels in both studies altered quickly before returning to baseline. A further study found microRNA changes in opposite directions between the ventral tegmental area and the prefrontal cortex (Zurawek et al., 2016). The lack of overlap and in some cases, opposite directionality, suggests that different brain regions respond differently to stress.

Stress in early life as well as antenatal parental stress is linked to the development of later depression-like and anxiety-like behaviour in offspring. Epigenetic mechanisms, like microRNAs, appear to play a role in this. Several studies have examined the effect of various early-life stresses on microRNAs with little overlap or consensus findings. However, these studies have used differing brain regions, which may go some way to explain this. For example, two studies using a maternal deprivation (MD) model where young pups are separated from their mothers found little overlap in microRNA expression in the hippocampus and medial PFC respectively (Bai et al., 2012, Uchida et al., 2010). Parental stress can also lead to alterations in microRNA levels. Maternal stress, where pregnant rats are

subjected to late-gestation stress, led to microRNA changes in both mothers and pups, as well as disrupting normal maternal behaviour in mothers (Zucchi et al., 2013). The microRNAs identified have been predicted to target genes involved in neurotransmission, neurodevelopment, cell signalling and stress response. This suggests that antenatal stress can lead to epigenetic modifications affecting both mother and offspring later in life. Exposure to paternal stress can also lead to changes in microRNA levels in mouse sperm, and intrazygotic injection of microRNAs altered by paternal stress has been shown to lead to the same phenotypic changes as paternal stress itself (Rodgers et al., 2013, Rodgers et al., 2015). A number of microRNAs were increased following paternal stress. Several of these microRNAs targeted a DNA methyltransferase critically involved in DNA methylation, one of the epigenetic mechanisms through which genes can be silenced. Offspring of these mice also had a blunted HPA-axis response to stress. Together, these findings indicate that early life stress leads to microRNA changes that may affect behaviour at the time, but also can lead to a heightened vulnerability to the effects of stress later in life (Zhang et al., 2013b).

1.3.4.2 Preclinical studies of microRNAs and depression

A handful of studies (see Table 1.3 below) to date have examined the relationship between microRNAs and depressive-like behaviour in animal models without any clear consensus as a result. Using sucrose preference as a proxy measure of anhedonia in a maternal deprivation model, let-7a upregulation was associated with anhedonia and a downregulation of the serotonin receptor-4 (Htr4) (Bai et al., 2014). These correlations were not seen for other stress models. This may indicate that different stressors have different molecular mechanisms.

The learned helplessness model (LH) is an animal model of stress-induced behavioural depression involving inescapable electric shocks (Czeh et al., 2016). In a study of LH and non-LH rats, LH rats had a blunted microRNA response in the frontal cortex. The microRNAs that were blunted were identified as regulators of Creb1, which in non-LH rats may form part

of an adaptive response to stress (Smalheiser et al., 2011). In a follow-up study, the same group showed that the antibiotic enoxacin reduced LH behaviour to inescapable shock. Enoxacin also increased levels of let-7a, miR-124, miR-125a-5p and miR-132 in the frontal cortex (Smalheiser et al., 2014b). Elsewhere, increased levels of miR-124a, a *BDNF*-targeting microRNA, were found after exposure to social defeat stress in the hippocampi of rats. Overexpression of miR-124a led to an increase in depression-like behaviour whilst knockdown of miR-124a lessened it (Bahi et al., 2014).

Author	Species/ model	Tissue	Analysis	Main Findings
Depressive-like behaviours				
Smalheiser et al. (2011)	Male Holtzman rats; LH	Frontal cortex	Micro-array	• LH ↑ miR-200b, miR-300, miR-miR-184, miR-106b*, miR-297a*, miR-136*, miR-496, miR-211, miR-214*, miR-369-3p, miR-18a*, miR-466d-3p, miR-467a*, miR-376a*, miR-142-3p, SNORD65, miR-22, miR-181a-1*, miR-29c*, miR-376a; ↓ miR-384-5p, miR-350
Bahi et al. (2014)	Male Wistar rats; Social defeat stress	Frontal cortex, hippo-campus	RT-qPCR	• Social defeat stress ↓ *BDNF* mRNA & ↑ miR-124a in hippocampus but not frontal cortex • Overexpression of hippocampal miR-124a ↑ depressive-like behaviour • Knockdown of miR-124a ↓ depressive-like behaviour
Bai et al. (2014)	Male Sprague Dawley rats; MD vs. CUPS	Hippo-campus	RT-qPCR; western blotting	• MD but not CUPS ↑ let-7a • Let-7a negatively correlates with sucrose preference rate and hippocampal Htr4 mRNA & protein
Yi et al. (2014)	Male ICR mice; CUPS; oleanolic acid	Hippo-campus	RT-qPCR; western blotting	• CUMS ↓sucrose preference • oleanolic acid ↑miR-132 and reversed ↓sucrose preference • miR-132 activates hippocampal BDNF signalling
Li et al. (2016)	Male Wistar rats; CUPS	Hippo-campus	RT-qPCR	• CUMS ↓BDNF protein and ↑miR-182 • Injection of BDNF and miR-182 silencer led to anti-depressant like effect • miR-182 downregulated BDNF protein in hippocampus • *BDNF* a functional target of miR-182

Antidepressants				
Baudry et al. (2010)	1C11 neuro-ectodermal cell line; Male Swiss-Kumming mice; Flx treatment	-	RT-qPCR	• ↑ miR-16 in NA neurons but not 5HT neurons • ↓ miR-16 in NA neurons ↑ SERT • Flx ↑ miR-16 & ↓ SERT in raphe nuclei in vivo • S100β ↓ miR-16 & ↑ SERT in NA neurons & in vivo • Overexpression of miR-16 in vivo ↓ depressive-like behaviours following CUPS
Launay et al. (2011)	Male Swiss-Kumming mice; Flx treatment	Hippo-campus	RT-qPCR	• Flx ↓ depressive-like behaviours following CUPS, ↓ hippocampal miR-16, ↑ SERT, ↑ neurogenesis • Knockdown of hippocampal miR-16 ↓ depressive-like behaviours • BDNF, Wnt2 & PGJ2 act synergistically to ↓ miR-16 & ↑ SERT in hippocampus
O'Connor et al. (2013)	Male Sprague-Dawley rats; MD; Flx, Ket or ECS treatment	Hippo-campus	Micro-array	• Flx, ECS & Ket ↑ miR-598-5p using microarray in non-stress animals • Flx & Ket ↑ miR-598 using RT-qPCR in non-stress animals • MD ↓ miR-451 using microarray and RT-qPCR • Flx, ECS & Ket reverse MD-induced ↓ miR-451 using microarray • Flx reverse MD-induced ↓ miR-451 using RT-qPCR • miR-451 mRNA targets: CREB5, GABAa receptor associated protein, muscarinic cholinergic receptor 5
Ryan et al. (2013)	Male Sprague-Dawley rats; acute and chronic ECS	DG, frontal cortex, cere-bellum, whole blood	RT-qPCR	• ↑ miR-212 in DG following both acute & chronic ECS • Positive correlation between miR-212 in DG & whole blood following chronic ECS

Yang et al. (2014)	Male Sprague-Dawley rats; primary hippo-campal neuronal cultures; Ket treatment	Hippo-campus	Micro-array	• Ket ↑ miR-30e-5p, miR-218a-5p, miR-181a-5p, miR-181c-5p, miR-136-5p, miR-487b-3p, miR-132-3p, miR-345-5p, miR-598-3p, miR-98-5p, miR-221-3p, miR-138-5p, miR-219a-5p, miR-495, miR-497-5p, miR-99a-5p, miR-29c-3p, miR-124-5p, let-7c-5p, miR-29a-3p, miR-488-3p, miR-365-3p; ↓ miR-150-5p, miR-344b-1-3p, miR-299a-5p, miR-206, miR-103-1-5p, miR-344b-2-3p, miR-935, miR-132-5p, miR-340-3p, miR-465-3p, miR-3557-5p, miR-22-5p, miR-485-3p, miR-1839-3p, miR-3568, miR-221-5p, miR-214-3p, miR-3596c • miR-206 ↓ following Ket • miR-206 overexpression attenuates Ket-induced ↑ BDNF
Liu et al. (2015)	Mice; CUPS; 7-CTKA	Hippo-campus	RT-qPCR, micro-array	• miR-34a-5p, miR-200a-3p, miR-144-3p, miR-1894-5p altered by CUPS • Increased sucrose preference following 7-CTKA treatment

Table 1.3 Pre-clinical studies of microRNAs and depression.

Abbreviations: Flx= fluoxetine; NA= noradrenergic; 5HT= serotonergic; SERT= serotonin transporter; Li= lithium; VPA= valproate; Ket= ketamine; RT-qPCR= real-time quantitative polymerase chain reaction; TST= tail suspension test; CMS= chronic mild stress; VEGF= vascular endothelial growth factor; BDNF= brain derived neurotrophic factor; ECS= electroconvulsive stimulation; MD= maternal deprivation; EE= environmental enrichment; DG= dentate gyrus; CUPS= chronic unpredictable stress; Htr4= serotonin receptor 4; LH= learned helplessness ; 7-CTKA= 7-Chloro-kynurenic acid.

Initial evidence for the interaction between depression and microRNAs came from two important studies of the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Baudry et al., 2010, Launay et al., 2011). Treatment with SSRIs typically requires weeks before symptomatic relief is achieved, suggesting that changes to serotonin signalling and downstream cascades are necessary for antidepressant action. In these studies, miR-16 was initially identified as a regulator of the serotonin transporter (SERT) through bioinformatic analysis. Using human neuroectodermal cell lines (IC11) and *in vivo* study of the raphe nuclei of mice, the authors showed that administration of fluoxetine led to increased levels of miR-16, previously blocked by Wnt signalling (Baudry et al., 2010). Increased miR-16 levels resulted in decreased serotonin transporter levels, with a net effect of raised serotonin signalling at the synapse. Also, miR-16 induced an adaptational change from noaradrenergic to serotonergic type in IC11 cells in locus coeruleus neurons. The authors also found that miR-16 altered depressive phenotypes in mice. Following on from this study, the same group examined the effect of miR-16 on murine hippocampal neurogenesis (Launay et al., 2011). Although fluoxetine increases miR-16 maturation in the raphe nuclei, it decreased miR-16 levels in the hippocampus and locus coeruleus. BDNF, the signalling protein Wnt2 and the anti-inflammatory prostaglandin 15d-PGJ2 mediated these changes.

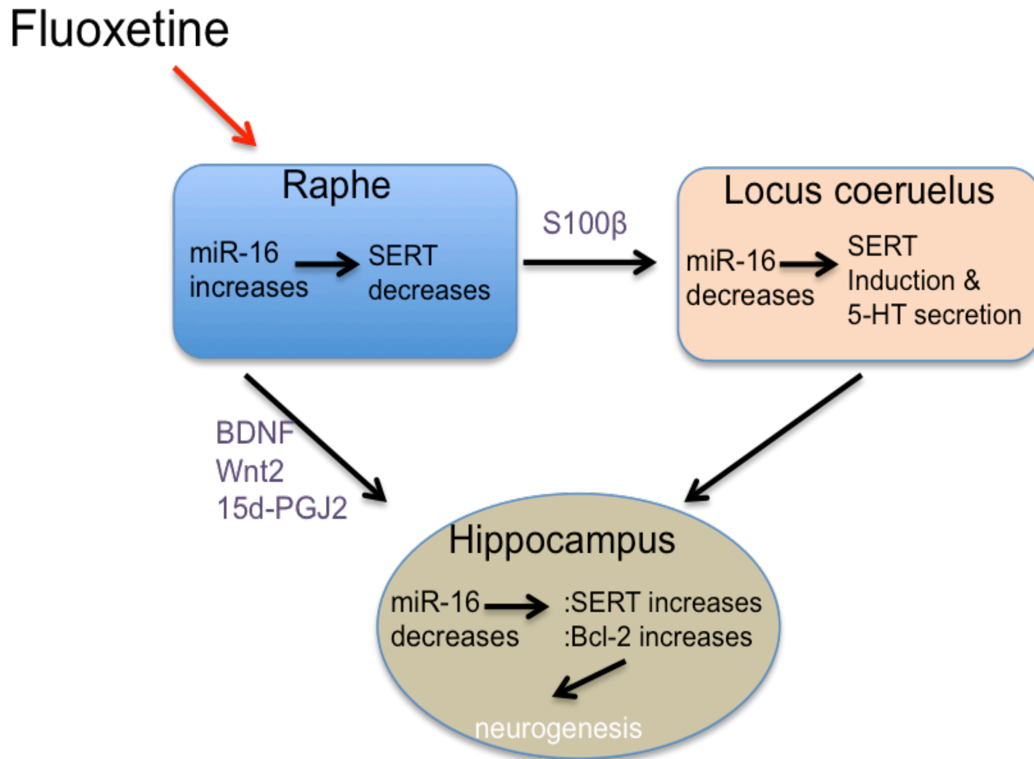


Figure 1.12 Fluoxetine mediated neurogenesis involves miR-16

Elsewhere, miR-182 has been identified as a potential negative regulator of *BDNF* in the hippocampus (Li et al., 2016). In this study, miR-182 was investigated using both a gain and loss of function approach. Virally-mediated overexpression of miR-182 led to a strengthening of stress-induced behaviours such as eating delay, decreased sucrose preference and performance on the forced swim test. These findings were reversed when miR-182 was silenced.

The effect of ketamine and ECS on microRNAs has also been investigated in separate studies (O'Connor et al., 2013, Ryan et al., 2013, Yang et al., 2014). In rat dentate gyrus, ECS was shown to increase levels of miR-212, which is associated with BDNF (Ryan et al., 2013). There was also a positive association between miR-212 levels in whole blood and in the dentate gyrus, which offers the possibility that monitoring microRNA levels peripherally may reflect microRNA levels in the brain. One further study using ECS, ketamine and fluoxetine in rats found that miR-451 reversed changes induced by a maternal deprivation model of depression (O'Connor et al., 2013). This microRNA is predicted to target genes in the CREB, GABA-ergic and cholinergic pathways. MiR-451 was however, not

identified in a screening study of microRNA changes in the hippocampus following treatment with ketamine (Yang et al., 2014). Another BDNF regulating microRNA, miR-206 was downregulated by ketamine, with an associated upregulation of BDNF, potentially identifying the mediating process between ketamine and its antidepressant effect. Another NMDA receptor antagonist is 7-Chloro-kynurenic acid (7-CTKA). This is also reported as having rapid antidepressant action, and in a mouse model reversed decreased sucrose preference following chronic unpredictable stress (CUPS) and mediated changes in microRNA levels in the hippocampus (Liu et al., 2015).

1.3.4.3 Clinical studies of microRNAs in depression

Research investigating microRNA involvement in depressive disorder is rapidly gathering pace with a number of clinical studies in recent years (see Table 1.4). A number of studies have investigated microRNA expression in the brain (Smalheiser et al., 2012, Smalheiser et al., 2014a, Lopez et al., 2014a, Issler et al., 2014, Lopez et al., 2014b, Maheu et al., 2015, Kohen et al., 2014). Others have focused on peripheral sources of microRNA, typically in the search of a biomarker for depression. These include studies investigating microRNA expression in cerebrospinal fluid (CSF) (Wan et al., 2015, Song et al., 2015, Launay et al., 2011), blood (Bocchio-Chiavetto et al., 2013, Belzeaux et al., 2012, Issler et al., 2014, Li et al., 2013, Lopez et al., 2014b, Wan et al., 2015, Song et al., 2015, Fan et al., 2014, Camkurt et al., 2015) and dermal fibroblasts (Garbett et al., 2015). Searching for rare variants that may be associated with depression has also yielded interesting results (Saus et al., 2010, Jensen et al., 2014, Guintivano et al., 2014, Xu et al., 2010b, He et al., 2012).

Author	Patients	Tissue	Analysis	Main findings
Post-mortem case-control brain studies				
Smalheiser et al. (2012)	Suicides=18 Controls=17	PFC (BA 9)	qRT-PCR	miR-142-5p, miR-137, miR-489, miR-148b, miR-101, miR-324-5p, miR-301a, miR-146a, miR-335, miR-494, miR-20a/b, miR-376a, miR-190, miR-155, miR-660, miR-130a, miR-27a, miR-497, miR-10a, miR-142-3p downregulated. Targeted genes/gene pathways: *CDK6, ELF1/6, NCOA2, DNMTB3, EZH2, MYCN, ICOS, SOX4, PTPRN2, MERTK, VEGFA, SLC16A1, SFRS11, TTK, AGTR1, BACH1, LDOC1, MATR3, TM6SF1, TAC1, CSF1, MAFB, MEOX2, HOXA1/5, SP1/3/4, RUNX1*
Smalheiser et al. (2014a)	Scz=15 BPAD=15 Dep=15 Controls=15	PFC (BA 10)	qRT-PCR	miR-508-3p and miR-152-3p downregulated
Lopez et al. (2014a)	Dep=15 Controls=16	PFC (BA44)	qRT-PCR	miR-34c-5p, miR-139-5p, miR-195, miR-320c upregulated Targeted genes/gene pathways: *SAT1, SMOX*
Lopez et al. (2014b)	Dep=64 Controls=40	PFC (BA44)	Micro-array qRT-PCR	miR-1202 downregulated Targeted genes/gene pathways: *GRM4*
Issler et al. (2014)	Dep=6 Control=11	Raphe nuclei	qRT-PCR	miR-135a, miR-16 downregulated
Kohen et al. (2014)	Scz=17 BPAD=16 Dep=17 Controls=29	Dentate gyrus	Deep sequencing	miR-182 signalling disrupted in MDD and schizophrenia.
Maheu et al. (2015)	Dep=16 Control=21	BLA	qRT-PCR	GDNF family receptor alpha 1 mRNA downregulated miR-511 upregulated

Peripheral tissue studies				
Launay et al. (2011)	MDD=9	CSF	qRT-PCR	Following fluoxetine administration, miR-16 targeting molecules BDNF, Wnt2, 15d-PGJ2 levels increased in CSF. Targeted genes/gene pathways: miR-16: *SERT, Bcl-2*
Bocchio-Chiavetto et al. (2013)	MDD=10	Whole blood	qRT-PCR	miR-130b*, miR-505*, miR-29-b-2*, miR-26a/b, miR-22*, miR-664, miR-494, let7d/e/f/g, miR-629, miR-106b*, miR-103, miR-191, miR-128, miR-502-3p, miR-374b, miR-132, miR-30d, miR-500, miR-589, miR-183, miR-574-3p, miR-140-3p, miR-335, miR-361-5p upregulated miR-34c-5p, miR-770-5p downregulated Targeted genes/gene pathways: *BDNF, NR3C1, NOS1, IGF1, FGF1, FGFR1, VEGFA, GDNF, CACn41C, CACNB4, SLC6A12, SLC8A3, GABRA4, 5HT-4*. Neuroactive ligand-receptor interaction, axon guidance, LTP, signalling pathways
Belzeaux et al. (2012)	MDD=9 Controls=9	PBMCs	Microarray qRT-PCR	miR-941, miR-589 upregulated
Li et al. (2013)	MDD=40 Controls=40	Serum	qRT-PCR	miR-132, miR-182 upregulated Targeted genes: *BNDF*
Lopez et al. (2014b)	MDD=32 Controls=18	Whole blood	qRT-PCR	miR-1202 downregulated
Issler et al. (2014)	MDD=11 Controls=12	Whole blood	qRT-PCR	miR-135a downregulated

Garbett et al. (2015)	MDD=16 Controls=16	Dermal fibroblasts	PCR array	miR-132, miR-421, miR-542, miR-450a, miR-16-2*, miR-424, miR-628-3p, miR-629-5p, miR-4293, miR-661, miR-3909, miR-33a*, miR-135b, miR-7, miR-4267, miR-548a-3p, miR-548d-3p, miR-613, miR-3714, miR-1294, miR-429 upregulated miR-122, miR-32, miR-196b*, miR-377, miR-193a-3-, miR-337-5p, miR-675*, miR-3176, miR-21*, miR-22, miR-425*, miR-185, miR-296-5p, miR-103a, miR-107, miR-186, miR-887 downregulated
Fan et al. (2014)	MDD=81 Controls=46	PBMCs	Microarray qRT-PCR	miR-26b, miR-1972, miR-4485, miR-4498, miR-4743 upregulated
Wan et al. (2015)	*Pilot* MDD=6 Controls=6 *Validation* MDD=32 Controls=21	CSF & Serum Serum	PCR Panel qRT-PCR	4/16 microRNAs differentially expressed in CSF followed same pattern in serum miR-34a-5p, miR-221-3p, let-7d-3p upregulated miR-451a downregulated Targeted genes: *AKT, 5HT2A, HTR2C, CRHR1, SCL1A2*
Song et al. (2015)	MDD=36 Controls=30	CSF and Whole blood		CSF levels of miR-16 downregulated, correlated to HDRS-24 scores No significant differences or correlations in blood
Camkurt et al. (2015)	MDD=50 Controls=41	Plasma	qRT-PCR	miR-320a downregulated miR-451a, miR-17-5p, miR-223-3p upregulated Targeted genes: *SCL17A7, GRIN2A, DISC1*

Genotyping studies				
Xu et al. (2010b)	MDD=1088 Controls=1102	DNA	miR-SNP	Positive association between SNP in miR-30e precursor and MDD
He et al. (2012)	MDD=314 Controls=252	DNA	miR-SNP	SNP in miRNA processing gene *DGCR8* increased frequency SNP in miRNA processing gene *AGO1* decreased frequency. Associated with suicide risk and treatment response
Jensen et al. (2014)	6725 subjects	DNA	Microarray	Association between SNP in target site of miR-330-3p in MDD
Guintivano et al. (2014)	**PM Brains** MDD=29 BPAD=40 Scz=29 Controls=70 **Blood** MDD=75 BPAD=15 Controls=308	DNA (PM Brain, Blood)	Microarray, pyrosequencing	Association between SNP in *SKA2* and suicide, possibly mediated by miR-301a

Table 1.4 Clinical studies of microRNAs in depression.

Up/down-regulation in all studies refers to cases (depressed) compared to controls. Abbreviations: BA= Brodmann's Area; BLA= Basolateral amygdala; BPAD= Bipolar affective disorder; DLPFC= Dorsolateral prefrontal cortex; HDRS-24= 24-item Hamilton Depression Rating Scale; LTP= Long-term potentiation; MDD= Major depressive disorder; PBMCs= Peripheral blood mononuclear cells; PFC= Prefrontal cortex; PM= post-mortem; qRT-PCR= Quantitative reverse transcriptase polymerase chain reaction; Scz=Schizophrenia; SNP= Single-nucleotide polymorphism.

1.3.4.3.1 Post-mortem studies

The first study to examine microRNA expression levels in human brain was in a sample of 18 antidepressant-free suicide and 17 matched non-psychiatric controls (Smalheiser et al., 2012). Using multiplex PCR the

authors found a downregulation of 21 microRNAs. Validated predicted targets of these microRNAs included *VEGFA*, B-Cell CLL/Lymphoma 2 (*BCL-2)* and DNA (Cytosine-5-)-Methyltransferase 3 Beta (*DMNT3B*), but when their respective protein levels were measured in the same cohorts, only DMNT3B was significantly up-regulated.

The same group went on to study the expression of microRNAs in a separate set of brain samples, along with patients with schizophrenia, BPAD and healthy controls (Smalheiser et al., 2014a). MiR-508-3p and miR-152-3p were both significantly downregulated but no correction for multiple testing was carried out so the results should be interpreted with some caution.

Building on previous work identifying the role of the polyamine genes Spermidine/Spermine N1-Acetyltransferase 1 (*SAT1)* and spermine oxidase (*SMOX)* in suicidal behaviour, Lopez et al. (2014a) investigated prefrontal cortex (PFC) levels of microRNAs predicted to target these genes. Four microRNAs were upregulated in suicide completers. Two of these, miR-34c-5p and miR-320c, had a significant negative correlation with mRNA levels of *SAT1*, and miR-139-5p and miR-320c had a significant negative correlation with *SMOX* mRNA levels. The protein products of these mRNA transcripts were not measured, and multiple comparison correction was omitted.

The same group went on to examine microRNA expression in a larger sample of brains from the Douglas-Bell Canada Brain Bank in Quebec (Lopez et al., 2014b). A microarray based approach tested for 866 microRNAs and found miR-1202 to be significantly decreased in the PFC of 14 depressed subjects compared to 11 controls. This microRNA is brain-enriched, and is only present in humans and primates. These findings were further validated with qRT-PCR in a sample of depressed subjects with a history of antidepressant use and controls. Interestingly, miR-1202 expression in those who had a history of antidepressant use was significantly different from those not exposed to antidepressants, with expression levels more similar to healthy controls. Glutamate Receptor, Metabotropic 4 (*GRM4)*, a glutamate receptor-encoding gene, was predicted in-silico to be targeted by miR-1202. *GRM4* levels were increased in these brain samples and negatively correlated with miR-1202 levels.

The neurotrophic factor GDNF has been identified as having a potential regulatory role in affective disorders, and its expression in serum is increased by ECT (Zhang et al., 2009). In 16 depressed subjects who had died from suicide, mRNA levels of one of the isoforms of GDNF receptors, but not *GDNF* itself, were decreased compared to 21 controls (Maheu et al., 2015). Conversely, miR-511, which was predicted to target this receptor was upregulated.

A final study, which initially investigated the role of miR-135 in mouse models went on to investigate levels of miR-135 and microRNA-16 in various subnuclei of the raphe in the brains of depressed suicide victims and controls (Issler et al., 2014). Significantly lower levels of miR-135 and miR-16 were found in the dorsal raphe and raphe magnus compared to controls.

Post-mortem studies have significant limitations, including the use of different brain regions, differences in post-mortem intervals, a lack of consensus as to the best statistical significance testing approach, correction for multiple testing, normalisation strategy and what endogenous controls are best suited to this type of analysis (Liu et al., 2014). To date, results from post-mortem studies in depressed cohorts suffer from the same lack of consistency of findings that has been observed to date in post-mortem studies of other psychiatric disorders (Kolshus et al., 2014).

1.3.4.3.2 Peripheral tissue studies

Correlating microRNA level changes before and after treatment and matching them to clinical outcomes offers an exciting potential for biomarkers as well as the molecular basis of depression. Initial work using CSF had highlighted that targets of miR-16, previously implicated in the action of the antidepressant fluoxetine (Baudry et al., 2010), were increased following fluoxetine treatment (Launay et al., 2011). This highlighted the translational potential of microRNAs.

The first study investigating the effect of blood microRNA changes following treatment involved 10 treatment-naïve depressed patients who showed a good response to twelve weeks of the antidepressant escitalopram (Bocchio-Chiavetto et al., 2013). Thirty microRNAs were significantly altered, and many of these are important gene expression

regulators in the brain and have been implicated in other psychiatric disorders.

A further study investigating transcriptional signatures at different time points in depression identified two microRNAs that were upregulated at remission compared to healthy controls (Belzeaux et al., 2012). A panel of eight microRNAs was also identified that matched clinical response. Another attempt at identifying a blood-based microRNA signature for depression identified five different microRNAs, none of which previously have been reported in the depression literature (Fan et al., 2014). Of note, neither of these studies corrected for multiple testing, despite screening for a large number of microRNAs.

Li et al. investigated levels of two microRNAs that are thought to regulate BDNF, miR-132 and miR-182 (Li et al., 2013). In depressed patients, compared to controls, BDNF serum protein levels were lower, and miR-132 and miR-182 were upregulated. However, only miR-132 was significantly negatively correlated with BDNF levels. Of note, there was a significant positive correlation between the levels of these microRNAs and scores on a self-rating scale of depression, but no correction for multiple testing was performed.

In the previous section, the involvement of miR-1202 in depression based on post-mortem findings was discussed (Lopez et al., 2014b). In a further experiment, miR-1202 blood levels were measured before and after treatment with the antidepressant citalopram. Compared to controls, miR-1202 levels were downregulated, matching the results seen in post-mortem brain samples. Furthermore, after classifying the patients into those who remitted, and those who did not, the lower miR-1202 levels were specific to remitters, indicating the potential of miR-1202 to identify those who will respond to citalopram treatment.

In a similar vein, researchers built on earlier work investigating the role of miR-135 and miR-16 in mouse models and went on to measure their levels in brain samples and in human blood (Issler et al., 2014). Compared to controls, miR-135a levels were significantly decreased. Of note, after three months of cognitive behavioural therapy (CBT) there was a significant increase in blood miR-135a levels compared to patients receiving the antidepressant escitalopram. Supporting earlier work regarding miR-16, a

further study found baseline CSF levels of miR-16 in depressed subjects to be significantly higher than in healthy controls, although blood levels did not reveal any significant differences (Song et al., 2015). This would suggest that blood levels of microRNAs do not always reflect CSF levels, and indeed a recent study found that of 16 microRNAs significantly dysregulated in depressed subjects, only four were also dysregulated in the serum of the same subjects (Wan et al., 2015). A further study of 50 depressed patients and 41 controls which included miR-16 as one of eight candidate microRNAs also failed to find any significant difference in miR-16 levels in plasma between depressed patients and controls (Camkurt et al., 2015).

Finally, both mRNA and microRNA levels in dermal fibroblasts were investigated using a PCR array examining 1008 microRNAs (Garbett et al., 2015). 38 microRNAs were differentially expressed. Using microRNA-targeting software, 89% of these 38 differentially expressed microRNAs targeted mRNAs for binding (see Figure 1.13). These mRNAs were in turn differentially expressed in the same samples, indicating a close relationship between microRNA and mRNA networks. There was no correction for multiple testing, and culturing of fibroblasts represents a more time-consuming and delayed source of biomarkers in comparison to blood.

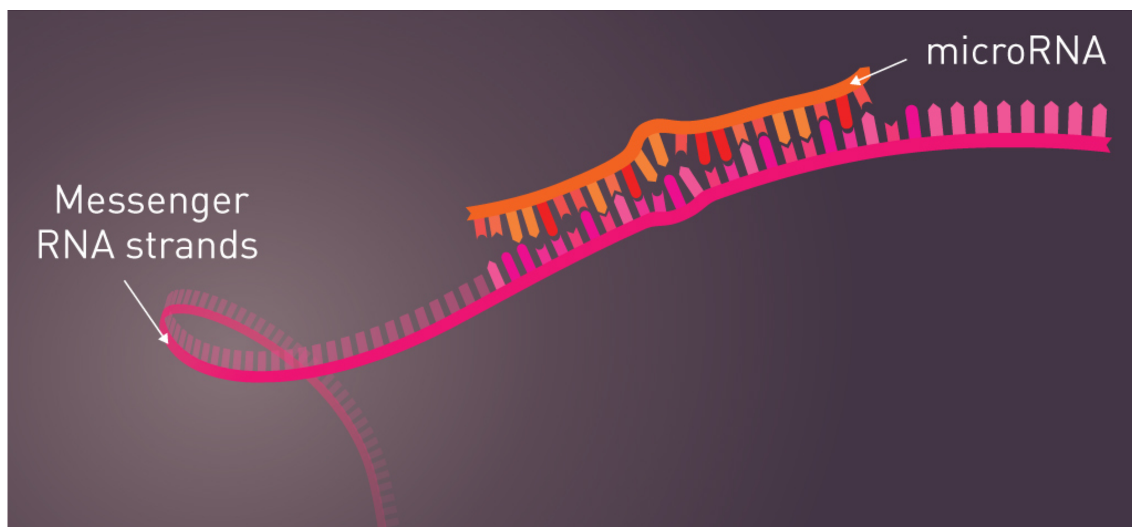


Figure 1.13 MicroRNA binding to a mRNA molecule.
Source: www.riken.jp - modification of original material by Kelvinsong (Licensed under CC BY 3.0).

These studies in general need to be validated, and many may have a high false discovery rate, with no correction for multiple testing performed. Some studies have investigated the role of specific microRNAs based on pre-clinical, in-silico or other tissues, such as miR-1202, miR-135a, miR-16 and miR-132. MiR-132 was also found to be upregulated in dermal fibroblasts in depressed patients, whereas the others have not been identified in general profiling studies. Questions also arise over whether changes in microRNA levels in the periphery give any helpful information about what is happening in the brain (Kolshus et al., 2014). However, as any clinically useful biomarker for depression is likely to come from these peripheral sources, continued efforts in this field would be welcomed.

1.3.4.3.3 Genotyping and rare variants

A number of studies have searched the human genome for single-nucleotide polymorphisms (SNPs) that may be associated with depression. One such large study of 1088 depressed patients and 1102 controls found a positive association between miR-30 and major depressive disorder (MDD) (Xu et al., 2010b). This was carried out in an ethnically homogenous Han Chinese population (Xu et al., 2010a).

A key symptom in affective disorders is disturbed sleep and disruption of circadian rhythms has been associated with depression (Germain and Kupfer, 2008). A SNP in miR-182 was found to be associated with late insomnia in 359 patients (341 controls) with MDD (Saus et al., 2010). Patients with this SNP had downregulated expression of genes previously associated with affective disorders and circadian rhythm such as *CLOCK* (Serretti et al., 2003).

SNPs in the genes involved in the microRNA processing machinery, like *DGCR8* and *AGO1*, were associated with increased risk of suicidal tendency and antidepressant treatment response in a sample of 314 patients and 252 controls (He et al., 2012).

Using previous GWAS data and microRNA target prediction software, a recent study identified a link between a miR-330-3p target site SNP (rs41305272) in mitogen-activated protein kinase 5 (*MAP2K5*) and restless

leg syndrome (Jensen et al., 2014). This disorder has been associated with anxiety (Sevim et al., 2004) and depression (Li et al., 2012b), and this SNP was therefore investigated in a separate GWAS dataset. This dataset consisted of 6725 unrelated drug-dependent subjects and non-drug dependent subjects recruited for a genetic study of dependence. There was an association between rs41305272 and depressive disorder (OR=2.64, p=0.01) in subjects of African-American descent. However, these results should be treated with caution, as comorbidity with other disorders was very common in this sample.

Finally, a study investigating epigenetic and genetic markers of suicide identified a potential role of miR-301a in suicide aetiology (Guintivano et al., 2014). Incorporating post-mortem and blood samples in various diagnostic groups and patient cohorts, the authors found an association between rs7208505, a SNP in a CpG site in the *SKA2* gene. In suicide cases, there was a higher level of DNA methylation of *SKA2*, as well as lower levels of *SKA2* gene expression, associated with the SNP. Further analysis of related regions identified a possible role for miR-301a in *SKA2* gene expression, albeit not in the suicide phenotype itself. MiR-301a has previously been found to be associated with suicide (Smalheiser et al., 2012).

1.3.4.3.4 Summary of microRNAs in depression

In summary, there has been a rapid growth in studies of microRNA involvement in depression as evidenced by expanding reviews in this field (Dwivedi, 2011, Mouillet-Richard et al., 2012, Maffioletti et al., 2014, Kolshus et al., 2014, Geaghan and Cairns, 2015, Dwivedi, 2016, Issler and Chen, 2015). Findings from preclinical studies are promising in terms of teasing out the contribution of microRNAs to depressive-like behaviours and the therapeutic antidepressant response. Moreover, the translational aspect of findings from preclinical studies is promising. However, despite a growth in microRNA studies in this field, there has been little progress in the way of validation and replication, and there are large variations in methodological approaches including normalisation strategies (Liu et al., 2014). It is encouraging to see studies beginning to explore correlations between the

brain and the periphery (Guintivano et al., 2014, Lopez et al., 2014b, Song et al., 2015). However, more work is clearly needed in this area in order to fully evaluate the role of microRNAs in depression and their potential to act as biomarkers for diagnosis and treatment.

1.3.5 MicroRNAs in other psychiatric disorders

MicroRNAs have been implicated in a broad range of psychiatric disorders (Kolshus et al., 2014). Their role has perhaps been best studied in schizophrenia although few have been replicated. Given microRNAs' role in brain development, from synaptic plasticity, neurogenesis and modulating neuronal function (Siegel et al., 2011, Sempere et al., 2004, Mouillet-Richard et al., 2012), there has been major interest in its potential role in the abnormal brain development seen in schizophrenia. Further support has come from the fact that patients with DiGeorge 22q11,2 deletion have a 30-fold increase in the risk of schizophrenia as well as deletions that affect *DGCR8*, which happens to be a key microRNA processing gene (Mellios and Sur, 2012, Stark et al., 2008). A "hotspot" for neuropsychiatric disorders, including autism and schizophrenia has also been described on chromosome 8. This area includes several microRNAs, lending support to their role in schizophrenia (Tabares-Seisdedos and Rubenstein, 2009).

It is not yet clear whether changes in microRNA levels in schizophrenia are due to global or individual expression changes. Nonetheless, some microRNAs have been repeatedly identified across several studies of schizophrenia, including in different tissues and patient cohorts and these are worth further investigation and replication. The miR-15 family, targeting genes implicated in schizophrenia such as *BDNF*, *DRD1*, *NRG1* and *EGR3* was upregulated in the frontal cortex in three separate studies (Mellios et al., 2009, Beveridge et al., 2010, Santarelli et al., 2011). Genotyping studies have identified variations in miR-137 as being strongly associated with schizophrenia (Ripke et al., 2011, Whalley et al., 2012, Potkin et al., 2009, Green et al., 2012). Carriers of this SNP have subsequently been found to be more likely to have mood-congruent psychotic symptoms compared to those without this SNP (Cummings et al., 2013). MiR-132 has been implicated in both pre-clinical and clinical studies

of schizophrenia (de Bartolomeis et al., 2015, Kim et al., 2010, Yu et al., 2015, Miller et al., 2012, Sun et al., 2015). A recent deep sequencing study has also identified miR-130b and miR-193a-3p as potential state-independent biomarkers in schizophrenia (Wei et al., 2015).

To date, microRNA studies in BPAD have been mostly limited to post-mortem studies, many of which included schizophrenia subjects (Kolshus et al., 2014). Others have focused on the impact of mood stabilizer treatment on microRNA levels. The majority of studies of microRNA expression in BPAD, although from a single brain bank, remain contradictory. There is an overlap with microRNAs implicated in schizophrenia. MicroRNAs also appear to be making a contribution to the actions of lithium and sodium valproate. One study of post-partum psychosis has identified miR-212 and miR-146a, both of which target inflammatory pathways, as being significantly altered in patients compared to controls (Weigelt et al., 2013).

Compared to other psychiatric disorders, there is a paucity of clinical studies of microRNAs in anxiety disorders, despite these being common with a high burden of disease. As in other disorders, genotyping rare variants (Donner et al., 2008, Muinos-Gimeno et al., 2009, Muinos-Gimeno et al., 2011, Hanin et al., 2014, Jensen et al., 2014) has been a productive approach to understand the genetic basis of anxiety disorders. Other studies have focused on establishing a blood biomarker in anxiety disorders (Katsuura et al., 2012, Honda et al., 2013, Zhou et al., 2014). There is little overlap so far in the microRNAs identified in these studies, but many studies have included either naturalistic models of anxiety or groups with multiple psychiatric disorders. Combining microRNA expression analysis and fMRI findings whilst undergoing a social stress task, an increase in miR-29c was associated with self-reported levels of stress as well as alterations in functional connectivity in the ventro-medial prefrontal cortex (Vaisvaser et al., 2016). This microRNA had previously been implicated in animal models of repeated restraint stress and paternal stress (Rinaldi et al., 2010, Rodgers et al., 2013). Two recent studies of microRNA expression included patients with co-morbid anxiety disorder and depression. One included patients with a diagnosis of post-traumatic stress disorder (PTSD) and depression (Wingo et al., 2015), whilst the other included patients in a primary care setting with a mix of depression, anxiety and adjustment

disorders (Wang et al., 2015). Given the amount of "noise" inherent in epigenetic mechanisms, like microRNAs, that can target hundreds of genes, it would seem sensible to start with well-defined patient groups with little comorbidity, although these can be hard to find. Few of the traditional brain banks have samples of anxiety disorders, which limit this area of study.

Studies investigating the role of microRNAs in other psychiatric disorders are either isolated to initial exploration studies or have not so far moved beyond animal models. Several animal studies in addiction disorders exist as well as one post-mortem microRNA brain expression study (Lewohl et al., 2011). Animal models have also shown upregulation of microRNAs in response to alcohol, and this may be a mechanism for neuronal adaptation (Pietrzykowski et al., 2008). Cocaine addiction and response has been the target of a number of animal studies but no human studies have yet been published. The data from animal studies implicate miR-212 (Hollander et al., 2010, Im et al., 2010), miR-124, miR-181 and let-7d in the response to cocaine (Chandrasekar and Dreyer, 2009, Chandrasekar and Dreyer, 2011, Eipper-Mains et al., 2011).

Other disorders where microRNAs have been implicated include Fragile-X (Edbauer et al., 2010), autism (Abu-Elneel et al., 2008, Talebizadeh et al., 2008) and Tourette's syndrome (Abelson et al., 2005).

1.3.6 Practical applications of microRNAs in disease and health

There have been few truly novel psychiatric therapeutic developments in recent years and some pharmaceutical companies have abandoned research efforts in the psychiatry field altogether (Miller, 2010). MicroRNAs harbour the possibility of opening up a genuinely new approach to therapeutics and are already in development in other fields. For example, Miravirsen, a miR-122-targeting locked nucleic acid antisense drug has been developed for hepatitis C. In a recent phase 2 clinical trial Miravirsen was well tolerated and resulted in a dose-dependent reduction in hepatitis-C mRNA levels (Janssen et al., 2013). Further work has shown that patients receiving Miravirsen have significantly lower levels of miR-212, without affecting the levels of other microRNAs, indicating that specific microRNAs can be

targeted in isolation (van der Ree et al., 2016). Existing drug treatments for psychiatric disorders have already been shown to alter microRNA expression. Targeting microRNAs may therefore represent a more direct route to treatment. Although delivering microRNA targeted therapies to the CNS poses a considerable challenge, they have been identified as therapeutic targets of great potential in other CNS disorders such as glioblastoma (Auffinger et al., 2013). One challenge is that, as post-mortem studies have shown, different regions of the brain express microRNAs at different, even opposing, levels. Techniques to target specific areas of the brain and not others, including outside the CNS, would therefore need to be developed to minimise inappropriate mRNA targeting.

However, it is the ability of a single microRNA to target whole networks of genes that may make it fruitful for psychiatric disorders where it is the sum of many genes of small effect that is thought to underlie heritability. Viral and non-viral vectors are being investigated for their potential to deliver microRNAs to the CNS (Zhang et al., 2013a) but a non-invasive method that has also shown some promise in this regard is intra-nasal delivery (Bortolozzi et al., 2012). MicroRNAs implicated in disease or therapeutic effects that are identified in clinical populations should be further investigated in animal and other pre-clinical models (Seong et al., 2002). This could involve overexpression or knockdown of a candidate microRNA in cell lines and measuring the target gene's protein expression levels as well as behavioural phenotype in animal models. Likewise, microRNAs identified in pre-clinical settings should be followed up in clinical samples. Identifying new rare variants through sequencing as well as genotyping larger cohorts for more common microRNA disease risk variants should also be explored. Studies correlating microRNA profiles with clinical outcomes would be helpful in the development of biomarkers.

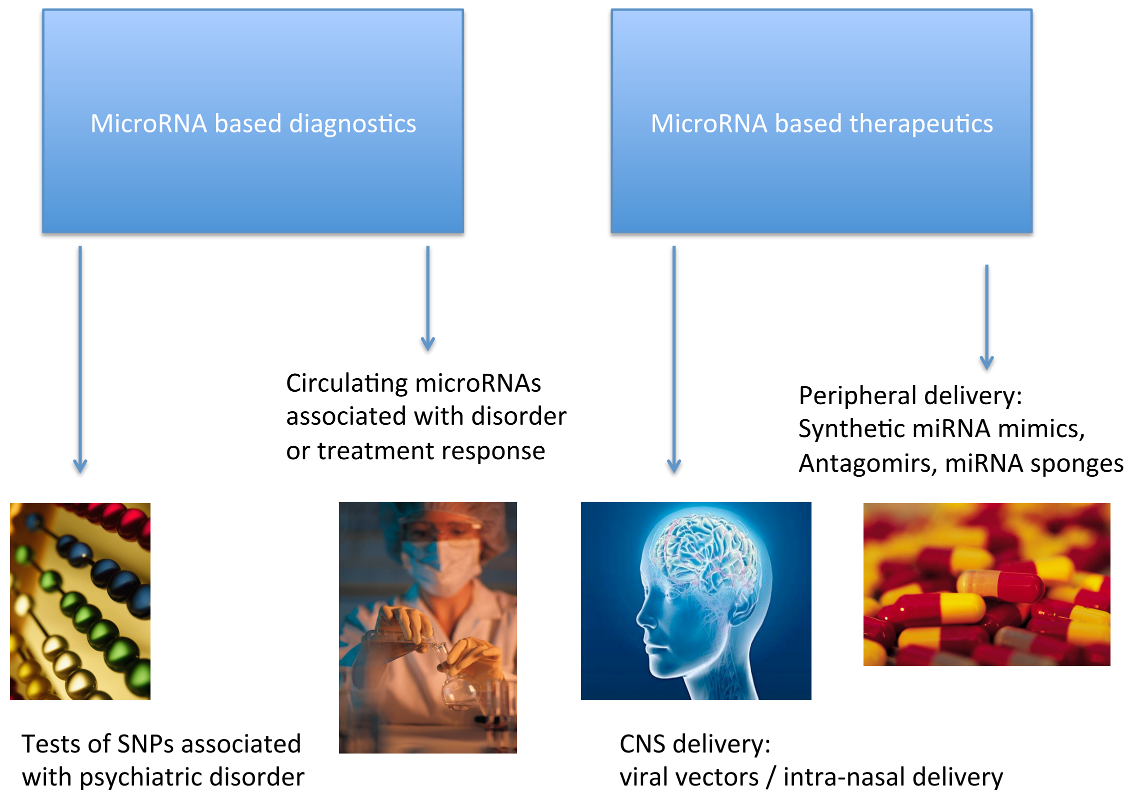


Figure 1.14 Applications of microRNAs in psychiatric disorders. Analyses of SNPs or circulating microRNA levels have biomarker potential. Potential therapeutic applications include using viral vectors to overexpress or knock down specific microRNAs in the CNS. Alternatively, synthetic microRNA mimics or microRNA silencers (e.g. oligonucleotides such as antagomirs or microRNA sponges) can be administered to increase or decrease microRNA activity respectively.

In summary, there is a growing realisation that microRNAs are central to the control of gene expression in the CNS and are altered in the development, presentation and response to treatment of psychiatric disorders. Although in its infancy, investigating these small molecules may lead to big developments in biomarkers and therapeutic targets for psychiatric disorders.

1.4 Objective

1.4.1 Aims

ECT remains the most powerful treatment available for depression, but our understanding of its molecular mechanisms, and of depression itself, remains elusive. The use of ECT is also limited by concerns over cognitive side effects, but variations in electrode placement and dose above seizure threshold may lessen these side effects. Emerging evidence suggests that alterations in microRNA abundance following ECT for severe depression could improve our understanding of the molecular neurobiology of depression and ECT. It could also potentially be informative for the development of clinical biomarkers for depression. MicroRNAs underpin molecular mechanisms of neuroplasticity that may also modulate response to treatment and cognitive side effects from ECT. To date, no-one has reported on microRNA expression in depressed patients undergoing ECT.

As part of a research team carrying out a randomised controlled trial of the effectiveness and cognitive side effects of high-dose (6 x seizure threshold) right unilateral ECT and standard dose (1.5 x seizure threshold) bitemporal ECT, I was in a position to obtain blood samples and clinical data from a well-phenotyped cohort. The aims of this project were therefore to explore microRNA expression changes in this cohort and their potential downstream mRNA targets, as well as incorporating trial data in a wider systematic review and meta-analysis of the two forms of ECT used in the trial.

1.4.2 Hypotheses

Five main hypotheses underlie this thesis:

1. High-dose (6 x seizure threshold) right unilateral ECT is equally as effective as standard dose (1.5 x seizure threshold) ECT but will have less cognitive side effects
2. Profiling of circulating microRNAs will identify alterations in peripheral microRNA levels in severe depression treated with ECT

3. Bioinformatic analysis of microRNAs with altered levels in severe depression treated with ECT will identify mRNA targets relevant to depression
4. mRNA species identified as targets of significantly altered microRNAs in severe depression treated with ECT will in turn have significantly altered levels in the same cohort
5. Changes in microRNA or mRNA levels in severe depression treated with ECT will correlate with clinical and cognitive outcomes following ECT

1.4.3 Objectives

The specific objectives are as follows:

1. To carry out a systematic review and meta-analysis of the effectiveness and cognitive side effects of high-dose right unilateral ECT with standard-dose bitemporal ECT
2. To perform a deep sequencing study of whole blood microRNA expression to characterize changes in peripheral microRNA abundance following ECT for severe depression (n=16). Candidate microRNAs identified by deep sequencing will be confirmed using quantitative real time polymerase chain reaction (qRT-PCR)
3. To validate confirmed microRNAs in a larger sample of patients treated with ECT (n=38) and healthy controls (n=34) using qRT-PCR
4. To carry out a bioinformatic analysis identifying experimentally validated mRNA targets of differentially expressed microRNAs in severe depression treated with ECT
5. To study the abundance of selected experimentally validated mRNA targets identified in Objective 4 relevant to depression / ECT in a sample of patients treated with ECT (n=97) and healthy controls (n=53)
6. To correlate any significantly altered microRNAs/mRNAs identified in Objectives 3-5 with clinical (HDRS-24 / remission status) and neurocognitive outcomes (executive functioning, delayed verbal memory, autobiographical memory, reorientation time and global cognition)
7. To assess the potential of significantly altered microRNAs/mRNAs identified in Objectives 3-5 as biomarkers in depression.

Chapter 2
Materials & Methods

2 Materials and methods

2.1 Laboratory materials

2.1.1 General consumables

Mini-tubes (0.5mL)	Sarstedt, IRL
Microtubes (1.5mL)	Sarstedt, IRL
Pipette tips	Sarstedt, IRL
BD Vacutainer Safety Lok Blood Collection Sets	Becton Dickenson, UK
BD Sharps Container 3L	Becton Dickenson, UK
BD Vacutainer K2 EDTA 10mL bottle	Becton Dickenson, UK
Plastic syringe (1,5,10mL)	Becton Dickenson, UK
Disposable centrifuge tubes (15, 50mL)	Fisherbrand, Fisher Scientific, UK

2.1.2 RNA extraction and quality analysis

PAXgene Blood miRNA Kit	Qiagen, UK
PAXgene Blood RNA tubes	Qiagen, UK
Bioanalyzer RNA 6000 Nano Kit	Agilent, IRE
Agilent 2100 Bioanalyzer	Agilent, IRE
NanoDrop-1000 UV-Vis Spectrophotometer	Fisher Scientific, UK
TURBO DNA free kit	Applied Biosystems, UK
RNase Zap Wipes	Ambion, UK

2.1.3 Deep sequencing

SOLiD Total RNA-Seq Kit	Applied Biosystems, UK
SOLiD RNA Barcoding kit	Applied Biosystems, UK
SOLiD Bead Enrichment kit	Applied Biosystems, UK
SOLiD Buffer kit	Applied Biosystems, UK
SOLiD ePCR kit V2	Applied Biosystems, UK
SOLiD Emulsion Tray	Applied Biosystems, UK
IKA Template prep tubes	Applied Biosystems, UK
XD Slide and Deposition kit V2	Applied Biosystems, UK
SOLiD Pre-deposition kit	Applied Biosystems, UK

SOLiD ToP Workflow Analysis Reagents	Applied Biosystems, UK
SOLiD ToP Instrument Buffer Kit	Applied Biosystems, UK
SOLiD ToP Fragment BC Sequencing Kit	Applied Biosystems, UK
Novex® 10% TBE-Urea Gels 1.0mm, 10 well	Invitrogen, IRE
Novex® TBE-Urea Sample Buffer (2×), 10mL	Invitrogen, IRE
Novex® TBE Running Buffer (5×)	Invitrogen, IRE
PureLink™ PCR Micro Kit	Invitrogen, IRE
10 bp DNA Ladder	Invitrogen, IRE
SYBR® Gold nucleic acid gel stain, 10,000×	Invitrogen, IRE
MinElute® PCR Purification Kit (50)	Qiagen, UK
DNA 1000 kit	Agilent, IRE
IKA ULTRA-TURRAX® Disperser	Sigma Aldrich, IRL

2.1.4 PCR

MicroAmp Fast 96 well optical reaction plates	Applied Biosystems, IRE
MicroAmp Fast 96 well support base	Applied Biosystems, IRE
TaqMan gene expression assays	Applied Biosystems, IRE
TaqMan Fast Advanced Master Mix	Applied Biosystems, IRE
TaqMan Universal PCR Master Mix (2x), no UNG	Applied Biosystems, IRE
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, IRE
RNase-free water	Sigma Aldrich, IRL
TaqMan® MicroRNA Reverse Transcription (RT) Kit	Applied Biosystems, IRE
TaqMan® High Capacity cDNA RT Kit	Applied Biosystems, IRE
Optical adhesive covers	Applied Biosystems, IRE
Human Random DNA Control Panel	Sigma Aldrich, IRL

2.2 Phenotyping materials

2.2.1 Demographic variables

Demographic data on patients and healthy controls were collected with regard to gender, age, marital status, educational attainment and socio-economic group. Educational attainment was measured as number of years in education. Socio-economic group was derived from the standard UK National Readership Survey classification model (National Readership Survey, 2015).

2.2.2 Clinical variables

2.2.2.1 Diagnostic assessment

The presence or absence of a major depressive disorder in all patients and controls was confirmed by administering the mood disorder component of the research version of the Structured Clinical Interview for DSM-IV Axis 1 Disorders (SCID-1) (First et al., 2002). This is a semi-structured clinical interview designed to diagnose mental illness based on DSM-IV criteria.

The research version is slightly longer and more complex than the standard clinician version and contains more subtypes (including psychotic and melancholic depression) and a severity rating. It also includes information on the duration of the current episode and details of past depressive episodes. Researchers are encouraged to select modules or sections that are appropriate to their area of research. The SCID-1 has been shown to have good inter-rater reliability (Lobbestael et al., 2011).

2.2.2.2 Depression rating

Depression severity at baseline and at end of treatment was measured using the 24-item version of the Hamilton Depression Rating Scale (HDRS) (Beckham and Leber, 1985). The semi-structured clinician rated HDRS-24 is one of the most commonly used rating scales in major depression and has been widely used in the electroconvulsive therapy (ECT) literature. It is not a diagnostic instrument but measures depressive symptom severity and can

track changes over time. The original HDRS recommended a 17-item version with additional items having been added over time (Hamilton, 1960, Williams, 2001). Although the HDRS-24 has come under criticism both on a psychometric and conceptual basis, it continues to remain the "gold standard" for the assessment of depression (Bagby et al., 2004).

The HDRS-24 takes approximately 20 minutes to complete and total score ranges from 0 to 77 with a high score reflecting a higher burden of depression severity. The HDRS-24 has been shown to have good concurrent and external validity as well as inter-rater reliability (Maier et al., 1988b, Maier et al., 1988a, Miller et al., 1985). When administered over the telephone it has also been shown to correlate strongly with face to face interviews (Potts et al., 1990).

Item	Score	Item	Score
1. Depressed Mood	0-4	14. Hypochondriasis	0-4
2. Anhedonia	0-4	15. Insight	0-2
3. Appetite	0-2	16. Psychomotor retardation	0-4
4. Energy	0-2	17. Agitation	0-4
5. Weight Loss	0-2	18. Diurnal variation	0-4
6. Libido	0-2	19. Dissociation	0-4
7. Insomnia early	0-2	20. Paranoia	0-3
8. Insomnia middle	0-2	21. Compulsivity	0-2
9. Insomnia late	0-2	22. Helplessness	0-4
10.Feelings of guilt	0-4	23. Hopelessness	0-4
11. Suicidality	0-4	24. Worthlessness	0-4
12. Anxiety Psychic	0-4		
13. Anxiety Somatic	0-4	**TOTAL**	**0-77**

Table 2.1 24-item HDRS

2.2.2.3 Antidepressant treatment resistance

There are varying definitions of what constitutes treatment resistance to physical, psycho- or pharmacotherapy. Many modern ECT research studies have used the Antidepressant Treatment History Form (ATHF) (Oquendo et al., 2003). The ATHF was therefore used to facilitate comparison with other ECT studies. The ATHF produces a binary yes/no outcome for treatment resistance with one failed trial deemed adequate for treatment resistance. Set criteria are in place in terms of what constitutes an adequate dose and duration.

2.2.2.4 Psychotic symptomatology

Psychotic psychopathology severity was measured using items from the Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham, 1962). The original BPRS was designed to characterise psychopathology and measure change in clinical research settings. It includes 18 (originally 16) psychopathological signs and symptoms based on a clinical interview with a patient (Overall and Gorham, 1962). In terms of psychotic symptoms the BPRS has more of a focus on positive symptoms and it has therefore been suggested that it is more applicable for patients outside the schizophrenia spectrum, such as depression (Zanello et al., 2013).

Five items from the BPRS - hallucinations, unusual thought content (delusions), suspiciousness, emotional withdrawal and blunted affect (BPRS-5) have been identified as having the highest clinical validity and responsiveness to change in psychotic depression (Ostergaard et al., 2014, Park et al., 2015). The BPRS-5 items, in conjunction with six items from the 17-item HDRS, form the 11-item Psychotic Depression Assessment Scale (PDAS) (Ostergaard et al., 2014).

2.2.3 Cognitive variables

2.2.3.1 National Adult Reading Test (NART)

The NART is commonly used as a proxy ("hold test") for premorbid intelligence and was most recently revised in 1991 (Nelson HE and J, 1991).

It has been widely used in the ECT literature. The test consists of subjects reading out loud a list of 50 phonetically irregular words, which require prior knowledge to pronounce correctly, e.g. 'demesne' or 'cellist'. It has been found to have a high correlation coefficient (0.73) with IQ at age 11 (Crawford et al., 2001).

2.2.3.2 Addenbrooke's Cognitive Examination-Revised (ACE-R)

The ACE-R was developed as a brief bedside cognitive test battery for dementia screening (Mioshi et al., 2006). It offers a global measure of cognition as well as scores on subscales such as attention and concentration, letter and category fluency, visuo-spatial performance, language and memory. Although it incorporates the Mini-Mental State Examination (MMSE) (Folstein et al., 1975) it offers a greater depth of cognitive assessment and has a much improved utility for detecting dementia and its subtypes, as well as separating those with late-onset depression from cognitive dysfunction due to dementia (Rotomskis et al., 2015).

The maximum score on the ACE-R is 100 points, with subscale maximums of 18 for attention/orientation, 14 for fluency, 16 for visuo-spatial, 26 for language and 26 for memory. Different versions exist to minimize the practice effect. Concurrent validity and internal consistency is good (Cronbach's alpha 0.8) (Mioshi et al., 2006).

2.2.3.3 Trail Making Tests

The Trail Making Tests were developed by U.S. Army psychologists in the 1940's and are freely available in the public domain (Lezak, 2012). It consists of two parts (TMT-A and TMT-B) with TMT-A (Figure 2.1) measuring attention and visuo-motor speed. In the TMT-A, subjects are asked to draw a continuous line through a set of numbers from 1-25 in consecutive order.

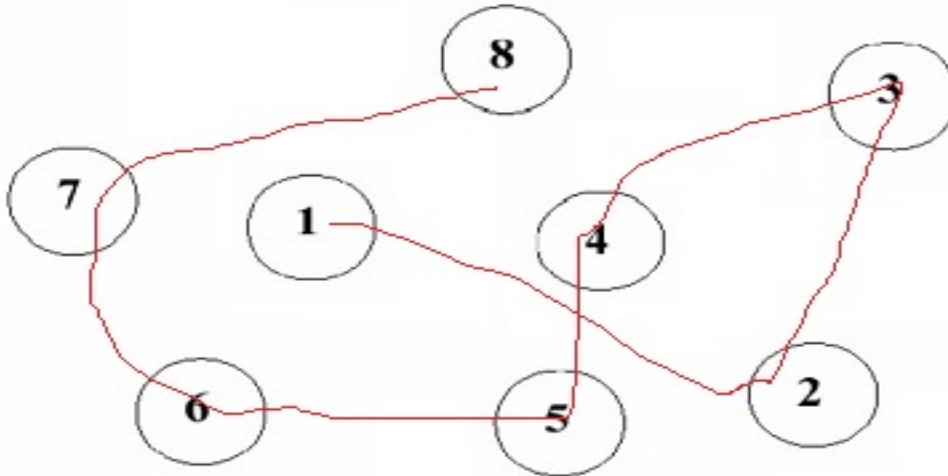


Figure 2.1 Example of TMT-A test

In TMT-B, subjects are required to alternate between consecutive sets of letters and numbers, which tests executive functioning and task shifting. The typical scoring mechanisms for both versions involve recording the time taken to complete the task (including time spent in correcting mistakes made underway). Reliability coefficients are good, with many above 0.8 (Lezak, 2012). Executive functioning is one area that seems particularly vulnerable to the short-term effects of ECT and we would therefore expect to see a larger difference on performance on the TMT-B compared to the TMT-A after ECT (Semkovska and McLoughlin, 2010).

2.2.3.4 Free and Cued Selective Reminding Test

The Free and Cued Selective Reminding Test (FCSRT) uses a repeated trial 16-word learning task to assess verbal memory and learning (Van der Linden and GREMEM, 2004). On subsequent recall subjects are assessed both on free and cued recall of learned words. It is a slightly modified version of the original 12-item version developed by Buschke (Buschke, 1984). It aims to differentiate between retrieval from short-term and long-term memory (Lezak, 2012). The FCSRT has high sensitivity and specificity when differentiating those with Alzheimer's dementia from healthy controls

and other dementias, and has good internal consistency (Cronbach's alpha >0.85) (Lemos et al., 2014).

2.2.3.5 Autobiographical memory

The Columbia Autobiographical Memory-Short Form (CUAMI-SF)) is the most widely used measure of retrograde autobiographical memory in the ECT literature (Sackeim, 2014). The CUAMI-SF produces a baseline score based on a series of questions about personal life events. At follow-up, scores are a percentage, representing the amount of questions answered correctly at baseline that are subsequently also answered correctly at a given time-point in the future. As such it is a measure of consistency with baseline, no matter how 'good' or 'bad' performance was at baseline. It also lacks any real normative data or measures of reliability, although it is sensitive to differences in autobiographical memory performance due to differences in electrode placement (Semkovska and McLoughlin, 2013, Sackeim et al., 2007).

2.3 Laboratory methods

2.3.1 Blood collection

Fasting blood samples were taken at baseline and end-of-treatment for patients receiving ECT. In the case of healthy controls, fasting bloods were taken at a single time-point. Ribonucleic acid (RNA) is vulnerable to degradation by enzymes (RNases) ex-vivo. As my research took place in a clinical setting, I was not in a position to analyse bloods for microRNA or messenger RNA (mRNA) at the time of sampling. To protect RNA from degradation until the time of batch analysis I used the PaxGene© system (Qiagen Inc., USA) to collect whole blood according to the manufacturer's instructions. PaxGene© tubes contain a reagent that disrupts blood cells, releasing intracellular RNA. This RNA is then immediately stabilised to prevent degradation of the intracellular RNA, to ensure that RNA levels measured reflect the actual intracellular state (Rainen et al., 2002). PaxGene© tubes were subsequently stored between -60° to -80°C.

According to the manufacturers, blood samples collected in these tubes show no changes in RNA transcript levels for at least fifty months when stored between -20° to -70°C.

2.3.2 MicroRNA and mRNA extraction from blood

MicroRNAs and mRNA were extracted from whole blood using the PaxGene© miRNA and PAXgene© Blood RNA kits according to the manufacturer's instructions (Qiagen Inc., USA). Before the commencement of RNA extraction, benchtops and equipment were cleaned using RNase Zap wipes which removes potentially degrading RNases.

Frozen tubes containing blood were then thawed at room temperature for a minimum of three hours, then centrifuged for ten minutes at 3,000 x g. The supernatant was then discarded by pipetting, and 4 mL RNase-free water was added to the pellet. The pellet was dissolved though vortexing and then centrifuged for a further ten minutes at 3,000 x g. The supernatant was discarded by pipetting and 350 µL of resuspension buffer 1 was added to the pellet. The pellet was dissolved through vortexing and added to a 1.5mL microcentrifuge tube together with 300 µL of binding buffer 2 and 40 µL of proteinase K. The addition of proteinase K removes any endonucleases that might contaminate the sample.

Following brief vortexing, the sample was incubated for ten minutes at 55°C in a shaker-incubator at 300 rpm. The samples were then homogenised through PAXGene© shredder spin columns centrifuged at 18,000 x g for three minutes. The flow-through supernatant was added to a new 1.5 mL microcentrifuge tube. In the case of microRNA analysis, 700 µL of isopropranolol (100% purity grade) was then added. In mRNA extraction 96% Ethanol was used in place of isopropranolol.

Following vortexing the sample was run through PAXGene© spin columns where RNA binds to silica membranes. The addition of 350 µL of wash buffer 3 to the spin columns, which were then centrifuged at 18,000 x g for one minute removed potential contaminants. The processing tubes with flow-through were discarded and the remaining spin columns were placed in new 2 mL processing tubes. 80 µL of DNase incubation mix (to remove DNA contamination) was then pipetted directly onto the spin

column membrane. The spin columns were left to incubate at room temperature for 15 minutes. The DNase was subsequently removed through a series of washes with wash buffers. For microRNAs these washes constituted of a 350 µL wash buffer 3 for fifteen seconds at 18,000 x g, followed by 500 µL of wash buffer 4 for 15 seconds at 18,000 x g and finally another 500 µL of wash buffer 4 for two minutes. For mRNA extraction the wash buffers and centrifuge speeds were the same, but the duration was one minute for the first two steps and three minutes for the final wash buffer step. The spin columns were then centrifuged for one minute at 18,000 x g to completely dry the columns. The processing tube containing flow-through was discarded and the spin column was placed in a new 1.5 mL microcentrifuge tube. 40 µL of elution buffer 5 was then added directly to the spin columns. These were then centrifuged for one minute at 18,000 x g and repeated with a second round of same procedure to release the RNA from the silico membranes. The RNA eluate was incubated for five minutes at 65°C in a shaker-incubator before being divided into aliquots and stored at -80°C.

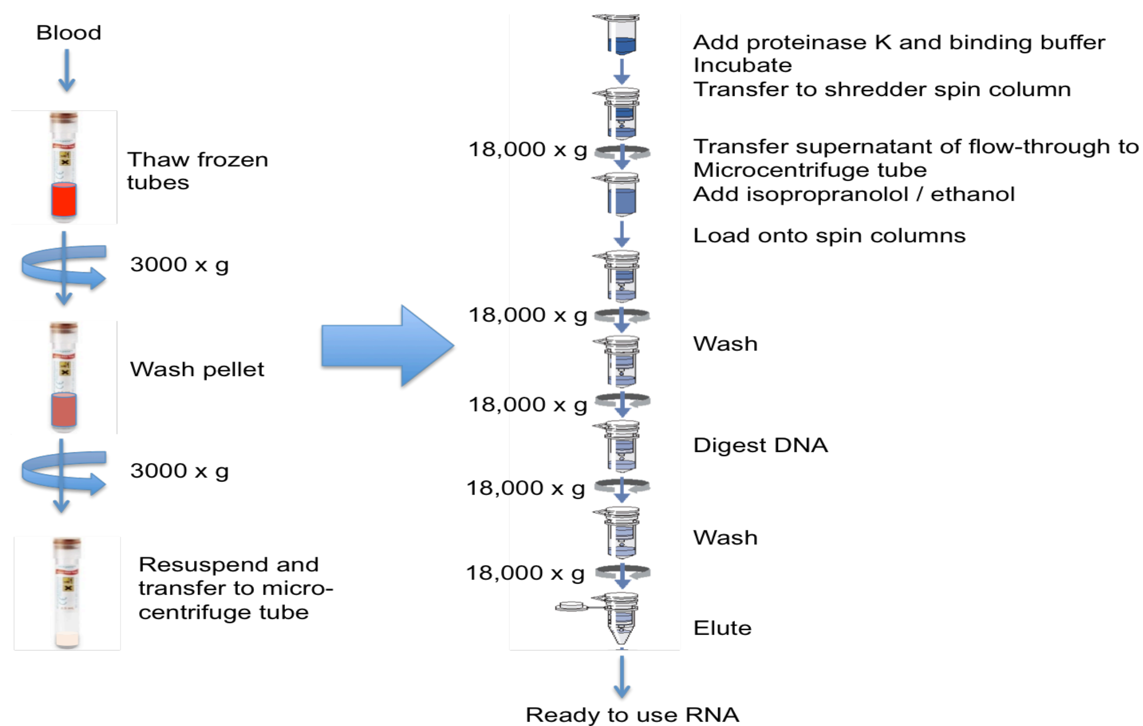


Figure 2.2 Overview of microRNA/mRNA extraction from blood. Adapted from PaxGene© Blood RNA Kit Handbook, Qiagen, UK.

2.3.3 RNA quantification and quality analysis

RNA quantification was performed using the NanoDrop© 1000 UV-Vis Spectrophotometer which gives a measurement of RNA concentration as well as an indication of the quality of the sample. Briefly, before quantification the spectrophotometer was blanked using 1 µL of elution buffer 5 from the PaxGene© kits. Then, 1 µL of the RNA sample was placed on the NanoDrop pedestal and the absorbance at 260 nm (A260) and 280 nm (A280) was measured. The concentration of an RNA sample is measured using A260, whilst the A260/A280 ratio gives an indication of the purity of the sample. In general, a A260/A280 ratio >1.8 is considered suitable for gene expression analysis (Becker et al., 2010). The instrument was cleaned between each sample using a tissue wipe. Samples were subsequently equalized to 2 ng/µL using RNase-free water as dilutant.

Total RNA quality was measured using the Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Assay Protocol according to the manufacturer's instructions. In this protocol small amounts of sample are added to a chip designed for the analysis of RNA fragments. These chips (Figure 2.2), which have 16 wells, contain interconnected microchannels that allow for the separation of nucleic acid fragments based on their size, which are then detected by laser-induced fluorescence. Initially, all reagents were allowed to equilibrate to room temperature for at least 30 minutes. 550 µL of RNA gel matrix was then pipetted into a spin filter and centrifuged at 1500 x g for ten minutes at room temperature. 65 µL aliquots of filtered gel were made, with extra aliquots stored at 4°C for up to four weeks. RNA dye concentrate was vortexed for ten seconds, and 1 µL of the dye was added to the filtered gel. This gel-dye mix was vortexed for ten seconds, and then spun at 13,000 x g for ten minutes at room temperature. A RNA 6000 Nano chip was placed in a chip priming station and 9 µL of the prepared gel-dye mix was pipetted into the indicated well. The plunger in the chip priming station was primed and pressed until held by a clip. After thirty seconds the clip was released and the plunger slowly pulled back to its original position. The chip priming station was opened, and 9 µL of the prepared gel-dye mix was pipetted into two further indicated wells. 5 µL of RNA 6000 Nano Marker was pipetted into the twelve sample wells and the

ladder well. 1 µL of RNA 6000 Ladder was pipetted into the ladder well, and 1 µL of sample was added to each of the twelve sample wells. The chip was vortexed for one minute at 2400 rpm and then run in the 2100 Bioanalyzer.

The 2100 Bioanalyzer provides an RNA integrity number (RIN), which reflects the state of degradation of a sample ranging from one (most degraded) to ten (most intact). It is based on an algorithm that includes more detail than just the A260/A280 ratio and has been found to be superior to using just one electropherogram feature (Schroeder et al., 2006).

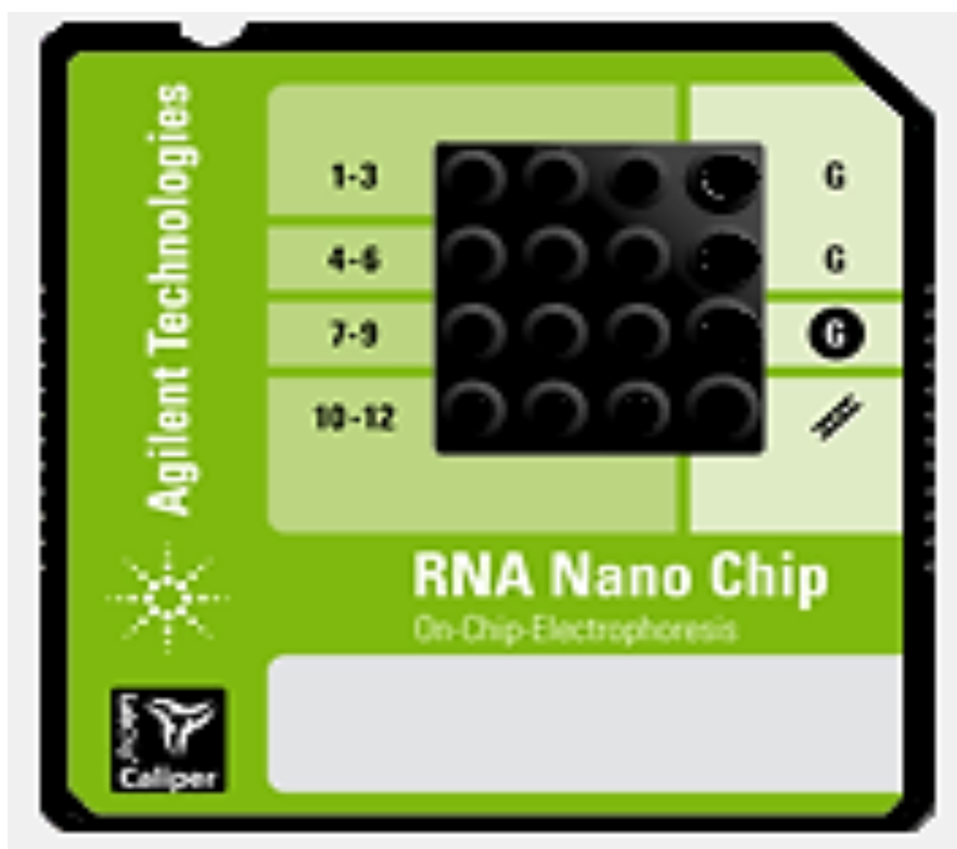


Figure 2.3 The Agilent RNA 6000 Nano Chip.
The Agilent RNA 6000 Nano Chip contains twelve sample wells, a ladder well and three gel-dye wells.

2.3.4 Deep sequencing

Deep sequencing (or next generation sequencing) was carried out on the SOLiD™ platform according to the manufacturer's instructions (Applied Biosystems Inc., USA).

2.3.4.1 Deep sequencing - library preparation

Initially, microRNA samples were converted to a library suitable for emulsion polymerase chain reaction (PCR) using the SOLiD Total RNA-Seq Kit© according to the manufacturer's instructions. Initially, a hybridisation mix was prepared on ice containing 3 µL of microRNA sample, 3 µL of hybridisation sample and 2 µL of SOLiD™ Adaptor Mix. This was gently mixed and then run in a thermal cycler for ten minutes at 65°C and five minutes at 16°C. 10 µL of 2 x Ligation Buffer and 2 µL of Ligation Enzyme Mix was then added. The tube, containing 20 µL of ligated microRNA, was gently mixed and incubated in a thermal cycler at 16°C for 16 hours with the lid open. The ligated microRNA was then reverse transcribed (RT) by first adding 19 µL of RT master mix to the sample. The RT master mix consisted of 11 µL nuclease-free water, 4 µL of 10 x RT Buffer, 2 µL of deoxyribose nucleoside triphosphate (dNTP) mix and 2 µL of SOLiD™ RT Primer. This was mixed gently, then incubated in a thermal cycler with a heated lid at 70°C for five minutes then snap-cooled on ice. 1 µL of ArrayScript™ Reverse Transcriptase was then added to each ligated RNA sample, gently mixed and incubated in a thermal cycler for 42°C for 30 minutes, generating complementary DNA (cDNA).

The cDNA was then purified using the MinElute® PCR Purification Kit. 40 µL of cDNA was transferred into a clean 1.5 µL microcentrifuge tube. 60 µL of nuclease-free water and 500 µL of Buffer PB were added and the tube was mixed well. 600 µL of the sample was then added to a MinElute column, which was spun at 13,000 x g for one minute. The flowthrough was discarded and the column was returned to the microcentrifuge tube. 750 µL of Buffer PE was added to the column which again was spun at 13,000 x g for one minute. The flowthrough was discarded and the column was re-spun for a further minute at 13,000 x g. The cDNA was then eluted in a new microcentrifuge tube by adding 10 µL of Buffer EB to the centre of the

column. After one minute, the column was spun at 13,000 x g for one minute.

The next step involved size-selecting the cDNA to separate library products from left over adaptors and primers using gel purification. First, a Novex 10% TBE-Urea Gel was prepared and 1000 µL of 1x TBE Running Buffer was added to the Upper and Lower Buffer Chamber. A 10-base pair (bp) DNA Ladder was diluted (24 µL RNase-free water to 1 µL 10-bp DNA Ladder). 5 µL of cDNA was mixed with 5 µL of 2x Novex TBE-Urea Sample Buffer. 5 µL of the diluted DNA ladder was also mixed with 5 µL of the 2x Novex TBE-Urea Sample Buffer. The cDNA and DNA Ladder was heated at 95°C for three minutes. The wells of the gel were then flushed several times with 1x TBE Running Buffer to remove urea. The cDNA and DNA Ladder were loaded onto the gel, avoiding lanes one and ten. The gel was run at 180V until the dye front passed the middle of the gel. 5 µL of SYBR Gold Nucleic acid gel stain was added to 50 mL of 1x TBE Running Buffer to stain the gel for five to ten minutes. The stained gel was illuminated and the segments containing 60 to 80 nucleotides were excised using a clean razor blade, see figure 2.4. The two gel slices from the middle of the lane (approximately 1 mm x 6 mm) were placed in individual microcentrifuge tubes. Each gel slice was then prepared for PCR by adding 98.0 µL of PCR Master Mix (see Table 2.2) to the tube along with 2 µL of barcoded SOLiD 3' PCR Primer.

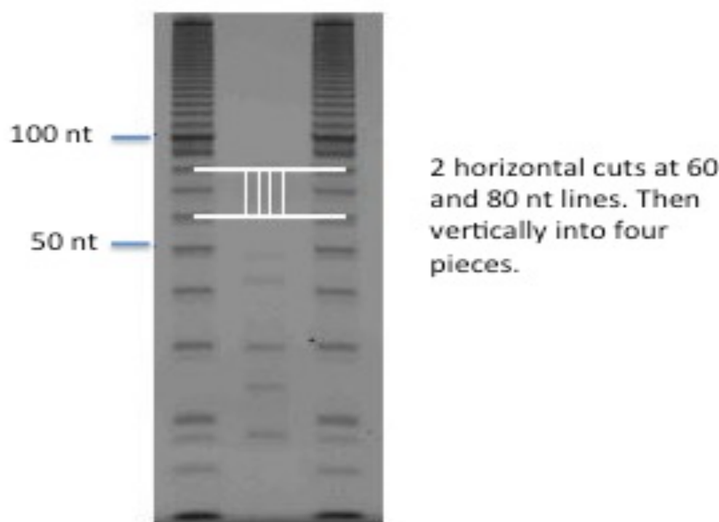


Figure 2.4 Size selection of cDNA from stained gel.

Component	Volume (µL)
Nuclease-free water	76.8
10x PCR Buffer	10.0
2.5 mM dNTP Mix	8.0
SOLiD 5′ PCR Primer	2.0
AmpliTaq DNA Polymerase	1.2
Total Volume	**98.0**

Table 2.2 cDNA amplification PCR Master Mix components.

The combined reagents were then run in a thermal cycler with the parameters listed in Table 2.3 below.

Stage	Temperature	Time
Hold	95°C	5 mins
Cycle (18 cycles)	95°C	30 seconds
	62°C	30 seconds
	72°C	30 seconds
Hold	72°C	7 minutes

Table 2.3 Thermal cycler parameters for cDNA amplification

Finally, the PCR product was purified prior to emulsion PCR. PCR purification was carried out using the PureLink PCR Micro Kit according to manufacturer's instructions. Briefly, the PureLink Micro Kit column was initially spun at 10,000 x g for one minute. 400 µL of Binding Buffer was added to the tube containing PCR product and mixed well. 500 µL of PCR sample and Binding Buffer was loaded on to the PureLink Micro Kit Column and spun at 10,000 x g for one minute. The flowthrough was discarded and the column was returned to a collection tube. 600 µL of Wash Buffer was added to the column, which was spun at 10,000 x g for one minute. The flowthrough was discarded and the column was returned to the collection tube and spun for a further minute at 14,000 x g. The column was then placed in a clean PureLink Elution Tube, 12 µL of Elution Buffer was added and then spun at 14,000 x g for one minute.

The purified product was then analysed on the 2100 Bioanalyzer using a DNA 1000 Kit to ensure the ratio of 120-130 bp DNA: 25-150 bp DNA was greater than 50%.

2.3.4.2 Deep sequencing template bead preparation

The DNA fragments generated by the previously described library preparation were clonally amplified using emulsion PCR. This is a specific form of PCR carried out in an emulsion where oil phase and aqueous phase is mixed. The aqueous phase forms droplets called microreactors. The desired microreactors contain a single SOLiD™ bead, and a single template, see Figure 2.5 below. The oil phase is prepared by first dispensing 1.8 mL of Emulsion Stabiliser 1 into a 50 mL conical tube. 400 µL of Emulsion Stabiliser 2 is then added slowly into the same tube. 37.8 mL of Emulsion Oil is then poured into the conical tube. The tube is then vortexed until the mixture is fully mixed and the emulsion stabilisers are incorporated into the Emulsion Oil. The mixture was allowed to degas for twenty minutes by slightly unscrewing the conical tube cap. A 10 mL syringe was primed by drawing about 2 mL of oil phase and dispensing back into the tube. Using the primed syringe, 9 mL of oil phase was then dispensed into a SOLiD ePCR tube.

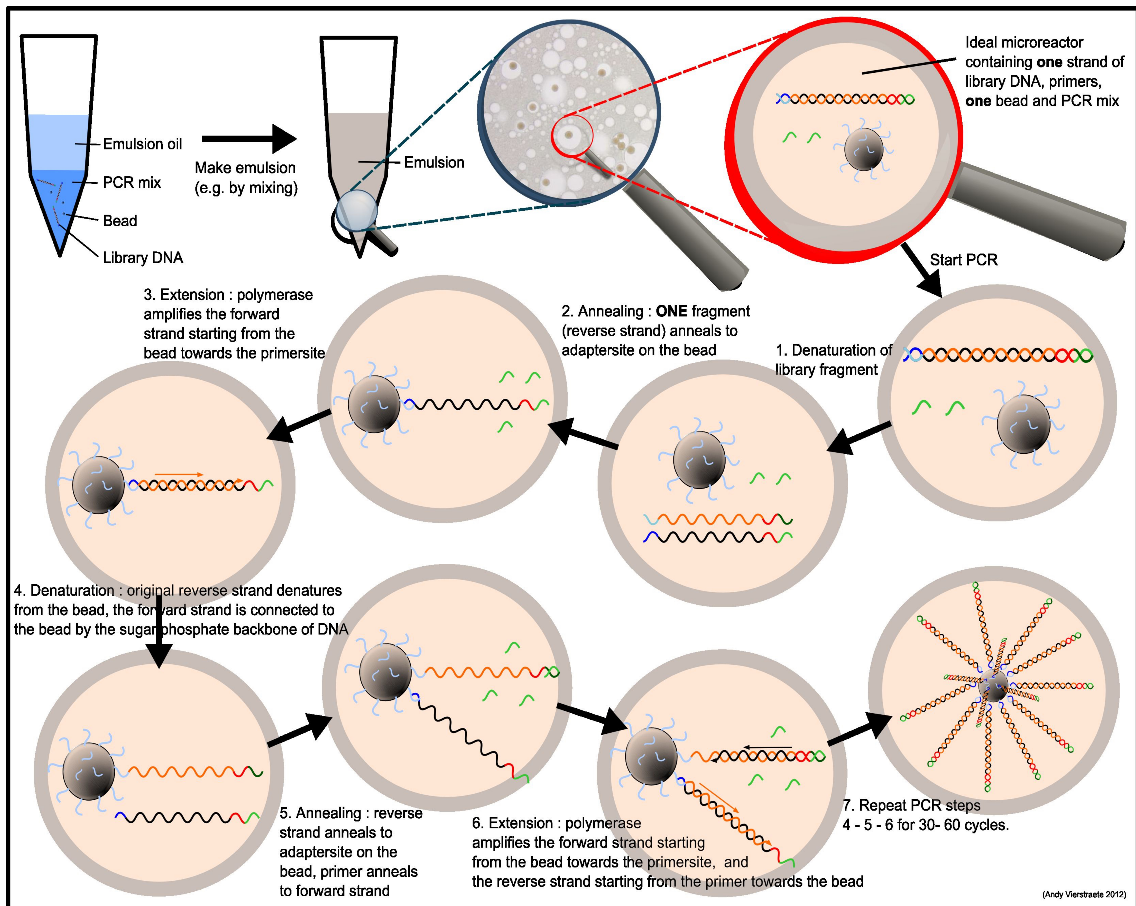


Figure 2.5 Overview of emulsion PCR (ePCR)

Source: Andy Vierstrate, "Next Generation Sequencing", http://users.ugent.be/~avierstr/nextgen/nextgen.html

For each ePCR reaction, 4 μL of a 10-μM working stock solution of ePCR Primer 1 was added to 36 μL of 1x Low TE buffer and mixed well. The library template was diluted to a concentration of 500 pM. The aqueous phase was prepared with the amounts and components listed in Table 2.4 below.

Component	Final concentration	Volume per reaction
10x PCR Buffer	1x	560 µL
dNTP Mix (100mM)	14 mM	784 µL
1M Magnesium Chloride	25 mM	140 µL
ePCR Primer 1 (10 µM)	40 nM	22.4 µL
ePCR Primer 2 (500 µM)	3 µM	33.6 µL
Template (500 pM)	0.5 pM	5.6 µL
Nuclease-free water	-	3294.4 µL
AmpliTaq Gold DNA Polymerase, UP (5U/µL)	0.54 U/µL	600 µL
TOTAL	-	**5440 µL**

Table 2.4 Aqueous phase components

A tube of SOLiD P1 DNA Beads was thoroughly vortexed and spun down. The tube was placed in a magnetic rack for one minute. When the solution cleared, the supernatant was removed and discarded. The beads were resuspended in 200 µL of Bead Block Solution. The solution was vortexed and spun down. The beads were sonicated using the Bead Block Declump program on the Covaris S2 System then spun down. The tube was placed in a magnetic rack for one minute and the supernatant was removed and discarded. The beads were then resuspended in 200 µL of 1x TEX Buffer, vortexed and spun down.

The SOLiD ePCR tube containing 9 mL of oil phase was placed on a ULTRA-TURRAX® device and locked into position. The SOLiD P1 DNA beads were sonicated using the Covalent Declump 1 program on the Covaris S2 system and spun down. 160 µL of SOLiD P1 DNA beads were immediately added to the aqueous phase, then mixed gently until uniformly dispersed. All of the aqueous phase (5.6 mL) and bead mixture was then filled in a 10 mL Combitip Plus pipette tip using an Xstream pipettor. The ULTRA-TURRAX Tube Drive was set to run for five minutes. Once the proper speed was reached, the Combitip Plus tip was placed in the centre of the sample-loading hole in the ULTRA-TURRAX cap. The aqueous phase and bead mixture was dispensed into the spinning oil phase until all contents was removed from the Combitip Plus tip. After removing a new Combitip Plus tip

from its packaging, the end was cut off at the bevel with a razor blade. This cut tip was then placed on an Eppendorf Repeater Plus Pipette. 150 µL of the emulsion was gently dispensed into each well of a 96-well PCR plate, and the plate was sealed. The ePCR reaction was then run on the GeneAmp PCR System 9700 with the parameters set out in Table 2.5 below.

Stage	Step	Temperature	Time
Hold	Denature	95°C	5 minutes
40 cycles	Denature	93°C	15 seconds
	Anneal	62°C	30 seconds
	Extend	72°C	75 seconds
Hold	Final extension	72°C	7 minutes
Hold	-	4°C	∞

Table 2.5 ePCR parameters

After the ePCR program was finished, the plate was examined to ensure the emulsions remained intact before proceeding. The SOLiD Emulsion Collection Tray was placed on top of the 96-well plate and the pieces were sealed together using tape. This was then flipped upside down and centrifuged for two minutes at 550 x g. The 96-well plate was gently removed from the collection tray, which was placed in a fume hood. 10 mL of 2-butanol was added to the collection tray and pipetted until the solution was homogenous. The solution was then transferred to a 50 mL conical tube and the reservoir was rinsed to ensure all residual beads were collected. The solution was vortexed, then centrifuged at 2000 x g for five minutes. The 2-butanol-oil phase was then gently decanted and any residual oil phase was removed by placing inverted tubes on paper towels for five minutes.

The template beads were washed by adding 600 µL of 1x Bead Wash Buffer and letting the mixture soak for two minutes. The pellet was resuspended by gentle pipetting and transferred to a 1.5 mL LoBind Tube. The bottom of the tube that contained the template beads was rinsed with an additional 600 µL of 1x Bead Wash Buffer, and transferred to the LoBind Tube. This tube was vortexed, then centrifuged at 21,000 x g for one

minute. The top oil phase was removed with a pipette and the supernatant was removed and discarded. The pellet was resuspended by adding 150 µL of 1x Bead Wash Buffer to the tube, which was then vortexed and spun down. The mixture was then transferred to a new LoBind Tube. The bottom of the original tube was washed again with an additional 150 µL of 1x Bead Wash Buffer. This wash was then transferred to the new LoBind Tube. A further 1 mL of 1x Bead Wash Buffer was added to the tube which was vortexed, then centrifuged at 21,000 x g for one minute. The beads were then resuspended in 200 µL of 1x TEX Buffer and placed in a magnetic rack for one minute. When the solution cleared, the supernatant was removed and discarded. The beads were subsequently resuspended in 200 µL of 1x TEX Buffer. The template beads were then stored at 4°C in 1x TEX Buffer prior to quantification.

Using a NanoDrop ND-1000 instrument and a SOLiD Bead Concentration Chart, the volume of the beads was adjusted until it was in the optimal range (750,000-1.25 million beads / µL) and ready for enrichment. For each ePCR reaction, 1.8 mL of Denaturing Buffer was added to a 15 mL conical tube. 200 µL of denaturant was then added to the tube, which was vortexed. 4 mL of nuclease-free water was added along with 6 mL of glycerol and vortexed.

Separately, the enrichment beads were vortexed and 650 µL was transferred to a 1.5 mL LoBind Tube. The tube was centrifuged for two minutes at 21,000 x g after which the supernatant was removed and discarded. The enrichment beads were resuspended in 900 µL of 1x Bind & Wash Buffer, then centrifuged at 21,000 x g, with the supernatant removed and discarded. The resuspension and centrifugation steps were repeated once. The enrichment beads were then resuspended in 350 µL of 1x Bind & Wash Buffer. 3.5 µL of 1mM Enrichment Oligo was added to the enrichment beads tube then vortexed and spun down. The tube was rotated at room temperature for 30 minutes then centrifuged at 21,000 x g for two minutes and the supernatant was removed. The enrichment beads were resuspended in 900 µL of 1x TEX Buffer then centrifuged at 21,000 x g for a further two minutes and the supernatant was discarded and removed. The resuspension and centrifugation steps were repeated once.

The enrichment beads were then resuspended in 150 µL of 1x Low Salt Binding Buffer. The tube was placed in a magnetic rack for one minute and once the solution cleared, the supernatant was removed and discarded.

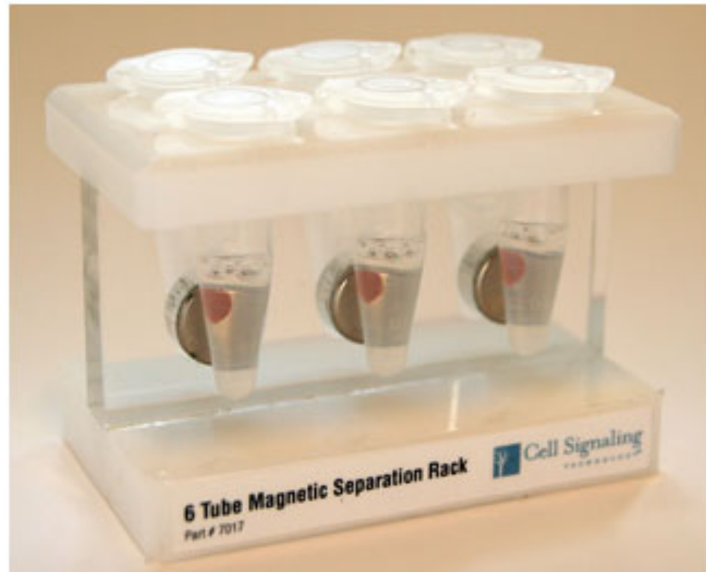


Figure 2.6 Magnetic rack.
This isolates beads from the remaining solution allowing for exchange or removal of solutions without loss of beads. Source: Cell Signalling Technology (www.cellsignal.com)

The template beads were resuspended in 300 µL of the prepared Denaturing Buffer and left for one minute. The tube was placed in a magnetic rack for one minute, once the solution cleared the supernatant was removed and discarded. This was repeated once and the template beads were resuspended in 300 µL of 1x TEX Buffer. The tube was again placed in a magnetic rack for one minute, and the supernatant removed and discarded once the solution cleared, and then repeated. The template beads were resuspended in 150 µL of 1x TEX Buffer then transferred into a new 0.5 mL LoBind Tube. The beads were then sonicated using the Covalent Declump 1 program on the Covaris S2 System. The enrichment beads were added to the template beads, vortexed and spun down. The Covalent Declump program 3 was run and the beads were spun down, incubated at 61°C for 15 minutes with a vortex and spin-down every five minutes during incubation. The beads were cooled on ice for two minutes. 600 µL of freshly

prepared 60% glycerol was added to a new 1.5 mL LoBind Tube and the bead mixture was gently pipetted on top of the glycerol solution. The tube was centrifuged at 21,000 x g for three minutes, after which the top layer of beads was transferred into a tube containing 1 mL of 1x TEX Buffer, avoiding the glycerol as much as possible. The tube was topped off with additional 1x TEX Buffer to the two mL mark and vortexed. The tube was centrifuged at 21,000 x g for one minute, the supernatant was removed and 400 µL of 1x TEX Buffer was added to the tube of beads and vortexed. The tube was then centrifuged for one minute at 21,000 x g after which the supernatant was removed and discarded.

400 µL of prepared Denaturing Buffer was used to resuspend the pellet and allowed to stand for one minute. The tube was placed in a magnetic rack for one minute until the supernatant was pure white or clear, at which stage the supernatant was removed and discarded. This was repeated until the supernatant was clear. The beads were resuspended in 400 µL of 1x TEX Buffer and placed in a magnetic rack for one minute and again the clear supernatant was removed. This was repeated once. The beads were then resuspended in 200 µL of 1x TEX Buffer, vortexed, spun down, and transferred to a new 1.5 mL LoBind Tube. The tube was rinsed with 200 µL of 1x TEX Buffer and the rinse placed in a new tube. The beads were sonicated using the Covalent Declump Program 1 and spun down. The tube was then placed in a magnetic rack for one minute, and the supernatant removed was when clear. The beads were resuspended in 400 µL of 1x TEX Buffer.

1 µL of 50 mM Bead Linker was added to 49 µL of 1x Low TE Buffer creating a 1 mM Bead Linker solution. The tube of P2 enriched beads were placed in a magnetic rack for one minute and the supernatant removed when clear. The beads were resuspended in 100 µL of 1x Terminal Transferase Reaction Buffer, and then transferred to a new tube, which was placed in a magnetic rack. After the supernatant was cleared and removed, the beads were resuspended in 100 µL of 1x Terminal Transferase Reaction Buffer and process repeated. The beads were then resuspended in 178 µL of 1x Terminal Transferase Reaction buffer and 20 µL of 1 mM Bead Linker solution was added. The beads were sonicated using Covalent Declump program 3 and 2 µL of Terminal Transferase was added. The tube was

vortexed and spun down and rotated for two hours at 37°C. The tube was then placed in a magnetic rack for one minute. When the supernatant cleared it was removed and discarded and beads were resuspended in 400 µL of 1x TEX Buffer, and the magnetic rack and resuspension procedure was repeated. The bead quantification procedure described earlier was repeated to ensure the concentration was within an accurate range and the beads were stored at 4°C in 1x TEX Buffer until bead deposition and sequencing took place.

2.3.4.3 Generation of sequencing data

The template beads were analysed on the SOLiD™ 4 platform, following a workflow analysis to determine the optimal library concentration and bead enrichment efficiency for the sequencing run. Briefly, the P2 enriched beads were sonicated using the Covalent Declump Program 1 on the Covaris S2 System. The tube of beads was then placed in a magnetic rack for one minute and the supernatant was removed once it cleared and the beads resuspended in 400 µL of SOLiD XD Slide Deposition Buffer v2, vortexed and spun down. The magnetic rack and supernatant clearing steps were then performed three times and resuspended in 400 µL of SOLiD XD Slide Deposition Buffer v2.

The slides were prepared and cleaned according to manufacturer's protocol and inserted into a SOLiD Opti Slide Carrier, which was then placed in the SOLiD Deposition Chamber. The Deposition Chamber was tightened with screws and the tabs flattened. The beads were sonicated using the Covalent Declump program 3, spun down and sonicated again. The template beads were then carefully deposited into the wells through the porthole. A 3 mm adhesive cover was placed over the portholes and the slid was centrifuged at 167 x g for ten minutes, then incubated at 37°C for one hour. The slide was then centrifuged for a further 10 minutes at 167 x g. The instrument reagents were then prepared and added.

Briefly, 1x Instrument Buffer, 1x T4 Ligase Buffer, Imaging Buffer, Universal Buffer and Cleave Solution 2.1 were installed in the SOLiD 4 Analyzer fluidics system. The instrument and Storage Buffer lines were primed and the slides were installed on the instrument following the

addition of enough SOLiD XD Slide Deposition Buffer v2 to cover the Deposition Chamber and enough Slide Storage Buffer to cover the slides. Once the slides were installed, reagent strips were thawed on ice, centrifuged at 160 x g for two minutes and placed in a chiller block once the temperature was below 10°C.

Using the SOLiD Instrument Control Software a run record was created, matching barcodes to libraries. Focus range was set to automatic. SOLiD sequencing is driven by cycles of ligation driven by a DNA ligase (Shendure and Ji, 2008). The template beads contain an adapter sequence. With sequencing by ligation, primers hybridise to the adaptor sequence on the template beads. Four fluorescently labelled two-base probes (octamers) are then added. These compete for ligation to the sequencing primer. The identity of specific positions on the octamer correlates with the colour of the fluorescent label. Once a fluorescent probe binds, the colour is acquired and the octamer is cleaved, removing the fluorescent label.

Repeated cycles of ligation take place which cover some of the positions on the template sequence (e.g. 2, 7, 12, 17 etc.) after which the extension product is removed and the template is reset, this time with a primer complementary to one position further back on the template (n-1, e.g. position 1, 6, 11, 16 and so on). This process is outlined in Figure 2.7 A-I below. These figures are courtesy of Andy Vierstrate (Source : http://users.ugent.be/~avierstr/nextgen/nextgen.html). Five rounds of resets are carried out to cover all bases of the template with two independent ligation reactions achieving very high accuracy (Shendure and Ji, 2008).

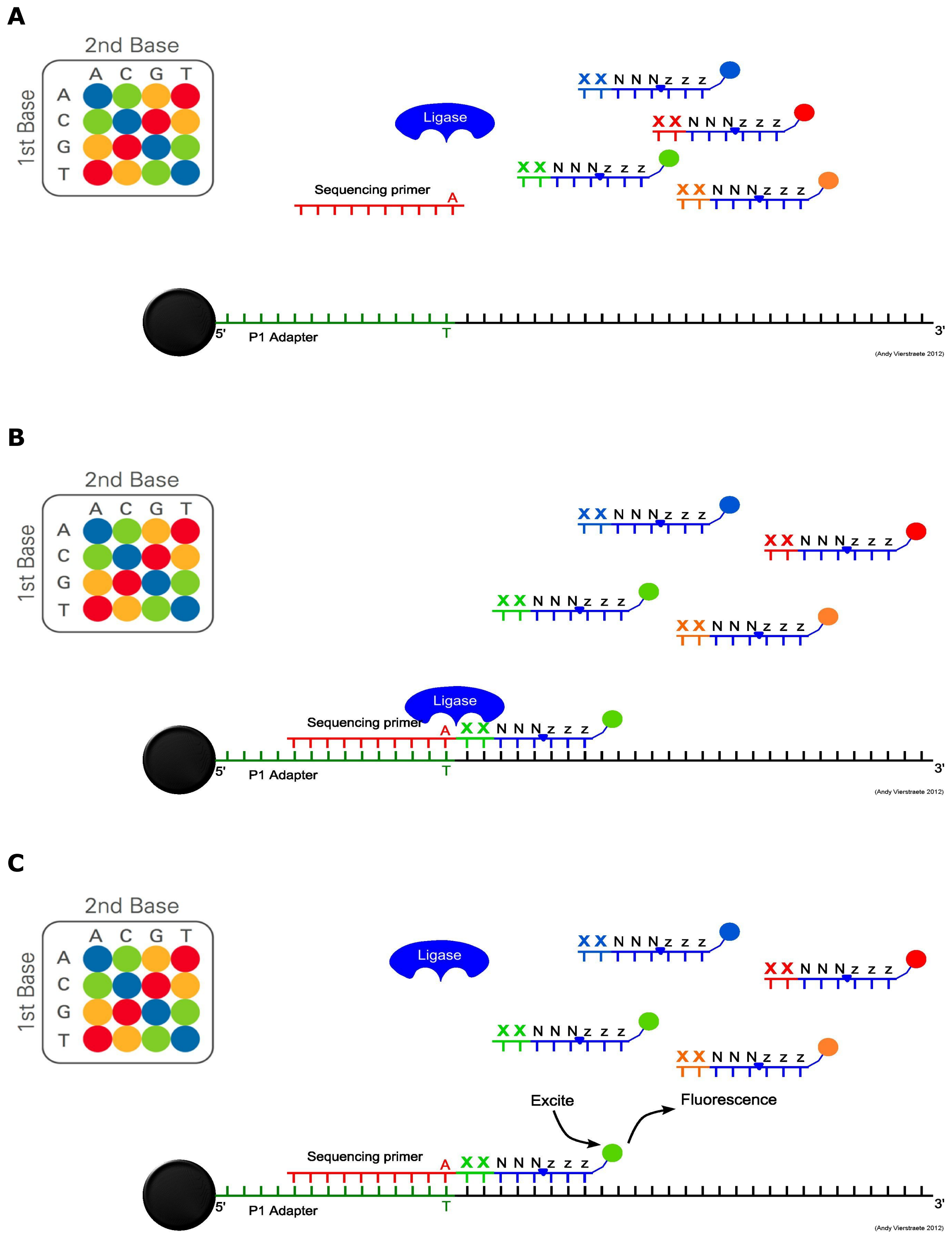
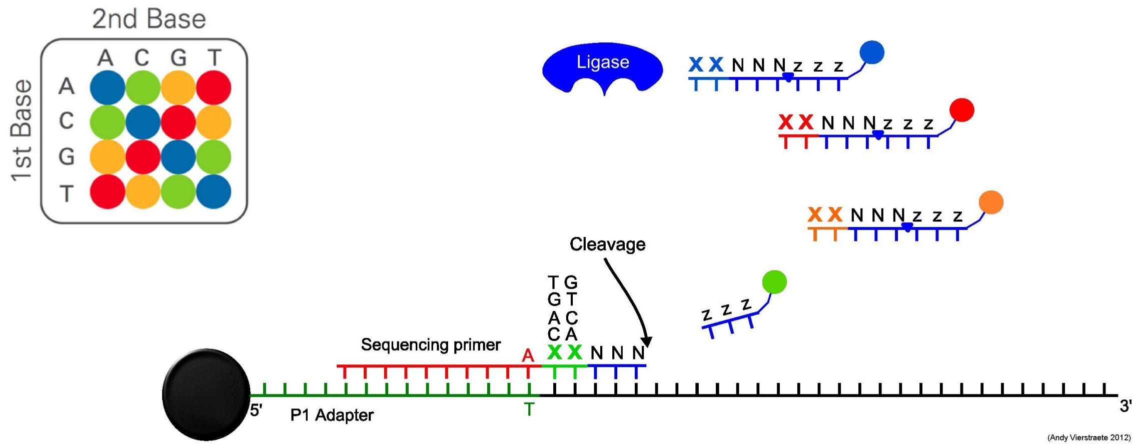


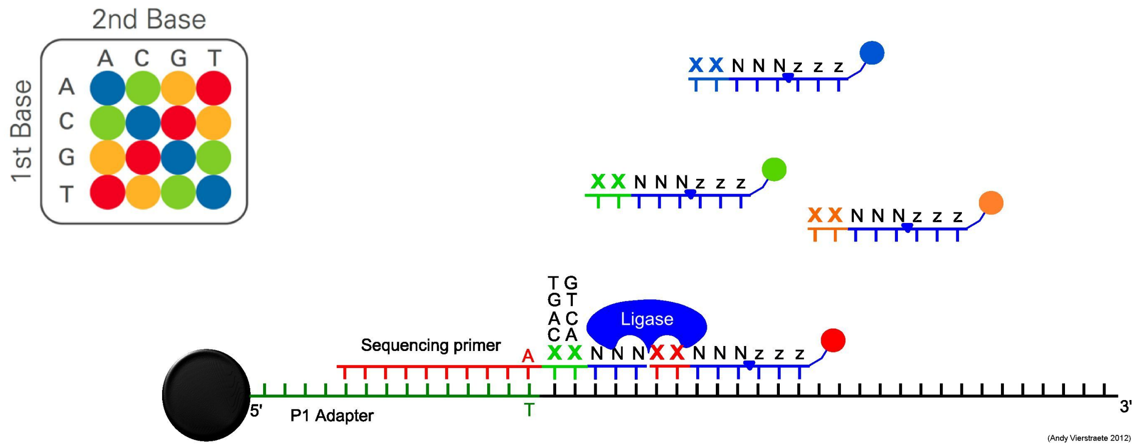
Figure 2.7 Sequencing by ligation

A: Templated beads, a sequencing primer, four fluorescently-labelled octamers and a DNA ligase in preparation for sequencing. B-C: DNA Ligase adds an octamer matching position 1-2 on template, the dye is excited and colour is acquired.

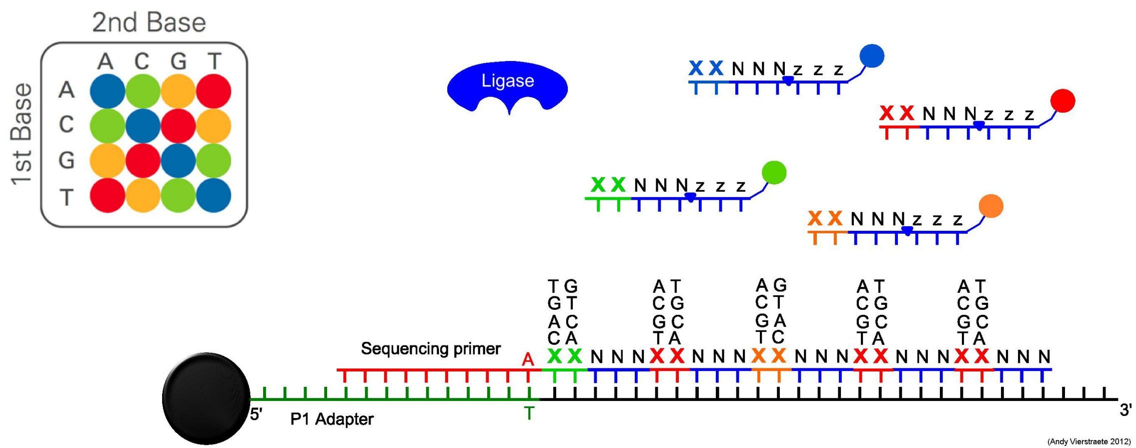
D



E

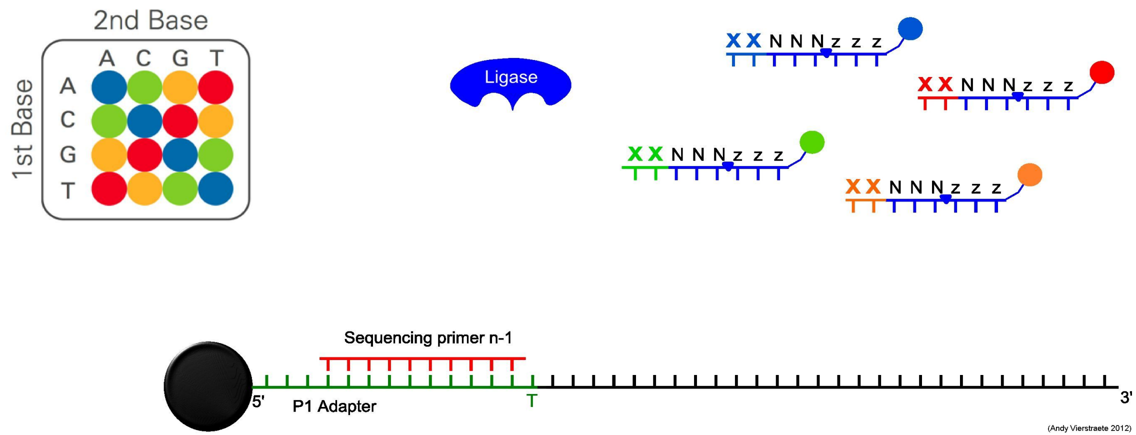


F

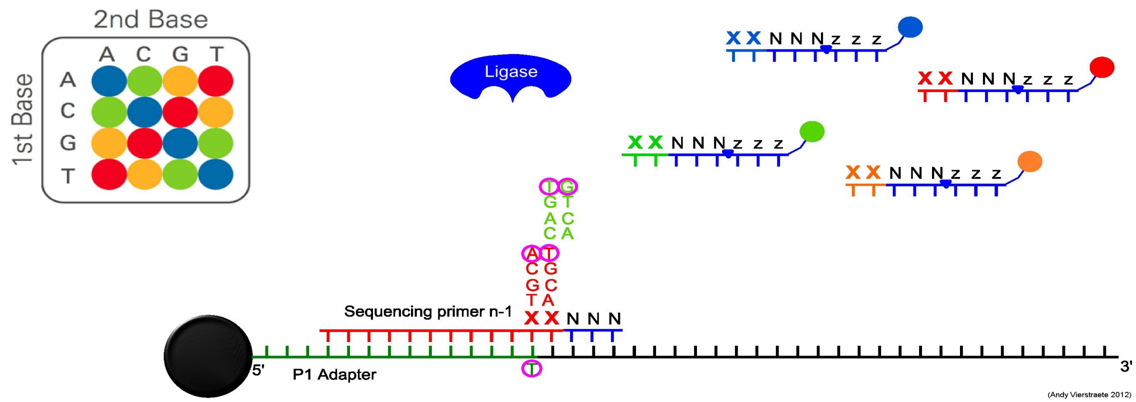


D: The octamer is cleaved off, and a new octamer probe ligates in position 6-7, the dye is excited and colour recorded. E-F: This is repeated for position 11-12, 16-17, 21-22 and template is reset.

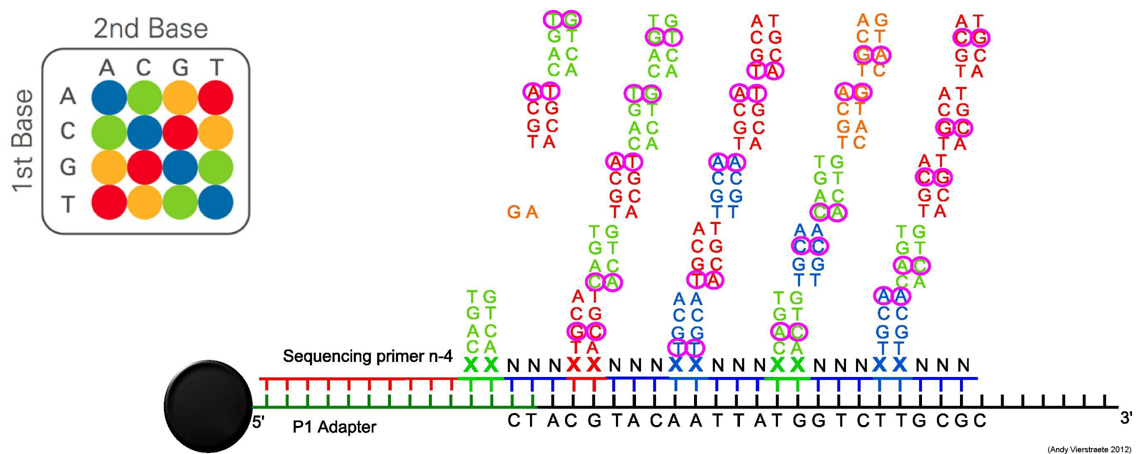
G



H



I



G: The template is reset and a new primer attaches at n-1 position. H-I: The cycle of ligation, fluorescence and cleavage is repeated as before, then again with primers at n-2, n-3, n-4. Two-base reading (based on colour chart) ensures high accuracy of sequencing.

2.3.5 microRNA cDNA synthesis

MicroRNAs are too short to accommodate standard primer pairs and probes for traditional PCR methods. They therefore need to be lengthened using a stem-loop primer to create cDNA fragments of >60 nucleotides (Kramer, 2011). This was achieved using stem-loop primers (Applied Biosystems, UK) and a TaqMan® microRNA reverse transcription kit (Applied Biosystems, UK) according to manufacturer's instructions.

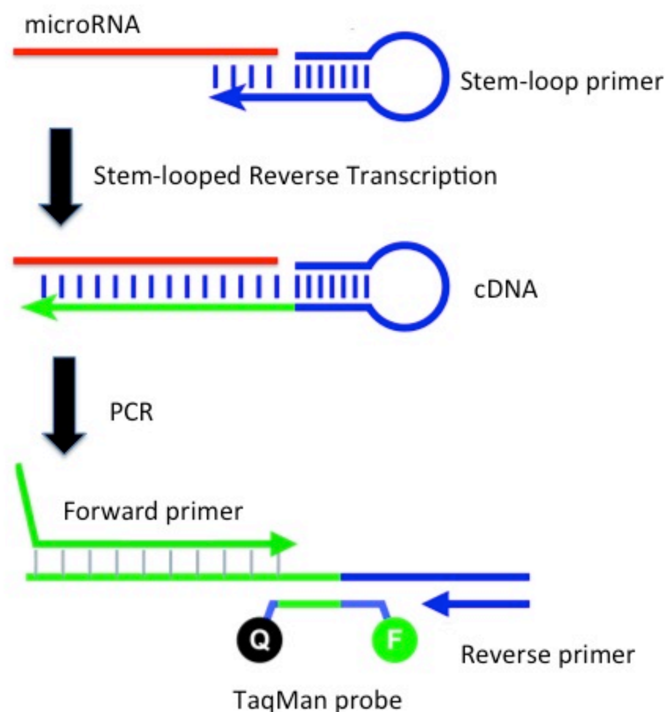


Figure 2.8 Stem-loop PCR

Stem-loop RT primers are annealed to microRNA segments and extended by reverse transcriptase. Once cDNA has been made, microRNA specific forward and reverse primer along with TaqMan probes are used for PCR reactions.

Initially, the TaqMan reagents were allowed to thaw on ice. A RT master mix was prepared in a polypropylene tube using the following components and volumes per sample; dNTP mix (100mM total) 0.075 µL, Multiscribe^{TM} RT enzyme (50 U/µL) 0.50 µL, 10x Reverse Transcription Buffer 0.75 µL, RNase Inhibitor (20 U/µL) 0.095 µL, Nuclease-free water

2.08 µL and 1.5 µL of 5x RT primer. The master mix was mixed gently and centrifuged. 5 µL of this master mix and 2.5 µL of microRNA were then added to each well in a 96-well PCR plate and the plates were run on a thermal cycler as outlined in Table 2.6 below. The end product was diluted with water in a 1:15 ratio.

Step	Temperature	Time
1	16°C	30 minutes
2	42°C	30 minutes
3	85°C	5 minutes
4	4°C	∞

Table 2.6 Thermal cycler parameters for microRNA cDNA synthesis

2.3.6 mRNA cDNA synthesis

Messenger RNA cDNA synthesis was performed on samples equalised with a RNA concentration of 10 ng/µL using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). All components of the kit were allowed to thaw on ice before preparing a master mix as outlined in Table 2.7 below.

Component	Volume (per reaction)
10xRT Buffer	2.0 µL
25c dNTP Mix (100mM)	0.8 µL
10x RT Random Primers	2.0 µL
Multiscribe™ Reverse Transcriptase	1.0 µL
Nuclease-free water	4.2 µL
Total	**10.0 µL**

Table 2.7 cDNA synthesis master mix components

An equal amount of master mix and sample (10 µL) were combined, vortexed and spun down. Two control reactions were also created, one with

no reverse transcriptase and one with no template (sample substituted with 10 μL of water). The samples were placed in the thermal cycler under the conditions outlined in Table 2.8 below. When complete, 20 μL of each sample was diluted with seventy μl of nuclease-free water and stored at -20°C until further analysis.

Step	Temperature	Time
1	25°C	10 minutes
2	37°C	120 minutes
3	85°C	5 minutes
Hold	4°C	∞

Table 2.8 cDNA synthesis thermal cycler parameters

2.3.7 Singleplex Real-Time PCR (RT-PCR)

For microRNA analysis, two-step singleplex quantitative RT-PCR was carried out on the StepOne Plus™ Real-Time PCR system (Applied Biosystems, UK) using TaqMan microRNA assays (Applied Biosystems, UK). TaqMan microRNA assays contain forward and reverse primers as well as a labelled probe for the target of interest, with a dye on the 5′ end and a quencher molecule on the 3′ end. During PCR the high temperature separates the DNA strands. As the temperature cools, the complimentary primers with fluorescent probes bind to the target sequence and Taq polymerase amplifies the target sequence. During the extension of the newly formed complimentary DNA the Taq polymerase cleaves off the fluorescent probe.

When released, the fluorescent dye, now less opposed by the quencher molecule, emits light, which is quantified by the computer. In each cycle this process repeats, increasing the level of fluorescence (Heid et al., 1996). When the level of fluorescence crosses a certain threshold, the machine will record the number of cycles required to reach this level, known as the cycle threshold (C_T) value. The StepOne Plus™ Real-Time PCR machine typically sets this threshold during the exponential phase, when the PCR reaction is most efficient.

In preparation for RT-PCR, the TaqMan assay and cDNA sample were allowed to thaw on ice, resuspended by gentle vortexing and spun down. A RT-PCR reaction mix was made to a total of 20 µL with the components outlined in Table 2.9 below.

Component	Volume per 20 µL reaction
TaqMan Small RNA assay (20x)	1.00 µL
cDNA product	1.33 µL
TaqMan Universal PCR Master Mix II, no UNG	10.00 µL
Nuclease free water	7.67 µL
Total volume	**20.00 µL**

Table 2.9 MicroRNA RT-PCR reaction mix components

20 µL of each reaction mix was then added to two wells on 96-well optical reaction PCR plate. The plate was covered with MicroAmp adhesive film, centrifuged for one minute and loaded into the StepOne Plus^{TM} PCR machine with the parameters listen in Table 2.10 below. At the end of RT-PCR the StepOne Plus^{TM} Software generated threshold cycle (C_T) values for the microRNAs of interest.

Controls with no template (cDNA product) were also added to each plate. In addition to microRNA targets of interest, a number of potential endogenous control assays were also run to facilitate the comparative C_T analysis.

Step	Temperature	Time
Enzyme activation	95°C	10 minutes
PCR (40 cycles)		
Denature	95°C	15 seconds
Anneal/extend	60°C	60 seconds

Table 2.10 MicroRNA Singleplex RT-PCR StepOne Plus^{TM} parameters

2.3.8 Multiplex RT-PCR

For mRNA gene expression analysis a two-step multiplex quantitative RT-PCR was carried out on the StepOne Plus™ Real-Time PCR system (Applied Biosystems) using TaqMan gene expression assays. Compared to singleplex RT-PCR, where only one target (be it the actual target of interest or endogenous control) are run on separate plates, multiplex RT-PCR has the advantage of analysing both the target of interest and an endogenous control in the same reaction. The TaqMan gene expression assays contain a primer for the target of interest containing a FAM™ labelled probe as well as a VIC™ labelled probe for the endogenous control glyceraldehyde-3-phosphatedehydrogenase (GAPDH).

Briefly, the components of the gene expression assay kit components were allowed to thaw on ice for at least thirty minutes. The multiplex RT-PCR reaction mix was the constituted as outlined in Table 2.11 below.

Component	Volume for one reaction
TaqMan Fast Advanced Master Mix 2x	5.0 µL
TaqMan Gene Expression Assay 20x (target gene assay and control)	2x 0.5 µL
cDNA product (diluted 2:7 with nuclease-free water)	4.0 µL
Total volume per reaction	**10 µL**

Table 2.11 mRNA multiplex RT-PCR reaction mix components

Step	Temperature	Time
UNG incubation	50°C	2 minutes
Enzyme activation	95°C	10 minutes
PCR (40 cycles)		
Denature	95°C	15 seconds
Anneal/extend	60°C	60 seconds

Table 2.12 mRNA multiplex RT-PCR StepOne Plus™ parameters

10 µL of the reaction mix was added to two wells on a 96-well plate and repeated for each cDNA product. The plate was covered with MicroAmp adhesive film, centrifuged for one minute and loaded into the StepOne Plus PCR machine with the parameters listen in Table 2.12.

At the end of RT-PCR the StepOne Plus™ Software generated threshold cycle (CT) values for the mRNAs of interest. Control samples with either no template, or no reverse transcriptase, as well as a random human DNA panel were run as additional controls.

2.3.9 Gene expression analysis

The comparative CT (or $2^{-\Delta\Delta CT}$) method was used to assess gene expression for both microRNA and mRNA analysis. There are two main methods of assessing gene expression, the comparative CT method and the absolute quantification method, where the PCR signal is related to a standard curve. The comparative CT method compares relative gene expression between two different samples, such as treated vs. controls or before and after treatment rather than absolute quantification (Livak and Schmittgen, 2001). The comparative CT method is convenient as no standard curve is required, it eliminates dilution errors, and uses up less well space. It assumes that the amplification efficiency of the target gene and the control gene is 100%. The comparative CT is derived from the following formulas:

$\Delta\Delta CT = \Delta CT(\text{baseline sample}) - \Delta CT(\text{end of treatment (EOT) sample})$

Where $\Delta CT(\text{any sample}) = CT(\text{reference gene}) - CT(\text{target gene})$

$\Delta\Delta CT = (CT(\text{reference gene baseline}) - CT(\text{target gene baseline})) - (CT(\text{reference gene EOT}) - CT(\text{target gene EOT}))$

The ratio of the target gene (from baseline to EOT) is calculated by taking $2^{-\Delta\Delta CT}$. This ratio is based on the premise that the amount of PCR product is proportional to 2^{CT}. The ratio of our target gene to our reference gene can therefore be written as $2^{CT(\text{Reference})} / 2^{CT(\text{Target})}$ which can also be

written as $2^{CT(Reference)} - 2^{CT(Target)}$. If we were to calculate a ratio between baseline and EOT samples, corrected for reference, this would be $2^{CT(Reference\ baseline)-CT(Target\ baseline)} / 2^{CT(Reference\ EOT)-CT(Target\ EOT)}$ which using the same formulas as above equates to $2^{-\Delta\Delta CT}$.

This ratio is also known as the fold change reflecting whether there is increased or decreased amount of a gene between two time-points, or between treatment and controls. The reference sample (housekeeping gene) always has a ΔΔCT value of zero. $2^{-\Delta\Delta CT}$ of zero =1, which is no fold change and the standard to which the other samples are referenced, or relatively quantified.

2.4 Phenotyping methods

2.4.1 Patient and control recruitment

The majority of the patients discussed in this thesis were participants in a randomised controlled trial of two forms of ECT, the EFFECT-Dep trial (Semkovska et al., 2016). In addition, healthy controls were recruited separately as part of other studies. Both patients and controls gave blood samples and underwent phenotyping focusing on depression and measures of cognition.

2.4.1.1 The EFFECT-Dep Trial

The EFFECT-Dep (Enhancing the eFFectiveness of ElectroConvulsive Therapy in severe Depression) Trial is a recently completed randomised controlled trial carried out in St. Patrick's University Hospital Dublin and Trinity College Institute of Neuroscience, Trinity College Dublin (Semkovska et al., 2016). Recruitment began in May 2008 and was completed in October 2012. The primary objective of the trial was to test the hypothesis that right unilateral (RUL) ECT, delivered at 6x seizure threshold, was not inferior to bitemporal (BT) ECT, delivered at 1.5x seizure threshold, for the treatment of moderate to severe depression. Depression severity was measured by the HDRS-24. Secondary objectives included whether RUL ECT had a cognitive advantage over BT ECT. Apart from the main trial, various

independent projects and studies have arisen from the large amounts of data, both clinical and genomic, generated from the trial. The studies in this thesis use data generated from the EFFECT-Dep trial but are not in themselves randomised controlled trials.

2.4.1.1.1 EFFECT-Dep Trial Design

The EFFECT-Dep Trial was a pragmatic, two-group, parallel, double-blind randomised controlled non-inferiority trial comparing high-dose right unilateral with standard-dose bitemporal ECT. It was pragmatic in that it followed 'real-world' practices such as maintaining patients on their psychotropic medications before and during the trial and letting treating clinicians and patients determine the total number of treatments. Patients, who were referred from three different sites, were randomly allocated to a course of BT ECT (1.5 x seizure threshold) or RUL ECT (6.0 x seizure threshold). Groups were stratified to ensure minimal differences between age, referral site and history of previous ECT. Referring clinicians, participants and raters were blinded to treatment.

2.4.1.1.2 EFFECT-Dep inclusion and exclusion criteria

Inclusion criteria for the EFFECT-Dep Trial were patients aged 18 or over diagnosed with major depressive disorder (DSM-IV) referred for ECT. Exclusion criteria included any condition rendering patients medically unfit for general anaesthesia or ECT, treatment with ECT in the past six months, a co-morbid axis 1 disorder, dementia, alcohol or other substance abuse in the preceding six months or being unable or unwilling to consent to either ECT or the trial itself. During the trial period 475 patients were assessed for eligibility, see Figure 2.9 below.

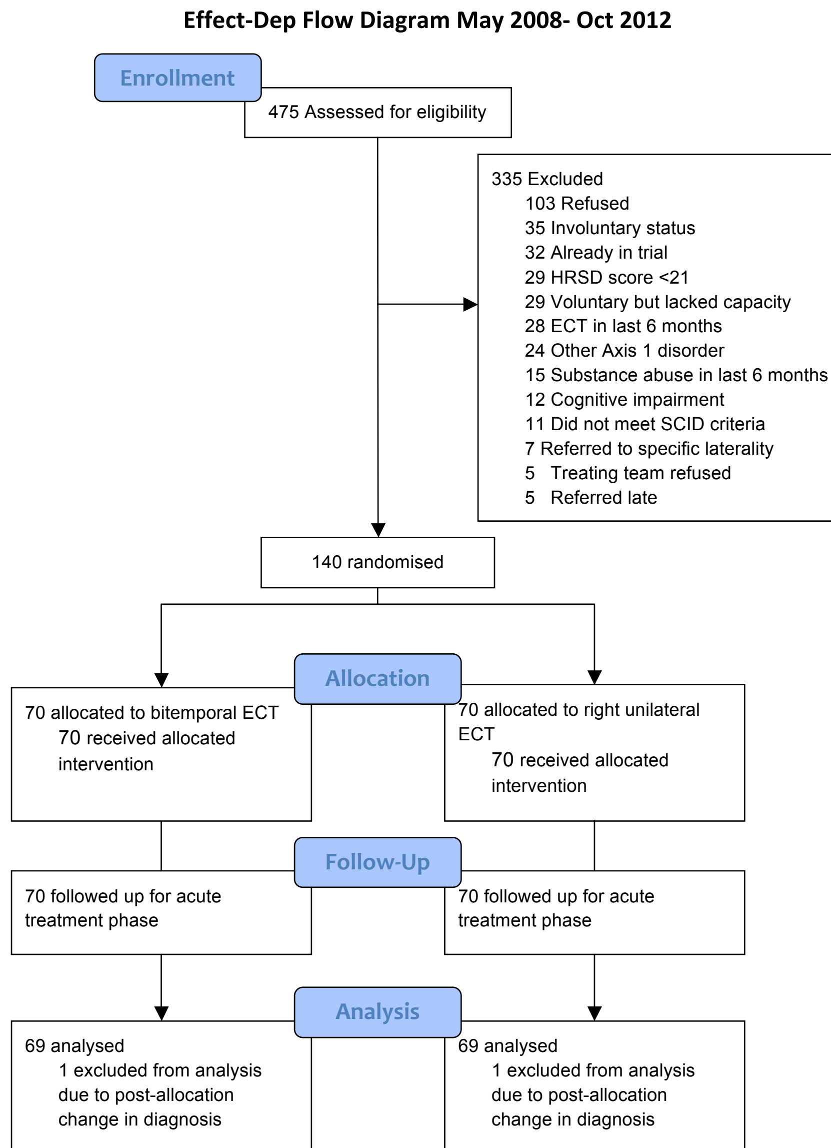


Figure 2.9 EFFECT-Dep Recruitment

2.4.1.1.3 EFFECT-Dep study site and population

The 138 patients who enrolled in the EFFECT-Dep trial all received ECT in St. Patrick’s University Hospital (SPUH), Dublin, Ireland. The hospital was founded in 1747 by Jonathan Swift and is the largest independent not-for-profit psychiatric hospital in Ireland. Patients were recruited from either St. Patrick’s University Hospital itself, St. Edmundsbury Hospital (also part of St. Patrick’s Mental Health Services) and from St. James’ Hospital, a public

hospital with whom SPUH has historic links. SPUH also receives tertiary referrals from hospitals nationwide including referrals from services that are not able to provide ECT. These patients were all admitted to SPUH and remained in-patients during their treatment period.

The ECT service in SPUH is accredited by the UK Royal College of Psychiatrists' ECT Accreditation Service (ECTAS) and has been awarded with a rating of excellence. SPUH carries out over a third of all ECT programmes nationally on an annual basis (Mental Health Commission, 2015).

2.4.1.1.4 EFFECT-Dep ECT administration

ECT in the EFFECT-Dep trial was administered twice weekly using hand-held electrodes on the Spectrum 5000M device (Mecta Corp., Oregon, USA) with a maximum output of 1200 milicoulombs (mC).

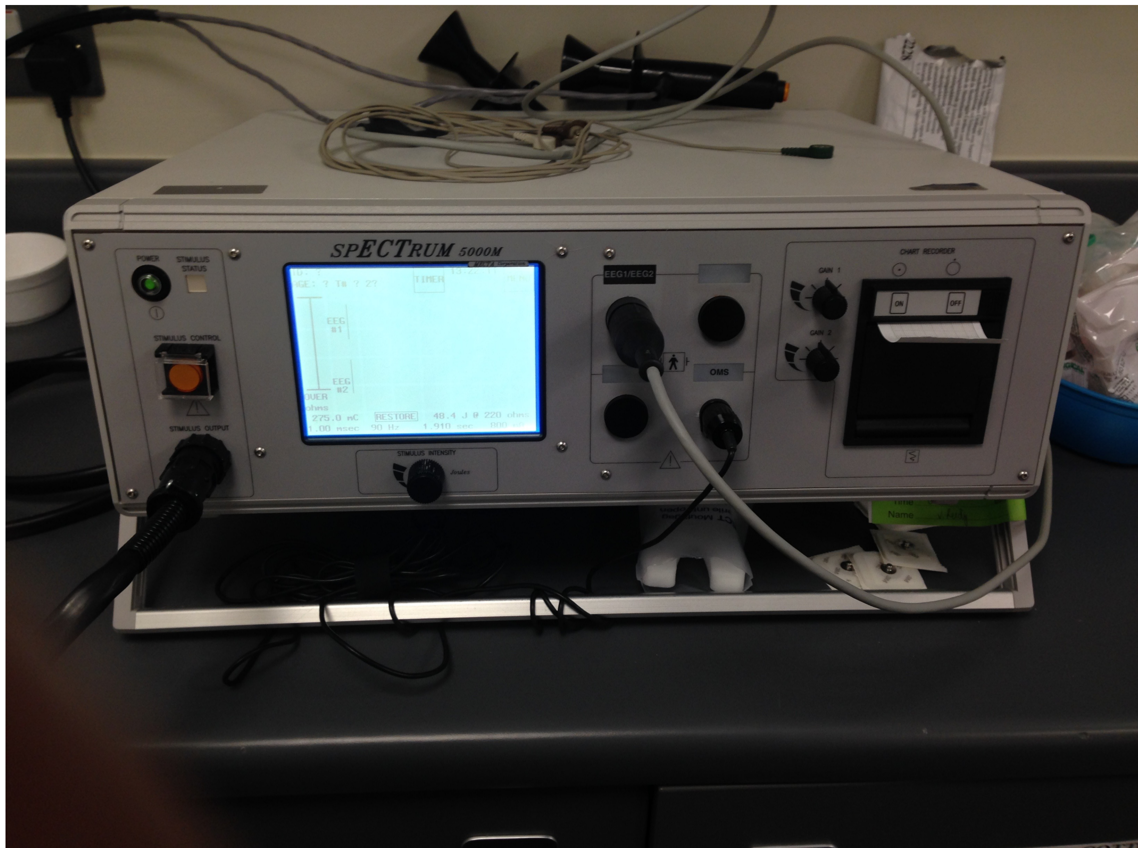


Figure 2.10 The Spectrum 5000M (MECTA corporation, USA)

The electrode positions were bitemporal and right unilateral (d'Elia) as described in Chapter 1, section 1.2.6.1. Methohexitone (0.75–1.0 mg/kg), a short-acting barbiturate was used for anaesthesia. Suxamethonium (0.5-1.0 mg/kg) was used as a muscle relaxant. Seizure duration was recorded both visually and by electroencephalogram (EEG). Seizure thresholds were established by means of a stimulus dosing protocol; see Table 2.13 below.

Level	Threshold Dose (mC)	1.5x Threshold Dose (BT ECT)	6x Threshold Dose (RUL ECT)
1	25	50	150
2	50	75	300
3	75	125	450
4	100	150	600
5	150	225	900
6	250	375	Max
7	350	550	Max
8	500	750	Max
9	750	1000	Max

Table 2.13 EFFECT-Dep Stimulus Dosing Protocol.

In the EFFECT-Dep Trial stimulus dosing protocol the first step involves establishing the Seizure Threshold Dose (minimum charge required to invoke a therapeutic seizure). The lowest charge available on the Spectrum 5000M device is 25mC. This would be the first step attempted unless the patient had certain treatment factors known to increase seizure threshold. For each of these factors the patient was moved up one level for their first stimulus. These factors were i) BT ECT, ii) Male sex, iii) Age >65, iv) Currently on anti-epileptic mediation, v) ECT in previous month. A patient could have three stimulations in one treatment session. Once the Threshold Dose was established, they were treated at the relevant Therapeutic Dose (1.5x or 6x Threshold Dose).

2.4.1.1.5 EFFECT-Dep quality control procedures

The EFFECT-Dep Trial recruited patients from May 2008 to October 2012. Over this time-period a number of group members were involved in assessment delivery. To ensure these assessments were carried out in a standardised fashion all group members underwent training in the relevant instruments prior to assessing patients. The primary outcome of the EFFECT-Dep Trial and the studies in this thesis was the HDRS-24. Particular effort was used for this outcome measure, including six-monthly inter-rater reliability assessments. This involved group members watching pre-recorded videos of HDRS-24 assessments and scoring this independently. Inter-rater reliability (Pearson’s r) for the study period was consistently above 0.86; see Figure 2.9 below. All raters were blind to treatment allocation. Several levels of data checking were employed to minimise data entry errors or miscalculation of scores. These levels included each rater first correcting their own assessments. A different individual then crosschecked each member’s assessments fortnightly. Any errors discovered at this level were discussed and corrected. Every four weeks the principal researcher ensured all these checks were completed and correct, and only these data were then entered into the final database. Six-monthly statistical checks were also carried out on the database identifying potential outliers or input errors.

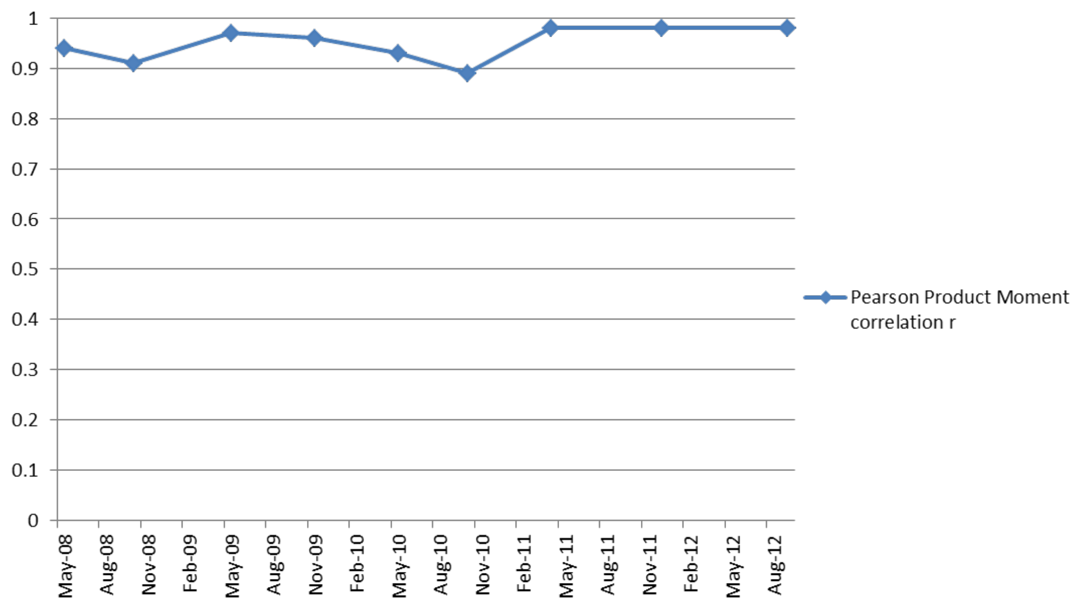


Figure 2.11 Inter-rater reliability for HDRS-24 (Pearson's r)

2.4.2 Healthy controls

Healthy controls were recruited by placing an advertisement in a national newspaper as well as on the research group's webpage and internally in Trinity College Dublin. Exclusion criteria for healthy controls were any history of any psychiatric disorder as well as any current haematological or inflammatory disorder. Depression severity was assessed as described earlier with HDRS-24. Cognitive measures included the ACE-R and the Trail-making Tests.

2.4.3 Ethics

The studies outlined in this thesis were undertaken according to the Declaration of Helsinki and Good Clinical Practice principles. The local research ethics committees approved both the EFFECT-Dep Trial and the stand-alone projects that make up this thesis. All patients and healthy controls provided written informed consent after receiving both written and oral information about their respective study. All patients and controls were free to withdraw from any of the study at any time.

The majority of assessments and blood collection took place in St. Patrick's University Hospital, but some were completed in the other EFFECT-Dep recruitment centres, and some were completed in the community where this was requested by the patient. After completing an assessment the original paper copy containing identifying information was stored in a locked cabinet in St. Patrick's University Hospital. All phenotypic data were entered into an electronic database where each patient was assigned a coded identification number. No identifying information such as address, hospital record number or date of birth was entered into the database. All blood samples were identified with the same coded identification number rather than names or other identifying information. Blood samples were stored in St. James' Hospital, Dublin or Trinity College Institute of Neuroscience, Dublin.

2.5 Statistical and bioinformatic considerations

2.5.1 Statistical analysis

Data were analysed using SPSS version 22.0 (IBM Corporation, NY), R version 3.1.3 (R Foundation for Statistical Computing, 2014) and GraphPad Prism 6 (GraphPad Software, CA). Baseline clinical and demographic factors are presented as means with standard deviation (SD), or number per group (% of group) where appropriate, unless otherwise specified. Categorical data were tested using chi-square test (χ^2) unless otherwise specified. All data were tested for normality and log-transformed where appropriate. Data with normal distribution were analysed using student's t-test or analysis of variance (ANOVA). Where covariates were identified that correlated with the outcome of interest, an analysis of covariance (ANCOVA) was run.

Changes over time were investigated with a mixed design ANOVA / General Linear Model (GLM) (repeated-measures) with time (baseline / end of treatment) and psychosis status (yes/no) or other categorical variable as factors. Non-parametric data were analysed with the Wilcoxon-Signed Rank test for paired comparisons or the Mann-Whitney U test for non-paired comparisons.

For correlational analysis of microRNA/mRNA levels and clinical and cognitive outcomes, data were analysed with Pearson's product-moment correlation coefficient (Pearson's r) for normally distributed variables, and Spearman's rho for non-parametric distributions. Baseline correlations were explored using multiple regression where appropriate, and end of treatment correlations were explored using ANCOVA to correct for baseline scores.

A p-value of less than 0.05 was considered statistically significant. However, where multiple hypotheses were applied to one dataset, they were corrected using Bonferroni or similarly conservative techniques.

2.5.2 Power

The EFFECT-Dep Trial was powered to detect (with 80% power, using a one-sided equivalence t-test at 5% level) that the mean reduction in HDRS-24 for RUL ECT was no more than four points less than the reduction seen

with BT ECT with 69 patients in each group. This was based on a previous large bitemporal ECT series (Petrides et al., 2001) and generally accepted levels of clinical relevance. Data from the EFFECT-Dep Trial were incorporated in the meta-analysis in Chapter 3, which had added power from the six additional studies identified in the systematic review, particularly for cognitive measures, which EFFECT-Dep was not necessarily powered to assess.

In Chapters 4 and 5, the studies of microRNA changes in peripheral blood following ECT had >80% power to detect a 2-fold change in microRNA levels between baseline and end of treatment (EOT). This was based on the only previous published study of changes in peripheral blood microRNA levels following antidepressant therapy (Bocchio-Chiavetto et al., 2013).

In Chapter 6, it is estimated that there is over 80% power to detect a statistically significant difference in mRNA levels between patients and controls with around 26-42 subjects in each group. For repeated measures tests, 16 subjects are required to have 80% power. This is based on results from two previous peripheral blood mRNA studies involving *VEGFA* (Berent et al., 2014, Iga et al., 2007).

With regard to potential correlational analyses in Chapters 5-6, our group has previously reported medium-large mean effect sizes (e.g. -1.1) for sub-acute impairments in episodic memory and executive functioning soon after completing ECT (Semkovska and McLoughlin, 2010). ECT has a similar therapeutic effect size (0.91) (UK ECT Review Group, 2003). Sample sizes of at least sixty per group are sufficient to achieve a power of 80% with up to six predictors to detect a medium to large correlation (Harris, 2001).

2.5.3 Bioinformatic analysis

2.5.3.1 Deep sequencing data analysis

The deep sequencing study (Chapter 4) provided millions of reads from the samples. A 'read' is a string of nucleotides (corresponding to the bases of the microRNA fragment that was sequenced) as well as a quality score reflecting the accuracy of the read. The accuracy of the reads was verified

and enhanced using the SOLiD Accuracy Enhancement Tool (Applied Biosystems, UK). This tool attempts to correct any misreads prior to read-mapping.

The goal of read-mapping is to match sequenced reads to a reference genome, identifying microRNAs in a sample. This was performed using the SHRiMP alignment package (Computational Biology Lab, University of Toronto, Canada). This package was specifically designed to analyse short reads like those generated by microRNAs, and is optimised for the SOLiD system. A number of the generated reads represented adaptor sequences or unwanted contaminants (e.g. mitochondrial DNA or other small non-coding RNA species). These unwanted sequences were matched to a 'junk' database and removed. Reads that remained after these processes was mapped against the full complement of non-coding RNA species in the human genome. This was obtained from ENSEMBL (Cunningham et al., 2015). ENSEMBL is a joint project between the Wellcome Trust Sanger Institute and the European Bioinformatics Institute (Cambridge, UK), maintaining a database of sequences for microRNAs as well as other gene sequences.

After microRNA species were identified through read-mapping, they were quantified through gene counting. Gene counting was performed using HTSeq (EMBL Heidelberg, Germany), a software tool to process data from high-throughput sequencing assays. HTSeq calculates the number of reads that map to certain genomic features, i.e. a set of microRNA genes, and presents these values as raw gene counts.

After raw gene counts were established, the next step involved a differential expression analysis to identify which microRNAs had levels that were significantly altered. This was performed using the Bioconductor® library DESeq (EMBL Heidelberg, Germany). The Bioconductor package (www.bioconductor.org) is a valuable open source resource for the analysis of high-throughput biology, most especially in the field of gene expression analysis. DESeq takes as input a table of raw gene counts as produced by HTSeq. Differential expression analysis was carried out successively on each pair of samples under comparison with a threshold of $\log_2$-fold change of one (this represents a doubling/halving of gene counts). Previous studies of peripheral microRNA expression found a significant change in the region of

ten to forty microRNAs in depression (Bocchio-Chiavetto et al., 2013, Belzeaux et al., 2012, Garbett et al., 2015). We therefore expected to identify at least twenty differentially expressed microRNAs in this sample of patients treated with ECT, the most powerful antidepressant treatment available.

Any microRNAs differentially expressed will be submitted for further analysis. Experimentally validated genes targeted by differentially expressed microRNAs will be obtained from the online databases listed in Table 2.14 below.

Database name	URL
TarBase 7.0	http://diana.imis.athena-innovation.gr/DianaTools/index.php
miRTarBase	http://mirtarbase.mbc.nctu.edu.tw

Table 2.14 Databases used to identify validated microRNA targets

The potential functions and processes of any experimentally validated gene targets were explored using Gene Ontology (GO) terms in the latest GO Consortium database (Gene Ontology Consortium, 2015). The Gene Ontology project is aimed at creating a set of consistent structured language or vocabulary (ontology) to describe gene products (Harris et al., 2004). The GO terms of experimentally validated genes targeted by differentially expressed microRNAs were then carried out using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) Gene List Analysis Tool (Mi et al., 2013). Subsequent analysis including entering lists of high and low confidence experimentally validated gene targets into The Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2007).

2.6 Contribution to studies

The studies that make up this thesis are separate from the larger EFFECT-Dep Trial in which nine different researchers have contributed to the overall data collection and analyses from 2008 to 2016. I joined the research team

in January 2011 and was not involved in the original design of the EFFECT-Dep Trial. However, since joining the team I was actively involved in patient recruitment, patient assessments, blood sampling, data analysis as well as delivery of ECT.

I recruited and carried out baseline assessments on 18.4% and administered ECT to 16.0% of the EFFECT-Dep patients whose data were included in this thesis. In addition, I collected nearly all of the blood samples used in Chapters 4 and 5, as well close to half of the blood samples used in Chapter 6. With regard to healthy controls, I recruited and carried out 62.1% of the assessments and collected 66.1% of the blood samples.

With regard to the laboratory work, I carried out all the experimental work described in this thesis, with the exception of sections 2.3.4.1-2.3.4.3, which was carried out by Dr Paul Smyth, Senior Medical Scientist, and the initial steps of section 2.5.3.1, which was carried out by Dr Gordon Blackshiels, Bioinformatician / Post-doctoral Research Fellow, both working with Professor Orla Sheils in the Department of Histopathology and Morbid Anatomy in St. James' Hospital, Dublin. Professor Sheils' team have particular experience in deep sequencing as well as access to the high performance computing platform required for the analysis of raw deep sequencing data, and I am very grateful for their guidance and support.

Chapter 3
Results

3 Bitemporal versus high-dose right unilateral electroconvulsive therapy for depression: a systematic review and meta-analysis

3.1 Introduction

Since its development in Italy in 1938, electroconvulsive therapy (ECT) has remained the most acutely effective treatment for severe depression (UK ECT Review Group, 2003). Depression is the second leading cause of years lived with disability worldwide (Vos et al., 2012). Pharmacotherapy and psychotherapy can be effective, but about 30% of patients do not respond to standard treatments (Rush et al., 2006). Many of these patients might benefit from ECT. Indeed, about 1.4 million people worldwide are treated annually with ECT, with treatment-resistant depression being the most common indication in Western industrialised nations (Leiknes et al., 2012).

Over the years ECT has been, and continues to be, refined with the aim of maintaining clinical effectiveness while minimising cognitive side-effects. Cognitive side effects continue to be the single most controversial aspect of ECT and remain an area of concern to psychiatrists and patients alike. Variations in ECT waveform, dose, frequency of administration and electrode placement may go some way to explain the differences that are seen in patient outcomes (Semkovska and McLoughlin, 2010).

Originally, ECT was delivered with a sine-wave stimulus with a long pulse-width (8.3ms). This is an inefficient form of electrical stimulation, using higher amounts of energy than required for neurons to discharge (Squire and Zouzounis, 1986). Sine-wave ECT was gradually replaced in most parts of the world by the square-wave brief-pulse (0.5-1.5ms) stimulus. The development of brief-pulse ECT led to a reduction in cognitive side-effects but maintained efficacy (Loo et al., 2012b).

A potential new refinement has been ultra-brief pulse (<0.5ms pulse-width) ECT. The ultra-brief pulse stimulus is closer in duration to the neuronal chronaxie (a measure of the length of stimulus required for a neuron to discharge) of neurons, which is estimated to be 0.1-0.2ms

(Squire and Zouzounis, 1986). A recent meta-analysis indicated that ultra-brief ECT may have an advantage in terms of cognitive side-effects, however, this was at a significant cost in antidepressant efficacy (Tor et al., 2015). Brief-pulse ECT is therefore likely to continue to remain a widely used form of ECT in the near future (Spaans et al., 2013).

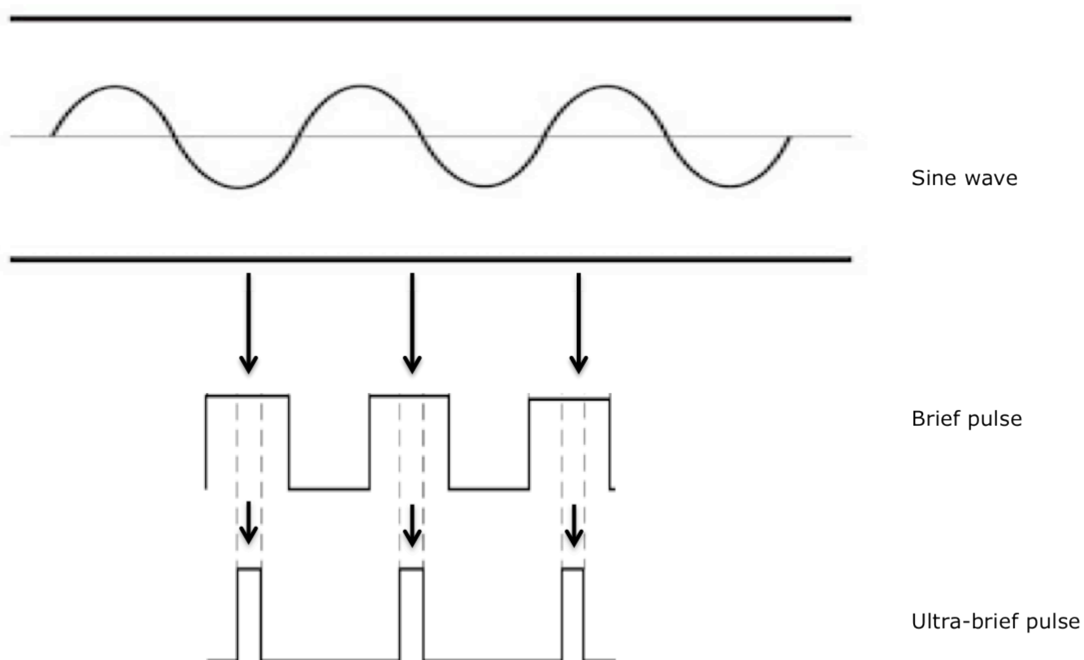


Figure 3.1 Different electrical stimuli used in ECT
From top: Sine wave ECT (8.3 ms), brief-pulse ECT (0.5-1.5 ms), ultra-brief pulse (<0.5 ms). Dotted line represents chronaxie.

With regard to electrode placement, although the bitemporal electrode placement remains most commonly used worldwide, right unilateral (d'Elia) placements is preferred in some countries (Leiknes et al., 2012). In 1949 Goldman first reported the use of right unilateral ECT in an attempt to spare the speech areas of the brain (Goldman, 1949). He found it to be equally as effective as bitemporal ECT, but also to cause markedly less confusion. The first controlled trial of right unilateral versus bitemporal ECT was not published until 1958 (Lancaster et al., 1958). This study found a significantly faster return of orientation and recall with right unilateral ECT with no significant difference in depression scores (Lancaster et al., 1958). Over subsequent years numerous studies with varying techniques and

procedures produced conflicting results and failed to settle the unilateral versus bitemporal 'controversy' (Janicak et al., 1985, Pettinati et al., 1986).

It was not until the publication of a series of studies from the USA, starting in 1987, that it became clear that the effectiveness of right unilateral ECT depends on the strength of the electrical dose above the seizure threshold (ST), being relatively ineffective at doses nearer threshold (i.e. 1.0-2.5 x ST) used in bitemporal ECT (Sackeim et al., 1987, Sackeim et al., 1993, McCall et al., 2000). This also demonstrated that generalised seizures were necessary but not sufficient for clinical response. The UK ECT Review Group (2003) concluded that high-dose ECT was more effective than low-dose ECT, but at that stage there were not enough studies to ascertain whether high-dose unilateral ECT was as effective as bitemporal ECT, or whether it was associated with less cognitive side-effects (UK ECT Review Group, 2003).

Only one randomised controlled trial at that time had compared high-dose (>6 x ST) right unilateral to bitemporal ECT (Sackeim et al., 2000). Since then however, several further randomised controlled trials have been carried out in this area. The underlying hypotheses of these trials were that high-dose unilateral ECT would match the efficacy of bitemporal ECT while maintaining a cognitive advantage (McCall et al., 2002, Ranjkesh et al., 2005, Sackeim et al., 2008, Sackeim et al., 2009, Kellner et al., 2010, Semkovska et al., 2016).

An alternative to unilateral or bitemporal electrode placement is bifrontal ECT, where the electrodes are placed over the frontal lobes, sparing both temporal lobes, (Letemendia et al., 1993). Meta-analytic evidence to date suggests bifrontal ECT is not more effective than the other placements and still requires further characterisation (Dunne and McLoughlin, 2012).

To date, there has been one meta-analysis comparing the cognitive effects of brief-pulse right unilateral compared to bitemporal ECT, but this did not examine clinical efficacy, and was not limited to randomised controlled trials (Semkovska et al., 2011). The United States Food and Drug Administration (FDA) carried its own systematic review and meta-analysis of various forms of ECT (Food and Drug Administration, 2011). However, the only comparison including high-dose right unilateral versus bitemporal

ECT was for depression scores; this only included four studies and the report was not published in a peer-review journal. The UK National Institute for Health and Clinical Excellence (NICE) have also published meta-analytic data comparing bitemporal and high-dose unilateral ECT as part of their latest depression guidelines (NICE, 2010). However, this meta-analysis combined studies of bifrontal and bitemporal ECT into a 'bilateral' group, as well as combining studies using brief pulse and ultra-brief pulse ECT. One of the studies included also compared ECT in a group of patients that had already failed to respond to moderate-dose right unilateral ECT (Tew et al., 2002). Together, these issues make it difficult to differentiate differences in efficacy and cognitive side effects that are due to pulse width from electrode placement.

3.1.1 Aims of the study

Given the new data from recent trials, and the lack of a complete meta-analysis separating out high from low to medium-dose right unilateral ECT, there is a need for a new review of this area. I therefore carried out a systematic review and meta-analysis to include randomised controlled trials comparing the efficacy and cognitive side-effects of brief-pulse high-dose right unilateral and brief-pulse bitemporal ECT for adults treated for depression.

3.2 Methods

3.2.1 Search strategy

The PubMed, PsycInfo, Web of Science, Cochrane Library and Embase databases were searched from inception up to the 1st March 2016 with the terms “electroconvulsive” or “electroshock” and “trial” with no language limits. Reference lists of relevant articles were manually searched for any further studies. In addition, The International Clinical Trials Registry Platform was searched, using the same terms as above, for any unpublished trials that may be underway. The studies were entered into bibliographic software (EndNote 7, Thomson Reuters, UK) for further analysis.

Inclusion criteria were (i) prospective randomised controlled trials comparing (ii) low to moderate dose (1.0-2.5 x ST) bitemporal ECT to high-dose (5-8 x ST) right unilateral ECT for (iii) unipolar or bipolar subjects diagnosed with a major depressive episode according to DSM-III, DSM-IV, or ICD-10 or primary depression according to Research Diagnostic Criteria (Feighner et al., 1972) (iv) aged 18 and above using (v) brief-pulse ECT and (vi) a standardised measure of depression for its primary outcome.

Trials of ultra-brief pulse ECT were excluded, as meta-analytic evidence suggests this differs from brief-pulse ECT both in terms of clinical efficacy and cognitive side-effect profile (Tor et al., 2015). Cognitive outcomes were included where trials adopted a pre-post design with an objective measure of cognitive performance. Reorientation time (which has no pre-ECT measure) was also included, as this has been shown to differ between electrode placements in previous trials (Kellner et al., 2010).

3.2.2 Data extraction

Following exclusion of duplicate records and studies that were clearly not eligible on abstract review, two reviewers independently screened the remaining full-text records. If inclusion criteria were met, data were independently extracted, cross-checked and any discrepancies resolved by consensus. Where possible, scores that had been adjusted for covariates that might influence outcomes, such as baseline depression severity was used. Outcomes where at least three studies reported data were included. In cases where data were not extractable, authors were contacted, or data were estimated from graphs. Risk of bias was assessed using The Cochrane Collaboration’s Risk of Bias Tool (Higgins et al., 2011).

3.2.3 Statistical analysis

All statistical analyses were performed using RevMan 5.3 (The Nordic Cochrane Centre, Copenhagen). For the variable reorientation time, which only had an outcome at end of treatment, effect sizes were based on raw mean differences. For continuous data, with both baseline and end of treatment data, mean change scores were used. Effect sizes were based on

standardised mean differences (SMD) as different versions of rating scales were employed across studies.

RevMan calculates SMDs based on Hedges's *g*, which provides a superior estimate of SMD in small sample sizes (Borenstein, 2009). For remission, response and relapse, risk ratios were created. Remission was defined as at least a 60% reduction on the Hamilton Depression Rating Scale (HDRS) scores with a final score below 10 (HDRS-24) or 12 (HDRS-21). Response was defined as at least a 60% reduction on the HDRS and a final score below 17 maintained for at least one week after the end of ECT. Relapse was defined as ≥10 points increase on the HDRS-24 compared to the end of treatment score plus a HDRS-24 score of ≥16. In the study using the HDRS-21, relapse was defined as more than 50% increase in HDRS scores from end of treatment and a score ≥15 on two consecutive occasions. In addition, this increase should be maintained over two interviews at least one week apart. Hospital admission for worsening of depressive symptoms also constituted a relapse.

As studies varied in terms of exact dose above threshold, frequency of ECT administration, maximal output of ECT machines and other treatment parameters (see Table 3.1 below), a random effects model with inverse variance was used throughout (DerSimonian and Laird, 1986). Heterogeneity was measured using the I^2 statistic (Higgins and Green, 2008). Where there was significant heterogeneity, a post-hoc sensitivity analysis was performed to identify the impact of individual studies on the whole group. This involved removing studies that differed from other studies on parameters that could be predicted a priori to affect outcome, such as dose above seizure threshold. Alternatively, studies that visually were outliers were removed on a one-study removed basis, and the effect on heterogeneity and pooled effect size were observed on an informal basis. I did not perform formal statistical analysis on these sensitivity analysis subgroups in line with recommended practice (Higgins and Green, 2008). A funnel plot analysis of publication bias was not carried out, as there were not enough studies to make this meaningful (Lau et al., 2006).

3.3 Results

3.3.1 Search results

The most recent search was completed on the 1st of March 2016. This search resulted in 13567 potentially relevant records after duplicate records were removed. Following abstract screening of these records, 147 full-text records were reviewed for inclusion. 140 records were excluded after further review, the reasons for which are summarised in Figure 3.2 below.

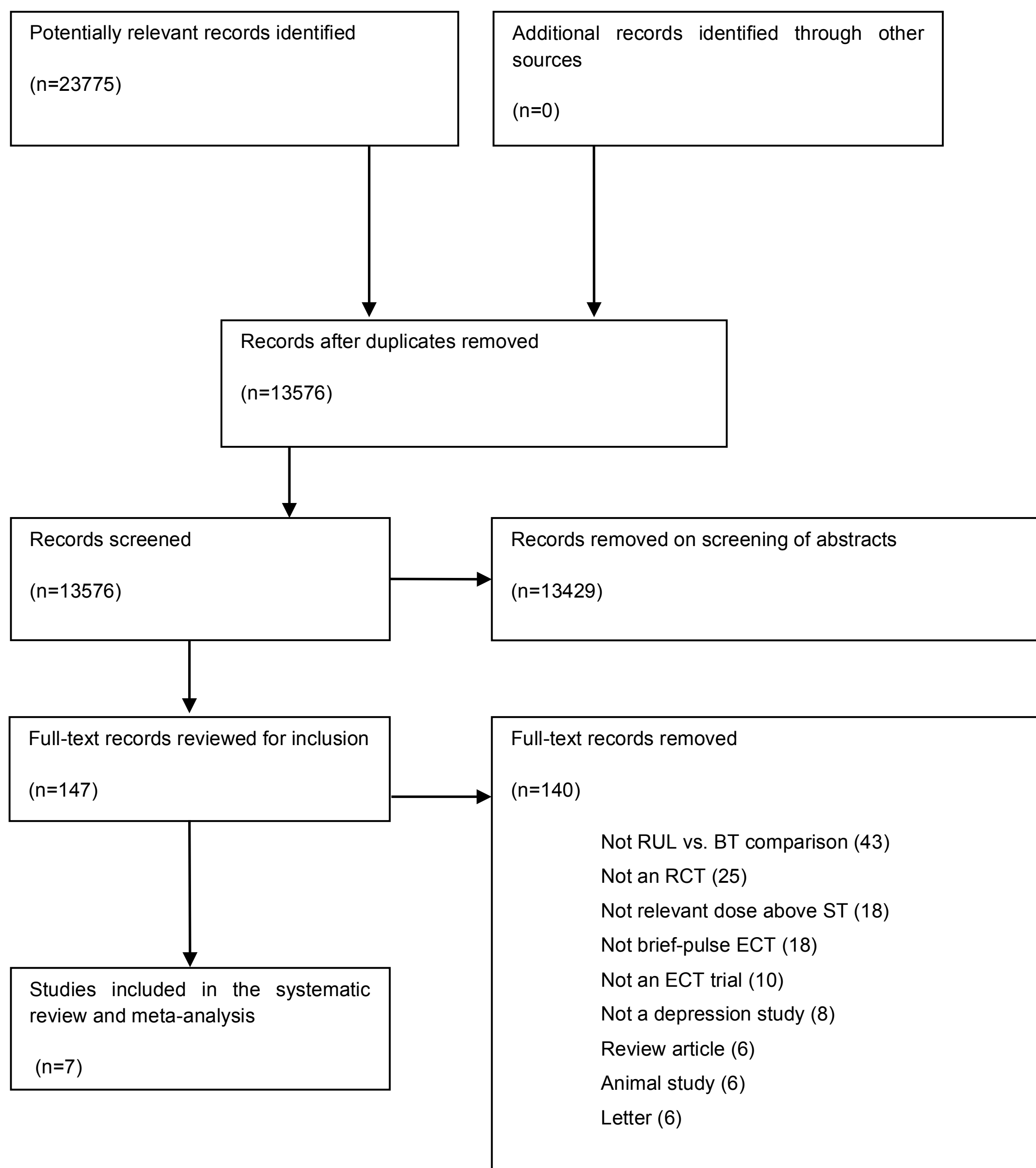


Figure 3.2 Flow diagram of search results

Flow diagram designed using PRISMA template (Moher et al., 2009).

This left a total of seven randomised controlled trials meeting the inclusion criteria (Sackeim et al., 2000, McCall et al., 2002, Ranjkesh et al., 2005, Sackeim et al., 2008, Kellner et al., 2010, Sackeim et al., 2009, Semkovska et al., 2016). A description of the seven studies can be found in Table 3.1 below. In five studies, where some data were not extractable, the original authors were contacted and four of these responded, providing the relevant data requested (Sackeim et al., 2000, Sackeim et al., 2008, Sackeim et al., 2009, Kellner et al., 2010).

	Sackeim 2000	McCall 2002	Ranjkesh 2005	Sackeim 2008	Sackeim 2009	Kellner 2010	Semkovska 2016
N=	40	77	26	45	319	149	138
Study country	USA	USA	Iran	USA	USA	USA	Ireland
Mean age (SD)	54.4 (15.9)	57.3 (16.4)	33.7 (12.4)	49.1 (16.5)	49.0 (15.7)	53.8 (15.0)	56.7 (14.8)
Female %	67.5%	63.6%	61.5%	57.8%	63.6%	63.1%	63.0%
% Bipolar	32.5%	NS	23.1%	35.6%	20.7%	18.8%	23.2%
Previous ECT	40%	NS	NS	31.1%	NS	NS	38.4%
Duration of illness in weeks (SD)	46.6 (35.9)	25.1 (20.4)	NS	102.1 (125.0)	37.9 (34.0)	127.8 (114.4)	31.6 (52.0)
Educational attainment (years)	14.6 (3.2)	12.7 (3.5)	NS	15.0 (3.0)	13.6 (2.9)	NS	13.1 (3.4)
Number of previous episodes	3.7 (3.3)	2.6 (1.7)	NS	3.0 (3.5)	NS	4.7 (12.1)	5.7 (4.8)
Treatment-resistant depression (%)	57.5%	80%	NS	NS	NS[¶]	NS	71%
Number of medication trials	6.3 (6.3)	NS	NS	5.5 (3.5)	5.2 (3.5)	NS	2.6 (1.5)
HDRS version (items)	24	21	24	24	24	24	24
Remission criteria (HDRS)	60% reduction and final score <10 (on 2 consecutive occasions)	60% reduction and final score <12	NS	60% reduction and final score <10 (on 2 consecutive occasions)	60% reduction and final score <10 (on 2 consecutive occasions)	60% reduction and final score <10 (on 2 consecutive occasions)	60% reduction and final score <10 (on 2 consecutive occasions)
Number of trial centres	1	1	1	1	3	4	1
Drug washout prior to ECT	Yes (BZD allowed)	Yes (BZD allowed)	Yes (BZD allowed)	Yes (BZD allowed)	Yes* (BZD allowed)	Yes	No
Days to assessment after last ECT	1-7	1-3	1	1-7	1-8	1-7	1-3
ECT Treatments per week	3	3	3	3	3	3	2

Mean number ECT sessions (SD)	8.3 (2.1)	5.8 (NS)	8.0 (NS)	7.3 (2.7)	8.1 (4.4)	NS	7.8 (2.5)
Multiple of seizure threshold • RUL • BT	6x 2.5x	8x 1.5x	5x 1.0x	6x 2.5x	6x 1.5x	6x 1.5x	6x 1.5x
Pulse width (ms)	1.5	1.0	1.0	1.5	NS	1.0	1.0
Anaesthetic medications	Atropine 0.4 mg Met 0.75 mg/kg Sux:0.75 mg/kg	Met 1.0 mg/kg Sux 1.0 mg/kg	NS	Atropine 0.4 mg Met 1.0 mg/kg Sux 0.75 mg/kg	Atropine 0.4-0.6 mg Gly 0.2-0.4 mg Met 0.75-1.0 mg/kg Sux 0.75-1.0 mg/kg	Etomidate Met,Prop Sux.Thiopental (Doses NS)	Met 0.75-1.0 mg/kg Prop 0.75-2.0 mg/kg Sux 0.5-1.0 mg/kg

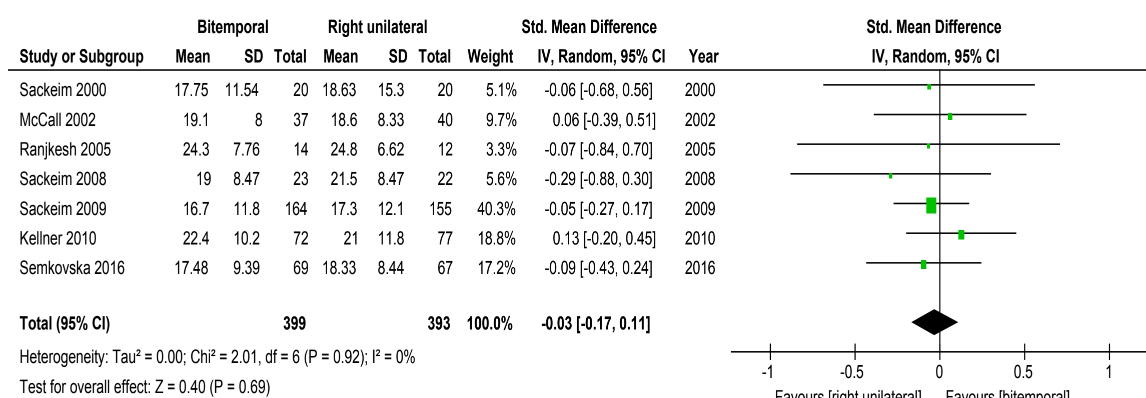
Table 3.1 Summary of studies included in the meta-analysis.

Data are presented as counts, means (standard deviations) or percentages (%). Treatment resistance was measured with versions of the Antidepressant Treatment History Form (ATHF). Abbreviations: BT: bitemporal ECT; BZD: benzodiazepines; ECT: electroconvulsive therapy; Gly: Glycopyrrolate; HDRS: Hamilton Depression Rating Scale; Met: methohexital; ms: milliseconds; NS: not stated; Prop: propofol; RUL: right unilateral ECT; Sux: Suxamethonium. ¶Total number of adequate medication trials (ATHF): 1.3; *After initial drug washout patients in this trial were randomised to concomitant pharmacotherapy with venlafaxine, nortriptyline or placebo.

3.3.2 Efficacy

3.3.2.1 Efficacy: change in depression rating scores

All included trials used a version of the Hamilton Rating Scale for Depression (HDRS) before and after either high-dose right unilateral (n=393) or bitemporal (n=399) ECT. Overall, there was no significant difference between the two treatments in pre-post HDRS change score (Hedges's g=-0.03, 95% confidence interval (CI)= -0.17 to 0.11, p=0.69, I^2=0%), see Figure 3.3.



	Bitemporal			Right unilateral				Std. Mean Difference	
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	Year
Sackeim 2000	17.75	11.54	20	18.63	15.3	20	5.1%	-0.06 [-0.68, 0.56]	2000
McCall 2002	19.1	8	37	18.6	8.33	40	9.7%	0.06 [-0.39, 0.51]	2002
Ranjkesh 2005	24.3	7.76	14	24.8	6.62	12	3.3%	-0.07 [-0.84, 0.70]	2005
Sackeim 2008	19	8.47	23	21.5	8.47	22	5.6%	-0.29 [-0.88, 0.30]	2008
Sackeim 2009	16.7	11.8	164	17.3	12.1	155	40.3%	-0.05 [-0.27, 0.17]	2009
Kellner 2010	22.4	10.2	72	21	11.8	77	18.8%	0.13 [-0.20, 0.45]	2010
Semkovska 2016	17.48	9.39	69	18.33	8.44	67	17.2%	-0.09 [-0.43, 0.24]	2016
Total (95% CI)			**399**			**393**	**100.0%**	**-0.03 [-0.17, 0.11]**	

Heterogeneity: Tau² = 0.00; Chi² = 2.01, df = 6 (P = 0.92); I² = 0%
Test for overall effect: Z = 0.40 (P = 0.69)

Figure 3.3 Forest plot: HDRS-24

Forest plot of standardised mean differences in HDRS-24 from baseline to end of treatment.

3.3.2.2 Efficacy: Remission, response and relapse

Six trials included data on remission status following high-dose right unilateral (n=383) or bitemporal (n=385) ECT. Three trials reported response rates (Sackeim et al., 2000, Sackeim et al., 2008, Semkovska et al., 2016). Overall remission rates were 51.7% (95% CI 46.7 to 56.7) in the high-dose right unilateral group and 53.2% (95% CI 48.3 to 58.2) in the bitemporal group. There was no statistically significant difference in the relative risk (RR) of achieving remission (RR 1.06; 95% CI 0.93 to1.20, p=0.41, I^2=0%), or response (RR 0.93; 95% CI 0.74 to 1.16) between the two treatments (see Figures 3.4-3.5 below).

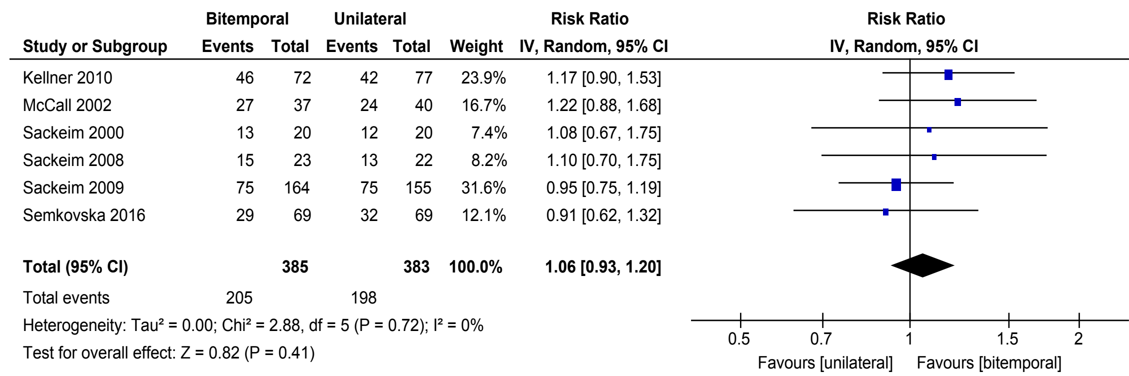


Figure 3.4 Forest plot: Remission at end of treatment

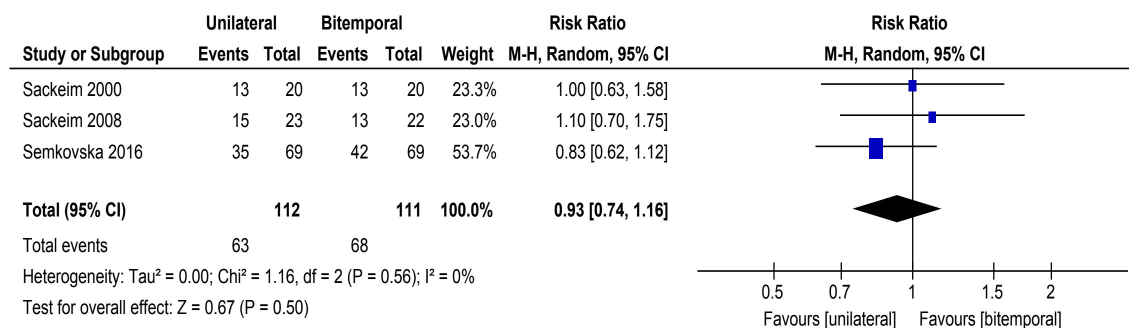


Figure 3.5 Forest plot: Response at end of treatment

With regard to relapse, two trials reported relapse rates at 12 months (Sackeim et al., 2000, Sackeim et al., 2008) and one further trial also monitored for relapse for one year following treatment (data not published) (Semkovska et al., 2016). Overall relapse rates within the first year following ECT were 34.6% (95% CI 22.6-48.7) in the high-dose right unilateral group and 49.1% (95% CI 35.6-62.8) in the bitemporal group. There was no statistically significant difference in the relative risk of relapse at 12 months after treatment between the two treatments (RR 0.78, 95% CI 0.56-1.08, p=0.13, I^2=0%), see Figure 3.6 below. Only two trials had extractable 6-month relapse rates and were therefore not analysed.

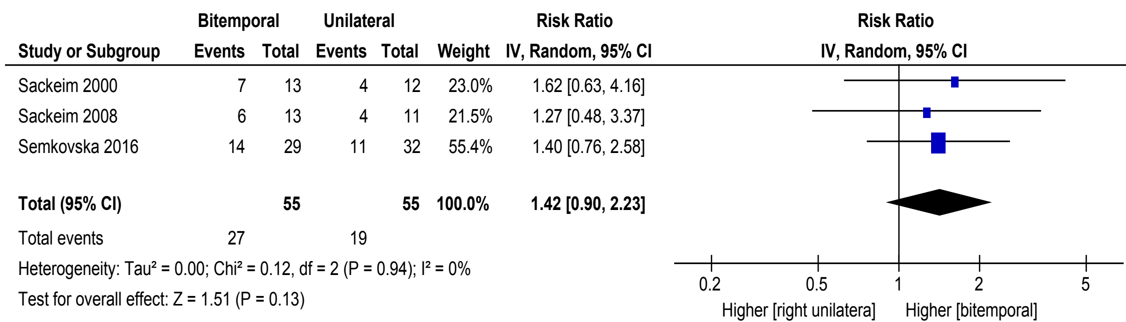


Figure 3.6 Forest plot: Relapse at 12 months

3.3.3 Cognitive side effects

3.3.3.1 Cognitive side effects: Overview

The cognitive side effects of ECT remain an area of concern to physicians and patients alike. Immediate disorientation following ECT is a recognised effect, typically resolving in the first hour after ECT (Sackeim et al., 1986). Meta-analytic evidence shows that in the first few days after brief-pulse ECT there is impairment in a wide range of anterograde cognitive tests, but these normalise and often improve after two to three weeks (Semkovska and McLoughlin, 2010). Bitemporal ECT has been found to have larger deficits in global cognition, delayed verbal memory as well as retrograde autobiographical memory when compared to unilateral ECT. Also, higher doses of unilateral ECT have been associated with decreases in verbal learning, delayed verbal memory, visual recognition and semantic memory retrieval (Semkovska et al., 2011). With regard to long-term retrograde memory less is known due to both a lack of randomised controlled trials with long-term follow-up but also the lack of an agreed measure of remote memory (Freeman, 2013, Semkovska and McLoughlin, 2013, Jelovac et al., 2015).

There was considerable variation in the cognitive assessments performed between the included studies, including number, cognitive domains and time points of tests, which restricted what could be meta-analysed. For example, six studies contained a measure of global cognition such as the Mini Mental State Examination (MMSE) at the end of treatment

(Sackeim et al., 2000, Ranjkesh et al., 2005, Sackeim et al., 2008, Sackeim et al., 2009, Kellner et al., 2010, Semkovska et al., 2016), whereas only one contained the n-back test (Sackeim et al., 2009). Outcomes assessed by fewer than three studies were excluded. In general, data was limited for long-term follow-up, and the results therefore reflect the short-term cognitive outcomes after ECT.

3.3.3.2 Cognitive side-effects: reorientation time

Three trials measured time taken to recover orientation following ECT sessions, see Figure 3.7 below. Reorientation was defined as correctly answering four out of five questions (name, location, age, date of birth and day of the week) after each ECT treatment (Sobin et al., 1995). Overall, patients receiving high-dose right unilateral ECT (n=106) recovered reorientation approximately 8 minutes quicker than those receiving bitemporal ECT (n=109), (mean difference = -8.28, 95% CI -12.86 to -3.70, p=0.0004). There was no significant heterogeneity (I^2=0%).

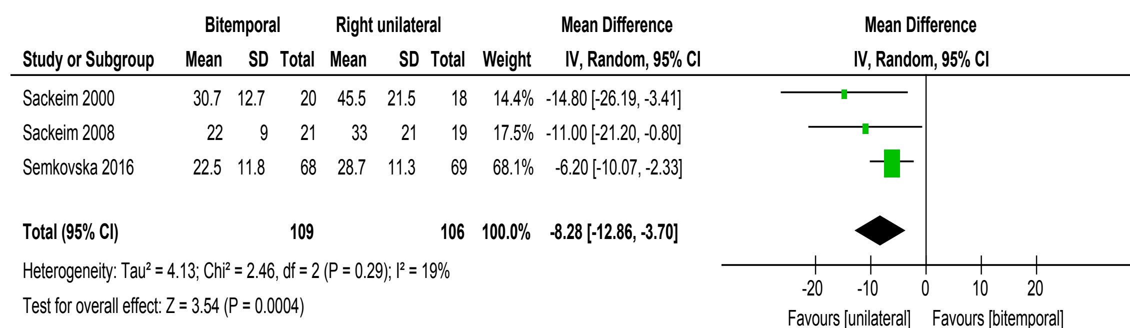


Figure 3.7 Forest plot: Reorientation times after ECT

3.3.3.3 Cognitive side-effects: Global cognition

Six trials measured global cognition, see Figure 3.8. Three trials (Ranjkesh et al., 2005, Kellner et al., 2010, Semkovska et al., 2016) used the original version of the Mini-Mental State Examination (MMSE) (Folstein et al., 1975). The other three trials (Sackeim et al., 2000, Sackeim et al., 2008, Sackeim et al., 2009) used a modified version of the MMSE (Mayeux et al.,

1981). There was no statistically significant difference between those receiving high-dose right unilateral (n=295) and bitemporal (n=281) ECT (Hedges's g=-0.03, 95% CI=-0.19 to 0.014, p=0.75). There was no significant heterogeneity (I^2=0%).

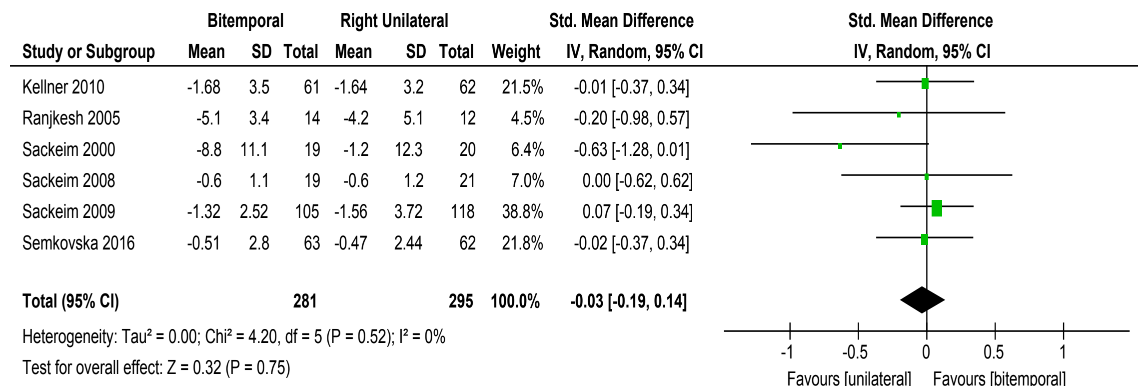


Figure 3.8 Forest plot: Measures of general cognition

3.3.3.4 Cognitive side-effects: Delayed visual recall in complex figure tests

Five trials provided data for performance on delayed complex figure tests, which reflect retrieval of visual memory (Figure 3.9). These tests involve copying a complex geometric figure and then reproducing it from memory either immediately or after a 20-30 minute delay (delayed recall) (Lezak, 2012). Different versions of complex figures including the Rey-Osterrieth Complex Figure test, the Taylor Complex Figure and the Medical College of Georgia Complex Figures were used to avoid practice effects (Lezak, 2012, Spreen and Strauss, 1998). There was no significant difference between those receiving high-dose right unilateral (n=178) and bitemporal (n=166) ECT (Hedges's g=-0.02, 95% CI=-0.24 to 0.20, p=0.84). There was no significant heterogeneity (I^2=1%).

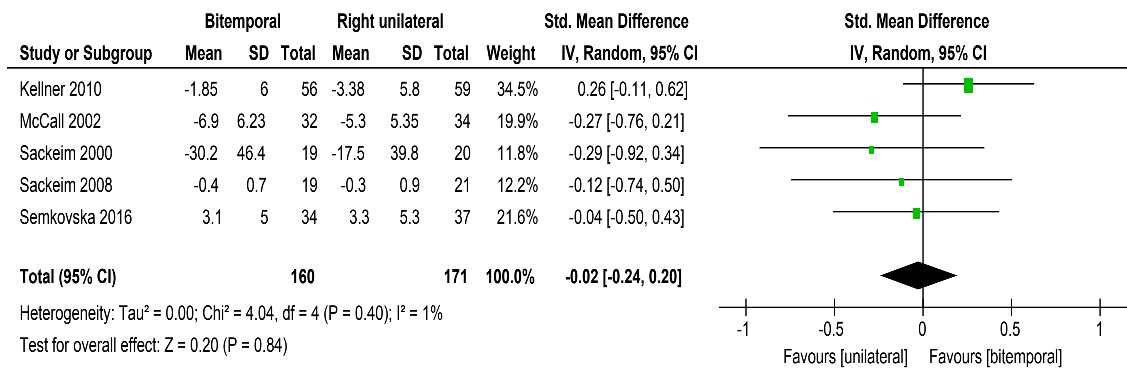


Figure 3.9 Forest plot: Measures of delayed visual recall

3.3.3.5 Cognitive side-effects: Delayed verbal memory

Five trials reported extractable data on tests of delayed verbal memory (Figure 3.10). These were tests of semantically unrelated lists of words that had to be learned and then recalled after an interval (Lezak, 2012). Two trials (Sackeim et al., 2000, Sackeim et al., 2008) used the Buschke Selective Reminding Test (Buschke, 1973, Hannay and Levin, 1985). Two trials (Kellner et al., 2010, McCall et al., 2002) used the Rey Auditory-Verbal Learning test (Ryan et al., 1986, Mungas, 1983, Rey, 1964). One trial, (Semkovska et al., 2016) used the Free and Cued Selective Reminding Test (Van der Linden and GREMEM, 2004).

There was no significant difference between those receiving high-dose right unilateral (n=183) and bitemporal (n=180) ECT (Hedges's g=-0.10, 95% CI=-0.39 to 0.19, p=0.49). Heterogeneity for this outcome was moderate (I^2=45%, p=0.12). Removing the Sackeim et al (2000) trial from the analysis reduced heterogeneity to 0%. This study used 2.5 x seizure threshold for bitemporal ECT, which may explain the advantage for right unilateral ECT seen in this trial. However, when the two trials (Sackeim et al., 2000, Sackeim et al., 2008) that used 2.5 x seizure threshold were analysed in a separate subgroup analysis there was no significant difference in performance on delayed verbal memory.

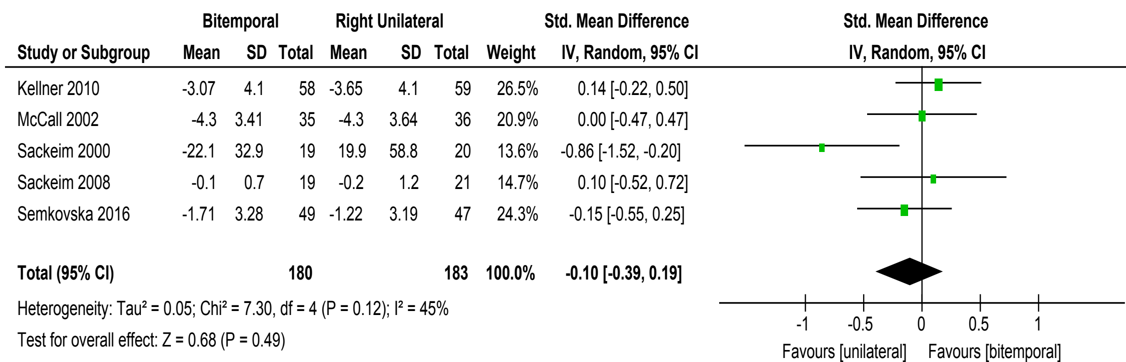


Figure 3.10 Forest plot: Measures of delayed verbal memory

3.3.3.6 Cognitive side-effects: Category fluency

Three studies (Figure 3.11) provided data on category (semantic) fluency, where participants are asked to produce as many words as possible from a chosen category, typically in one minute. It is one measure of executive functioning (Lezak, 2012). There was no significant difference between those receiving high-dose right unilateral (n=145) and bitemporal (n=141) ECT (Hedges's g=0.03, 95% CI=-0.20 to 0.26, p=0.79). There was no significant heterogeneity (I^2=0%).

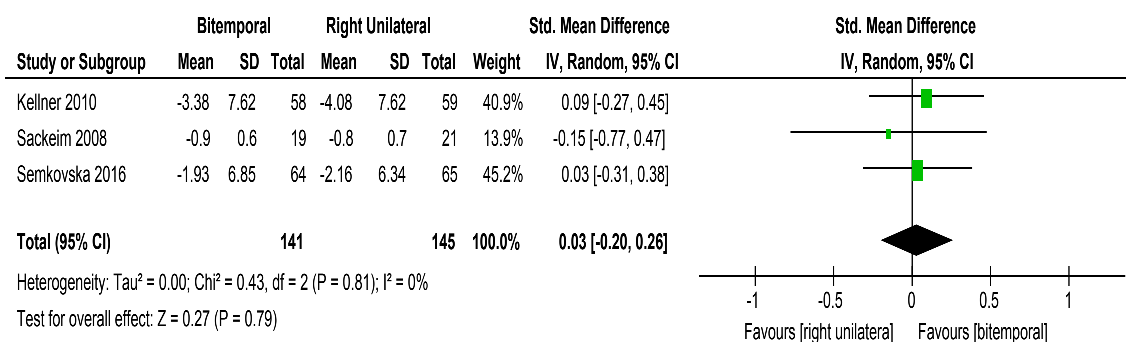
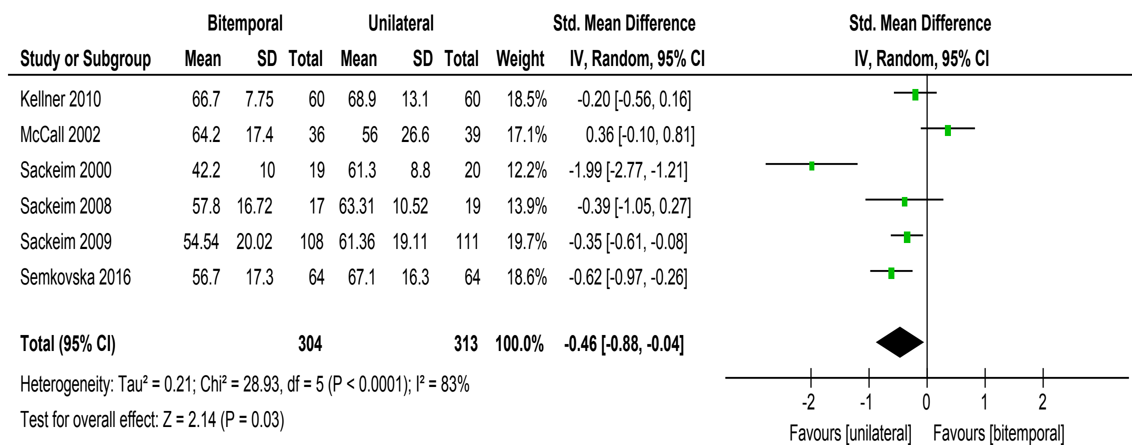


Figure 3.11 Forest plot: Measures of category fluency

3.3.3.7 Cognitive side-effects: Autobiographical memory

Six trials reported data on measures of retrograde autobiographical memory (Figure 3.12). Autobiographical (or personal) amnesia refers to difficulties after completing a course of ECT in recalling memories of personal facts (semantic autobiographical memory) or events (episodic autobiographical memory) that occurred before starting ECT (Semkovska and McLoughlin, 2013). Three trials (Sackeim et al., 2000, McCall et al., 2002, Sackeim et al., 2008) used the long form of the Columbia University Autobiographical Memory Interview (CUAMI) (McElhiney et al., 1995). Three trials (Sackeim et al., 2009, Kellner et al., 2010, Semkovska et al., 2016) used the short version of the CUAMI (CUAMI-SF) (McElhiney et al., 2001). The CUAMI/CUAMI-SF scores are percentages representing the amount of questions answered correctly at baseline that are subsequently answered correctly at end of treatment. As such, the comparison made for autobiographical memory is not a direct measure of change as was the case for the other outcomes, but rather a measure of consistency in recall. Overall, patients receiving high-dose right unilateral ECT (n=323) performed better that those receiving bitemporal ECT (n=304) (Hedges's *g* =-0.46, 95% CI=-0.88 to -0.04, p=0.03). Given the level of heterogeneity (I^2=83%, p<0.0001) a sensitivity analysis was performed. Removing Sackeim et al. (2000) reduced heterogeneity to I^2=65%. The next most influential study in terms of heterogeneity was McCall et al (2002). Removing this study further reduced heterogeneity to I^2=0%. Of note, the McCall et al. study was the only study using 8x (rather than 6x) seizure threshold in the RUL group and was also the only trial that found a trend for a disadvantage in this group. Conversely, Sackeim et al. used 2.5x (rather than the standard 1.5x) seizure threshold in the BT group in their 2000 trial. This could potentially have disadvantaged the bitemporal group in terms of CUAMI/CUAMI-SF performance. However, a 2.5x ST was also used in the Sackeim et al. (2008) trial without such an effect being observed. A further sensitivity analysis including only the three trials (Sackeim et al., 2009, Kellner et al., 2010, Semkovska et al., 2016) that compared bitemporal ECT at 1.5x ST with right unilateral ECT at 6x ST found that

right unilateral ECT at this dose maintained an advantage over bitemporal ECT on consistency of autobiographical memory recall after ECT.



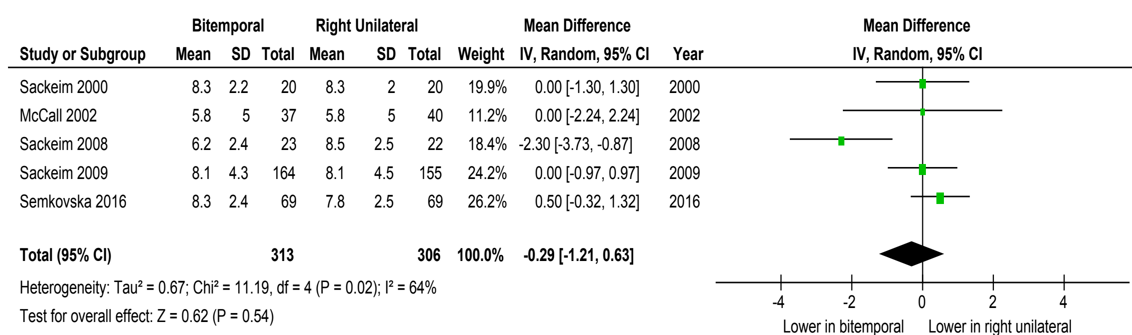
Study or Subgroup	Bitemporal Mean	Bitemporal SD	Bitemporal Total	Unilateral Mean	Unilateral SD	Unilateral Total	Weight	Std. Mean Difference IV, Random, 95% CI
Kellner 2010	66.7	7.75	60	68.9	13.1	60	18.5%	-0.20 [-0.56, 0.16]
McCall 2002	64.2	17.4	36	56	26.6	39	17.1%	0.36 [-0.10, 0.81]
Sackeim 2000	42.2	10	19	61.3	8.8	20	12.2%	-1.99 [-2.77, -1.21]
Sackeim 2008	57.8	16.72	17	63.31	10.52	19	13.9%	-0.39 [-1.05, 0.27]
Sackeim 2009	54.54	20.02	108	61.36	19.11	111	19.7%	-0.35 [-0.61, -0.08]
Semkovska 2016	56.7	17.3	64	67.1	16.3	64	18.6%	-0.62 [-0.97, -0.26]
Total (95% CI)			**304**			**313**	**100.0%**	**-0.46 [-0.88, -0.04]**

Heterogeneity: Tau² = 0.21; Chi² = 28.93, df = 5 (P < 0.0001); I² = 83%
Test for overall effect: Z = 2.14 (P = 0.03)

Figure 3.12 Forest plot: Autobiographical memory

3.3.4 ECT parameters

Right unilateral ECT has been associated with the need for a higher number of treatments, especially when used at doses nearer threshold (Fink, 2014). Five trials provided information on mean number of trials by electrode placement (Sackeim et al., 2000, McCall et al., 2002, Sackeim et al., 2008, Sackeim et al., 2009, Semkovska et al., 2016). Overall, there was no significant difference in the mean number of treatments (mean difference -0.29, 95% CI= -1.21-0.63, p=0.54), see Figure 3.13 below. There was significant heterogeneity, I^2=64%. Removing Sackeim et al. (2008) reduced this to I^2=0%.

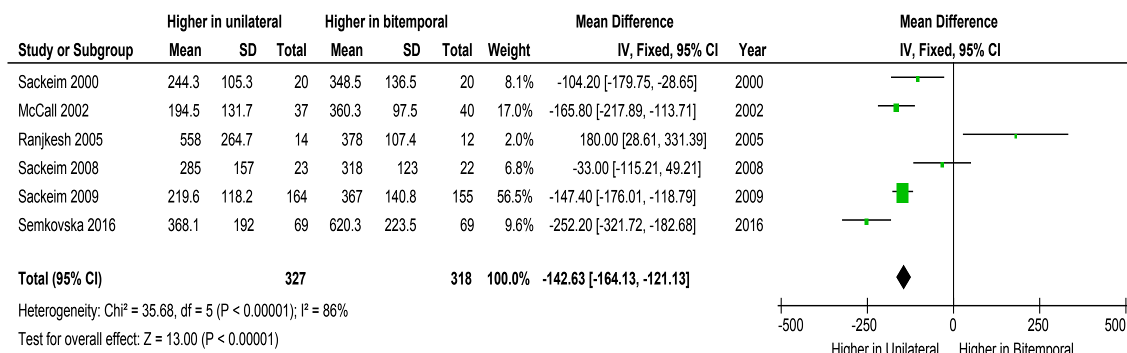


Study or Subgroup	Bitemporal Mean	Bitemporal SD	Bitemporal Total	Right Unilateral Mean	Right Unilateral SD	Right Unilateral Total	Weight	Mean Difference IV, Random, 95% CI	Year
Sackeim 2000	8.3	2.2	20	8.3	2	20	19.9%	0.00 [-1.30, 1.30]	2000
McCall 2002	5.8	5	37	5.8	5	40	11.2%	0.00 [-2.24, 2.24]	2002
Sackeim 2008	6.2	2.4	23	8.5	2.5	22	18.4%	-2.30 [-3.73, -0.87]	2008
Sackeim 2009	8.1	4.3	164	8.1	4.5	155	24.2%	0.00 [-0.97, 0.97]	2009
Semkovska 2016	8.3	2.4	69	7.8	2.5	69	26.2%	0.50 [-0.32, 1.32]	2016
Total (95% CI)			**313**			**306**	**100.0%**	**-0.29 [-1.21, 0.63]**	

Heterogeneity: Tau² = 0.67; Chi² = 11.19, df = 4 (P = 0.02); I² = 64%
Test for overall effect: Z = 0.62 (P = 0.54)

Figure 3.13 Forest plot: Mean number of ECT sessions per course

With regard to mean charge in millicoulombs (mC) over the course of ECT there was a significant difference between electrode placements in the six trials that provided this information (Sackeim et al., 2000, Sackeim et al., 2008, Sackeim et al., 2009, Ranjkesh et al., 2005, McCall et al., 2002, Semkovska et al., 2016). The mean charge (mC) in high-dose right unilateral ECT was higher than in bitemporal ECT (mean difference 142.6, 95% CI= 121.1-164.1, p<0.001, I^2=86%), see Figure 3.14. As one study (Ranjkesh et al., 2005) used 5x seizure threshold and was also a visual outlier this was removed in a sensitivity analysis. However, heterogeneity remained high at I^2=78% even after removing this.



	Higher in unilateral			Higher in bitemporal				Mean Difference	
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Fixed, 95% CI	Year
Sackeim 2000	244.3	105.3	20	348.5	136.5	20	8.1%	-104.20 [-179.75, -28.65]	2000
McCall 2002	194.5	131.7	37	360.3	97.5	40	17.0%	-165.80 [-217.89, -113.71]	2002
Ranjkesh 2005	558	264.7	14	378	107.4	12	2.0%	180.00 [28.61, 331.39]	2005
Sackeim 2008	285	157	23	318	123	22	6.8%	-33.00 [-115.21, 49.21]	2008
Sackeim 2009	219.6	118.2	164	367	140.8	155	56.5%	-147.40 [-176.01, -118.79]	2009
Semkovska 2016	368.1	192	69	620.3	223.5	69	9.6%	-252.20 [-321.72, -182.68]	2016
Total (95% CI)			**327**			**318**	**100.0%**	**-142.63 [-164.13, -121.13]**	

Heterogeneity: Chi² = 35.68, df = 5 (P < 0.00001); I² = 86%
Test for overall effect: Z = 13.00 (P < 0.00001)

Figure 3.14 Forest plot: Mean differences in charge in a course of ECT

3.3.5 Risk of bias

A risk of bias summary is included in Figure 3.15 below. Apart from allocation concealment, the majority of the information was from trials with low risk of bias. The only area where there was any study with a high risk of bias was with regard to incomplete outcome data (Kellner et al., 2010). 63 out of 230 participants (27.4%) dropped out of this trial prior to completion. The high dropout rate may have been due to those not achieving remission being classed as dropouts if they did not complete ten treatments. Of note, the adjudged high risk of bias in this trial is in reference mainly to cognitive outcomes, as mood outcomes had better rates of completion.

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)
Kellner 2010	+	?	+	+	–	+
McCall 2002	?	?	+	+	+	?
Ranjkesh 2005	?	?	?	+	+	?
Sackeim 2000	+	+	+	+	+	?
Sackeim 2008	+	?	+	+	+	+
Sackeim 2009	+	+	+	+	?	+
Semkovska 2016	+	+	+	+	+	+

Figure 3.15 Risk of bias summary

Cochrane Risk of Bias Summary for studies included in meta-analysis. Colour code; Green=low risk; Yellow=unclear risk; Red=high risk.

3.4 Discussion

3.4.1 Discussion - clinical efficacy

In the last 15 years there have been seven randomised controlled trials of high-dose right unilateral versus low-moderate dose bitemporal ECT. Although there was some variation in the outcomes used in these studies, it was possible to meta-analyse the major clinical and cognitive outcomes. In terms of clinical efficacy no significant difference between high-dose right unilateral ECT and bitemporal ECT was found, either on standardised depression rating scales or on categorical remission/response classification at end of treatment as well as at 12 months after completing ECT. This is in contrast to previous reviews that did not separate high-dose from low to medium-dose right unilateral ECT (UK ECT Review Group, 2003). It is in line with the US FDA review discussed earlier, but I was able to include over twice the number of patients in our meta-analysis (Food and Drug Administration, 2011).

No significant difference in the mean number of treatments used between the treatments was found. Some have argued that the most severely ill patients may benefit more, or at least respond quicker, with bitemporal ECT (Kellner et al., 2010). As the most severely ill patients are typically not recruited to randomised controlled trials, our meta-analysis indicates that for patients eligible to participate in a randomised controlled trial, both forms of treatment were equally efficacious.

3.4.2 Discussion - cognitive effects

With regard to cognitive outcomes, there was an advantage for high-dose right unilateral ECT on measures of retrograde amnesia for autobiographical memory (AMI) as well as reorientation time in the week following ECT. Measuring retrograde memory impairment may be less reliable than anterograde memory (Ingram et al., 2008). Although the CUAMI/CUAMI-SF have their limitations, it is sensitive to differences in autobiographical memory performance attributable to differences in electrode placement (Semkovska and McLoughlin, 2013, Sackeim, 2014). The only study that favoured bitemporal ECT (although not statistically significant) in terms of

autobiographical memory used right unilateral ECT at 8x seizure threshold (McCall et al., 2002). This may indicate that there is no cognitive advantage in going beyond 6x seizure threshold for right unilateral ECT.

Reorientation times were better for high-dose right unilateral ECT although this finding was limited to three trials only. Prolonged reorientation time at the time of ECT has been reported to be a predictor of subsequent retrograde amnesia after a course of ECT (Sobin et al., 1995, Martin et al., 2015).

For the other cognitive outcomes no advantage of one form of ECT over the other in the week following ECT was found. It therefore appears that some, but not all, of the cognitive advantage of right unilateral ECT is lost when given at a sufficient dose to achieve equal clinical efficacy with bitemporal ECT.

3.4.3 Limitations

Only seven randomised controlled trials that met inclusion criteria were identified, many with small numbers of patients. This prevented the analysis of publication bias. On the other hand, pooling the studies gave increased power to detect meaningful differences that were not necessarily apparent in the individual trials themselves.

Although all trials used a version of the HDRS to measure efficacy, cognitive outcome measures often varied widely from trial to trial which limited the data I could meta-analyse.

With the exception of relapse rates at one year, outcomes were limited to the short-term (first week after ECT). Only some trials (Sackeim et al., 2008, Semkovska et al., 2016, Sackeim et al., 2000, McCall et al., 2002) studied the longer-term cognitive outcomes of the two forms of ECT. However, as the trials differed with regard to the time of follow-up these results could not be pooled.

Whether the short-term differences in reorientation and retrograde autobiographical memory seen after ECT have a long-term impact therefore remains unclear. Although relapse following ECT is a concern (Jelovac et al., 2013), only three trials monitored patients for a year after ECT (Sackeim et al., 2000, Sackeim et al., 2008, Semkovska et al., 2016).

Care should be taken with extrapolating these results to non-depression groups such as schizophrenia or mania. Based on this meta-analysis, cognitive outcomes that differ on the basis of electrode placement in brief-pulse ECT include reorientation time and retrograde autobiographical memory. However, many trials had included cognitive outcomes that could not be meta-analysed due to heterogeneity between different instruments or a simple lack of trials. As recruiting patients to ECT trials is difficult (O'Connor et al., 2010), the use of common cognitive measures that may facilitate future meta-analysis would be helpful.

3.5 Conclusions

Based on this systematic review and meta-analysis of seven randomised controlled trials, high-dose brief-pulse right unilateral ECT appears to be as effective as brief-pulse bitemporal ECT for the treatment of depression, and appears to have some cognitive advantages. Although brief-pulse bitemporal ECT remains the most common form of ECT worldwide, these findings indicate that high-dose right unilateral ECT may represent a superior alternative for many patients. Although ultra-brief pulse ECT may have a further cognitive advantage, current evidence suggests this is at a slight disadvantage in terms of clinical response (Tor et al., 2015).

Evidence-based alternatives in electrode placement and pulse-width are now available for the clinician prescribing ECT. It may be that there is currently no "gold standard" form of ECT that suits every patient's need, but this analysis suggest that high-dose brief pulse right unilateral ECT represents an acceptable middle ground for many as a first line form of ECT.

Although these findings clarify some of the clinical questions around electrode placement the mechanism of action and neurobiology of depression remain unclear. It is likely that they involve molecular and cellular changes within the brain, which in turn may be reflected in peripheral blood. Changes in microRNA expression and function represent one such possibility. In the next chapter, using blood samples obtained from depressed participant in the EFFECT-Dep Trial, I explore the role of microRNAs in depression treated with ECT.

Chapter 4
Results

4 A deep sequencing study of whole blood microRNA expression following ECT for severe depression

4.1 Introduction

Major depression will be the second leading contributor to the global burden of disease by 2020. The lifetime prevalence is 12-20%. It is the costliest brain disorder in Europe, accounting for 1% of the European economy (Gustavsson et al., 2011). 30% of patients do not respond to multiple trials of antidepressants (Rush et al., 2006). However, electroconvulsive therapy (ECT) offers 50-60% of such patients remission (Eranti et al., 2007, Dierckx et al., 2012, Tokutsu et al., 2013). Like the biology of depression itself, the mechanism of action of ECT is not yet fully understood. Continued research into novel approaches for depression is therefore needed, and understanding the mechanisms underlying ECT and other effective treatments is likely to help identify new therapeutic targets and clinical biomarkers (Krishnan and Nestler, 2008).

Early models of major depression postulated a "chemical imbalance" in monoamine neurotransmitters. Current theories propose that antidepressants act by inducing transcriptional and translational processes, culminating in neuroplastic changes, e.g. hippocampal neurogenesis, synaptogenesis, increased dendritic spines and dendrites (Krishnan and Nestler, 2008). In animal models, ECT induces such neuroplastic changes more robustly than antidepressants, possibly accounting for its more powerful therapeutic effect (Malberg et al., 2000).

The importance of neuroplasticity is emphasized by evidence of loss of neurons and glia in the hippocampus and pre-frontal cortex in depression (Krishnan and Nestler, 2008). Brain-derived neurotrophic factor (BDNF) is widely expressed throughout the brain, promoting neuronal survival and maturation, synaptic plasticity and synaptic function (Lu et al., 2013, Park and Poo, 2013). Low levels of BDNF have been found in post-mortem brains of depressed patients, and BDNF can exert antidepressant activity (Krishnan

and Nestler, 2008). Other potential therapeutic targets include vascular endothelial growth factor (VEGF), cAMP response element-binding protein (CREB), a transcription factor involved in synaptic plasticity and memory formation, and LIM domain kinase 1 (LIMK1), an enzyme regulating dendrite size (Schratt et al., 2006). However, the precise mechanisms by which these factors are associated with major depression remain unknown. Recent findings implicate microRNAs as potential "micro-managers" of these changes (O'Connor et al., 2012, Kolshus et al., 2014).

MicroRNAs are short (21-23 bases), non-coding RNA molecules. Over 2,500 human microRNAs have been reported, targeting at least 50% of the genome (Griffith-Jones, 2015, Friedman et al., 2009). MicroRNAs suppress gene expression by binding to mRNA transcripts, preventing their translation or inducing their degradation (see section 1.3.2). A single microRNA can target hundreds of genes. They have been shown to regulate a number of neuroplastic processes relevant to psychiatric disorders (Kolshus et al., 2014). Our research group found increased levels of miR-212, a BDNF-associated microRNA, in rat dentate gyrus following acute and chronic electroconvulsive stimulation (Ryan et al., 2013).

MicroRNAs are present in a remarkably stable form in human blood and can be useful in the study of brain health and dysfunction (Kolshus et al., 2014, Ryan et al., 2016). Interestingly, microRNAs specific to brain diseases can be detected in peripheral blood and peripheral blood levels can correlate with brain levels (Gladkevich et al., 2004). Peripheral leucocytes in depressed patients undergo transcriptional alterations in the same molecular pathways involved in neuroplasticity. Also, lithium treatment in bipolar affective disorder leads to alterations in plasma microRNA levels that track treatment response (O'Connor et al., 2012). Together, these findings support the usefulness of whole blood microRNA expression analysis to study psychiatric disorders and treatments (Bocchio-Chiavetto et al., 2013). The genomics field has been advanced by recent technological advances including deep sequencing (also known as next generation sequencing). Deep sequencing allows for quantification of every microRNA in a sample (Metzker, 2010). Such analysis enables robust quantification of known microRNAs as well as potential novel microRNA discovery.

4.1.1 Aims of the study

Currently, there is scant knowledge regarding microRNA involvement in depression in general. There have been no studies examining the effect of ECT, the most powerful antidepressant available, on human microRNA expression. The purpose of this study was therefore to perform a discovery phase deep sequencing study of whole blood microRNA expression, characterising microRNA abundance before and after ECT for severe depression. The underlying hypothesis is that profiling of circulating microRNAs will identify alterations in microRNA levels in depression treated with ECT. Strict correction for multiple testing will be used.

Candidate microRNAs identified by deep sequencing were then confirmed using quantitative real time polymerase chain reaction (qRT-PCR).

4.2 Methods

4.2.1 Participants

This study included sixteen patients with moderate to severe depression enrolled in the EFFECT-Dep Trial, described in detail in Chapter Two (section 2.4.1.1). The presence or absence of a major depressive disorder was confirmed by administering the mood disorder component of the research version of the Structured Clinical Interview for DSM-IV Axis 1 Disorders (First et al., 2002). Depression severity at baseline and end of treatment (EOT) was measured using the 24-item version of the Hamilton Depression Rating Scale (HDRS-24) (Beckham and Leber, 1985). The patients selected for this study were all responders to ECT, defined as at least a 50% reduction in HDRS-24 score from baseline. Exclusion criteria included substance abuse in the last six months; premorbid existing cognitive impairment; other Axis I disorder; ECT in the past 6 months; inability to consent; current inflammatory, infectious, or haematological disorder. The EFFECT-Dep trial was a pragmatic trial, and as such, patients remained on medications prescribed by their treating teams throughout the trial period and no drug washout prior to ECT was required.

As these patients have shown the greatest therapeutic response to ECT, I expected that changes in microRNA levels in this enriched group would be the most informative about the effect of ECT treatment on microRNA levels. Written informed consent was obtained from all participants and ethical approval was granted from the local research ethics committee in St. Patrick's University Hospital.

4.2.2 Blood sampling and microRNA extraction

Blood sampling and microRNA extraction and quality analysis was carried out as described in detail in Chapter Two (section 2.3.1-2.3.3). Briefly, fasting blood samples were taken at baseline and EOT using the PaxGene© system (Qiagen Inc., USA) to collect whole blood. MicroRNA was extracted using PaxGene© microRNA kits according to the manufacturer's instructions (Qiagen Inc., USA). Quality analysis was carried out on the Bioanalyzer© 2100 (Agilent Technologies, Ireland).

4.2.3 Deep sequencing

Deep sequencing was carried out on the SOLiD™ platform according to the manufacturer's instructions (Applied Biosystems Inc., USA) as described in detail in Chapter Two (section 2.3.4). Briefly, extracted microRNA was converted into a library of 60-80 nucleotide length complementary DNA (cDNA) suitable for emulsion PCR. The cDNA fragments were attached to clonal bead populations. Following denaturing, the complementary template was then sequenced. Fluorescently labelled di-base probes compete for ligation to the sequencing primer. Multiple cycles of ligation, detection and cleavage were performed to ensure accuracy of 99.9%. Bioinformatic analysis was then used to analyse the millions of reads generated by deep sequencing. A read is a string of nucleotides as well as a quality score reflecting the accuracy of the read. Initially reads were analysed to identify microRNAs, which were then counted before undergoing a differential expression analysis. Differential expression analysis was carried out successively on each pair of samples under comparison with a threshold of log2-fold change of 1 (this represents a doubling/halving of gene counts).

4.2.4 qRT-PCR

Confirmation of candidate microRNAs derived from deep sequencing was confirmed using stem-loop reverse transcription and qRT-PCR as described in detail in Chapter Two (section 2.3.5-2.3.7). I used stem-loop primers (Applied Biosystems, UK) and a TaqMan® microRNA reverse transcription kit (Applied Biosystems, UK) according to the manufacturer's instructions. qRT-PCR was carried out using TaqMan® microRNA assays on a StepOnePlus™ instrument (Applied Biosystems, UK) according to the manufacturer's instructions.

MicroRNA/control	Sequence
hsa-miR-942-3p	CACAUGGCCGAAACAGAGAAGU
hsa-miR-942	UCUUCUCUGUUUGGCCAUGUG
hsa-miR-126	UCGUACCGUGAGUAAUAAUGCG
hsa-miR-126*	CAUUAUUACUUUUGGUACGCG
hsa-miR-130a	CAGUGCAAUGUUAAAAGGGCAU
hsa-miR-130a*	UUCACAUUGUGCUACUGUCUGC
hsa-miR-106a	AAAAGUGCUUACAGUGCAGGUAG
hsa-miR-106a*	CUGCAAUGUAAGCACUUCUUAC
hsa-miR-1249	ACGCCCUUCCCCCCCUUCUUCA
hsa-miR-766	ACUCCAGCCCCACAGCCUCAGC
RPL21	CTTAATGATGACTGTTTTTTTTGATTGCTTGAAGCAATG TGAAAAACACATTTCACCGGCTCTGAAAGCT
RNU49	CACTAATAGGAAGTGCCGTCAGAAGCGATAACTGACG AAGACTACTCCTGTCTGATT
RNU6B	CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATAT TTTT

Table 4.1 TaqMan microRNA/control assay primers

Candidate microRNAs for confirmation are in white boxes. Candidates for endogenous controls are in shaded grey boxes.

The comparative C_T method was used to assess gene expression for all qRT-PCR analyses with normalisation to endogenous controls to minimise quantification errors. The comparative C_T method compares the C_T values of one target gene to another (e.g. an internal control). A range of different candidates for endogenous control (see Table 4.1) was explored using RefFinder, a web-based tool for evaluating candidate reference genes (Xie et al., 2011). The RefFinder tool incorporates a number of computational tools to assess candidate reference genes. Each computational tool gives a ranking to each candidate gene, which is used to assign a candidate gene an appropriate weight. The RefFinder tool then calculates a geometric mean of these weights to give an overall comprehensive ranking.

4.2.5 Data analysis

Data analysis was performed as described in Chapter Two (section 2.5) using SPPS version 22.0 (IBM Corporation, NY), R version 3.1.3 (R Foundation for Statistical Computing, 2014) and GraphPad Prism 6 (GraphPad Software, CA). Deep sequencing data was analysed using The Bioconductor package (www.bioconductor.org). As deep sequencing tests for a large number of microRNAs, correction for multiple testing is important and this was accomplished using the Benjamini-Hochberg (BH) procedure of false discovery rate adjustment (Benjamini and Hochberg, 1995, Li et al., 2012a). In the BH algorithm, p-values for all tests are calculated and then ranked in ascending order. The most significant p-value is left unadjusted, but then, starting with the second largest p-value a corrected p-value is derived for the remaining genes. The following formula is used, where the p-value of a given gene is multiplied by the total number of genes divided by its rank:

p-value x (n/n-1)

In a hypothetical example of a study of a 100 genes, the adjustment to the second-ranked gene would therefore be

p-value x (100/99)

The third p-value is multiplied in a similar fashion, adjusting for its rank and so on.

p-value x (n/n-2)

In our hypothetical example of a 100 genes above, the adjustment to the third p-value would therefore be

p-value x (100/98)

4.3 Results

4.3.1 Participant details

Demographic and clinical details of the sixteen responders to ECT are summarised in Table 4.2 below. The participants had a high mean baseline severity of depression (30.4, (SD=5.7) on HDRS-24), which had been greatly reduced at EOT (7.1, (SD=4.1)). As the participants were taking part in a pragmatic randomised controlled trial, their medications were not washed out prior to ECT.

Variable	Deep Sequencing Group (n=16)
Age, range	63.7 (13.1), 40-88
Gender, female n (%)	8 (50.0%)
Education completed, n (%)	
Primary	6 (37.5)
Secondary	6 (37.5)
Tertiary	4 (25.0)
HDRS Baseline	30.4 (5.7)
HDRS End of Treatment	7.1 (4.1)
Unilateral ECT, n (%)	10 (62.5)
Episode duration, weeks, range	34.5 (31.8), 8-111
Treatment-resistant*, n (%)	10 (62.5)
Psychotic subtype, n (%)	4 (25.0)
Bipolar depression, n (%)	4 (25.0)
Previous ECT, n (%)	6 (37.5)
Medications, n (%)	
SSRI	7(43.8)
SNRI	7(43.8)
TCA	3(18.8)
Mirtazapine	11(68.8)
Agomelatine	2(12.5)
Lithium	6(37.5)
Antipsychotics	11(68.8)
Benzodiazepine	7(43.8)
Pregabalin	2(12.5)
Hypnotic^	2(12.5)

Table 4.2 Demographic and clinical details of participants

Data are presented in means (SD) or n(%). *Treatment resistance measured using the Antidepressant Treatment History Form (ATHF). ^Zopiclone, Zolpidem, Lormetazepam or Triazolam; HDRS: Hamilton Depression Rating Scale; SNRI: Serotonin and noradrenaline reuptake inhibitor; SSRI: Selective serotonin reuptake inhibitor; TCA: Tricyclic antidepressant.

4.3.2 microRNA quality assessment

Table 4.3 below contains microRNA quality analysis data for the sixteen patients (baseline and EOT) selected for deep sequencing. The SOLiD platform manufacturers (Applied Biosystems) recommend using samples with a RNA Integrity Number (RIN) >6. The other columns contain the percentage of microRNA in the sample: (miRNA concentration pg/uL) / (total RNA concentration ng/uL). Ideally this should be >0.5%. The three samples that were below 0.5% were purified to >0.5% using Invitrogen PureLink miRNA Isolation Kit™. The final column gives the ratio of absorbance at 260nm and 280nm, which reflects the purity of RNA and a ratio of ~2.0 is generally accepted as 'pure' for RNA. The samples therefore show good quality and are suitable for a deep sequencing experiment.

Patient	RIN	miRNA conc (pg/uL)	Total RNA conc (ng/uL)	miRNA%	Nanodrop 260/280
1084 BAS	8.4	48.1	16	0.30	1.89
1084 EOT	7.4	274.7	22	1.25	1.99
1089 BAS	8.8	620.3	70	0.89	2.07
1089 EOT	7.3	460.5	49	0.94	2.09
1101 BAS	8.4	231.0	17	1.36	1.82
1101 EOT	6.3	236.7	13	1.82	1.84
1102 BAS	8.0	317.8	37	0.86	2.03
1102 EOT	7.2	356.6	38	0.94	2.05
1108 BAS	7.7	246.0	43	0.57	1.99
1108 EOT	9.4	289.4	62	0.47	2.16
1111 BAS	8.8	465.2	47	0.99	2.02
1111 EOT	9.3	792.0	84	0.94	2.01
1118 BAS	7.9	589.0	90	0.65	2.09
1118 EOT	6.0	322.4	17	1.89	2.06
1122 BAS	7.2	565.1	44	1.28	2.03
1122 EOT	7.8	264.2	38	0.70	2.11
1123 BAS	8.8	300.0	35	0.86	1.93
1123 EOT	7.9	298.2	29	1.03	2.09
1124 BAS	8.7	230.3	90	0.26	2.03
1124 EOT	9.1	441.9	132	0.33	2.07
1126 BAS	8.2	148.4	17	0.87	2.09
1126 EOT	7.9	222.5	17	1.31	1.98
1129 BAS	7.5	312.5	44	0.71	2.03
1129 EOT	8.3	377.1	34	1.11	1.94
1134 BAS	8.5	709.8	77	0.92	2.08
1134 EOT	8.0	593.6	65	0.91	2.09
1135 BAS	8.4	316.4	48	0.66	2.09
1135 EOT	8.7	435.8	73	0.60	2.10
1137 BAS	8.7	671.7	96	0.70	2.02
1137 EOT	8.8	434.8	82	0.53	2.01

1141 BAS	7.7	154.4	10	1.54	2.35
1141 EOT	8.2	272.3	29	0.94	2.02

Table 4.3 MicroRNA extraction data for deep sequencing group
BAS: Baseline; EOT: End of treatment.

4.3.3 Deep sequencing

4.3.3.1 MicroRNA expression pattern in whole blood

Deep sequencing yielded over thirty-two million reads of non-coding RNAs that mapped onto the human genome (mappable reads) using the SHort Read Mapping Package (SHRiMP) alignment package (Computational Biology Lab, University of Toronto, Canada). This process eliminates sequences that are unwanted, such as adaptor sequences, mitochondrial DNA and so on. These mappable reads contained various forms of small and large non-coding RNA species (Table 4.4). MicroRNAs represented 73.0% of mappable reads.

No novel microRNAs were identified in the study. The number of mappable reads that were mappable to microRNAs varied greatly from a minimum of one to several millions of reads. However, the latter typically represented microRNA families that contained several individual microRNAs. The median number of mappable reads per microRNA was 14. Details of the ten top most frequently mapped microRNAs are listed in Table 4.5 and Figure 4.1 below. These frequencies only refer to microRNA abundance, and do not reflect differential expression.

Read class	Number of reads
Short non-coding reads	
microRNAs	23834659
miscellaneous other RNA	6232547
small nucleolar RNA	145237
small nuclear RNA	25495
ribosomal RNA	859152
Total short non-coding RNAs	31097090
Long non-coding reads	
long intergenic non-coding RNA	62610
antisense long non-coding RNA	1351331
Sense intronic long non-coding RNA	127297
Sense overlapping long non-coding RNA	374
3-prime overlapping non-coding RNA	34
Processed transcripts	20262
Total long non-coding RNAS	1499298
Total all non-coding RNAs	32596388

Table 4.4 Composition of non-coding RNA library

Rank	MiRNA name/family	Chr	Function
1	MicroRNA 144 Family MicroRNA-144-3p/5p MicroRNA-451a/b MicroRNA-732-3p/5p	17q11.2	Upregulated by lithium and sodium valproate (Zhou et al., 2009)
2	MicroRNA 191 family MicroRNA-191-3p/5p MicroRNA-425-3p/5p	3p21.31	Regulates BDNF (Varendi et al., 2014)
3	MicroRNA-484	16p13.11	
4	MicroRNA-223-3p/5p	Xq12	Targets glutamate receptors (Harraz et al., 2012)
5	MicroRNA-17-92 cluster host gene MicroRNA-17-3p/5p MicroRNA-92a-1-5p MicroRNA-92a-3p MicroRNA-18-3p/5p MicroRNA-19a-3p/5p MicroRNA-20a-3p/5p	13q31.3	Downregulated in suicide completer brains (Smalheiser et al., 2012)
6	MicroRNA-140-3p/5p	16q22.1	Upregulated in depression treated with escitalopram (Bocchio-Chiavetto et al., 2013)
7	MicroRNA-185-3p/5p	22q11.21	Lower levels in depressed patients vs. controls (Wan et al., 2015)
8	MicroRNA-425-3p/5p	3p21.31	Lower levels in depressed patients vs. controls (Belzeaux et al.,

			2012)
9	MicroRNA 103a-2-3p/5p	20p13	Upregulated in depressed treated with escitalopram (Bocchio-Chiavetto et al., 2013)
10	MicroRNA-150-3p/5p Family	19q13.33	

Table 4.5 Most common microRNAs and function

Top ten most expressed microRNA in deep sequencing sample. MicroRNAs are listed in in descending order by read count. Chr: Cytogenetic location.

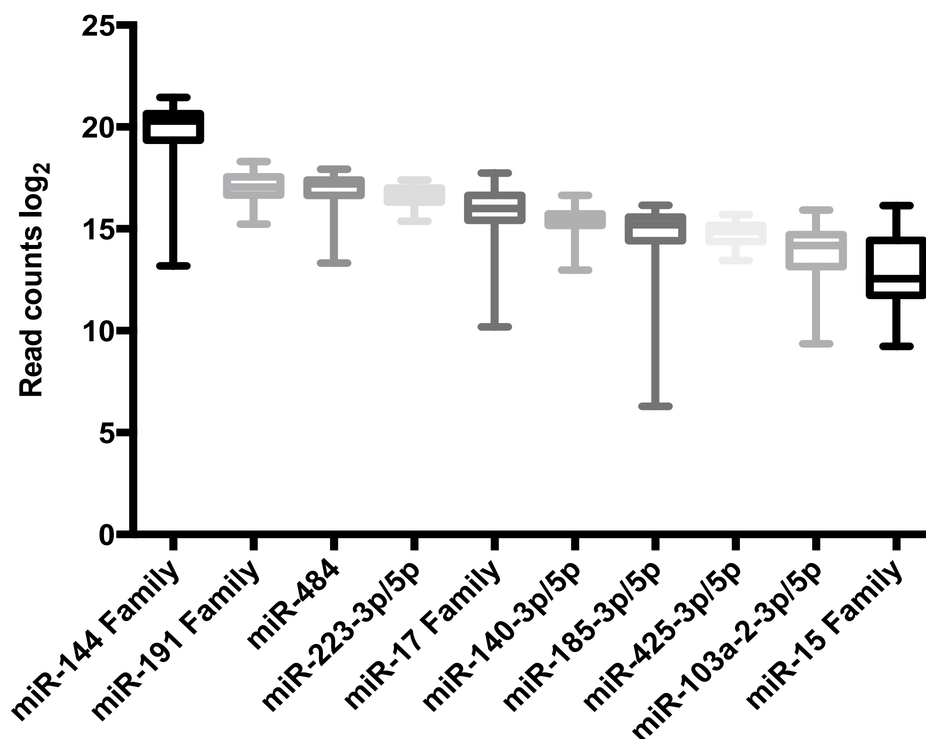


Figure 4.1 Distribution of most commonly detected microRNAs

Ten most highly expressed microRNAs/microRNA families. Data presented as log_2-transformed read counts in box and whisker plots (whiskers represent range), n=16.

The mean number of mappable reads per patient was 2114107 (SD= 812158.9, see Figure 4.2 below.

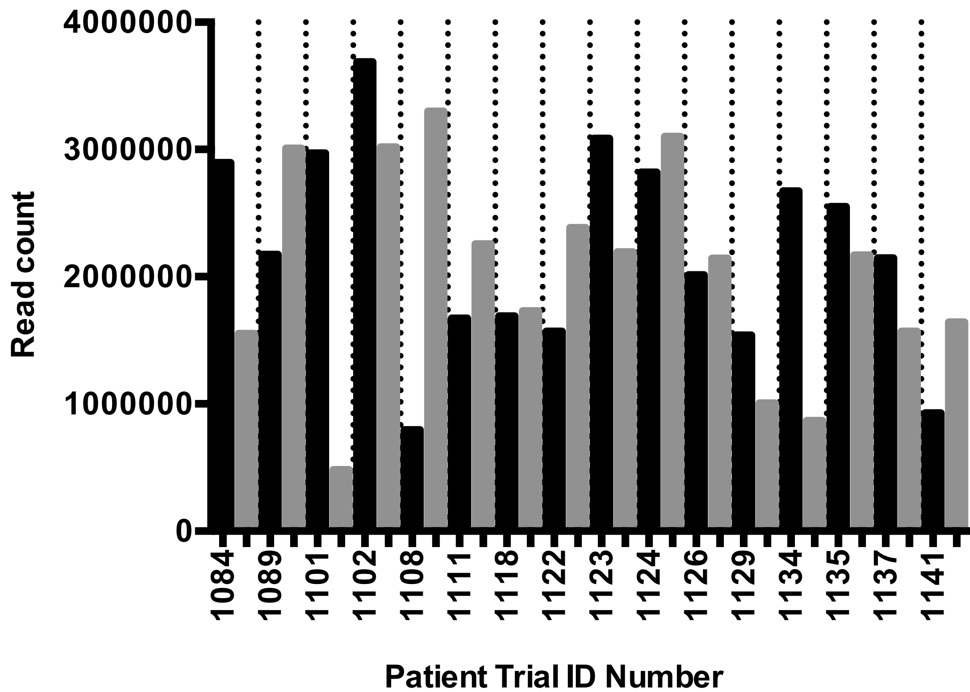


Figure 4.2 Mean number of mappable reads per patient

Each bar within the dotted columns represents baseline (black bar) and end of treatment (grey bar) mappable reads for the 16 patients in this study. Reads are raw counts mappable to ENSEMBL.

4.3.3.2 Differential expression in main cohort

No statistically significant differences in microRNA expression from baseline to end of treatment were found when the group as a whole (n=16) was examined. As a next step, patients were split into groups post-hoc based on diagnostic subtypes (bipolar/unipolar, psychotic/non-psychotic) and ECT electrode placement (unilateral/bitemporal) to explore potential meaningful expression changes that may have been limited to these sub-groups.

4.3.3.3 Differential expression in unipolar and bipolar patients

There are epidemiological, psychopathological and genetic differences between unipolar and bipolar depression (Smith and Craddock, 2011). A post-hoc analysis of those with and without bipolar depression was therefore carried out. In the bipolar depression group (n=4), 987 microRNA species were identified. The top 100 most expressed microRNAs are displayed in the heatmap below (Figure 4.3). In bipolar depressed subjects, one microRNA (miR-130a) was differentially expressed (see Table 4.6 and Figure 4.3-4 below).

In the twelve unipolar subjects 1297 microRNA species were identified. There were no significant differences in microRNA expression levels between baseline and EOT in this patient group.

Name	Log_2FC	LFCSE	Stat	Adjusted p-value	Ensembl Transcript ID
miR-130a	1.7478	0.4260	4.1026	0.04037	ENST 00000385274

Table 4.6 Differentially expressed microRNAs in bipolar patients

P-values were adjusted with Benjamini Hochberg correction. Log_2FC: Log2 Fold Change - the level of differential expression, expressed in log2 notation; LFCSE: logFoldChange Standard Error; Stat: Wald Statistic. n=4

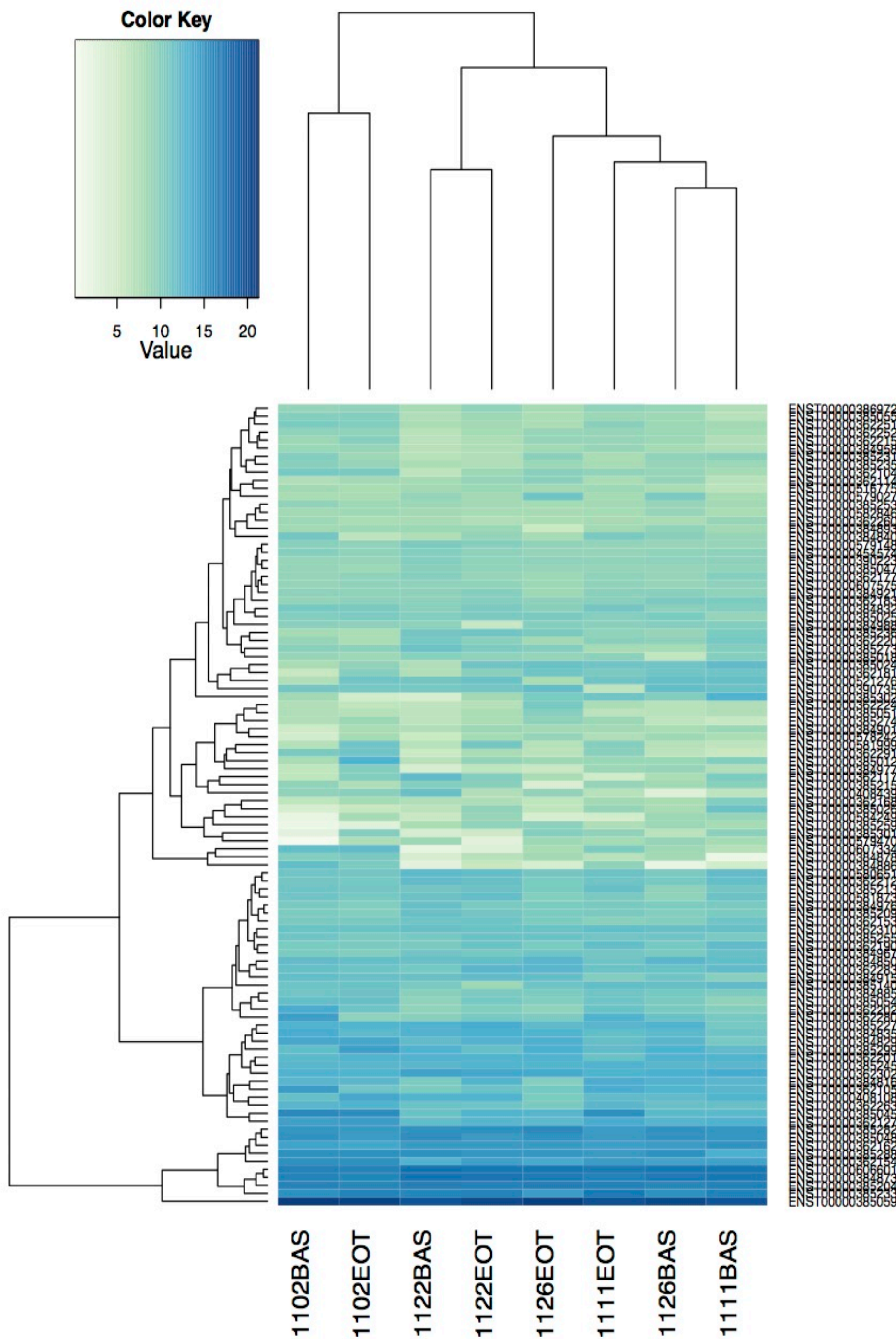


Figure 4.3 Heatmap of microRNA distribution - bipolar

Heatmap of 100 most expressed microRNAs in patients with bipolar depression. Patient trial ID number and time of blood collection (BAS or EOT) are on x-axis. MicroRNA ENSEMBL transcript IDs are on the right y-axis. Dark blue indicates high levels, green intermediate and white low expression levels. n=4

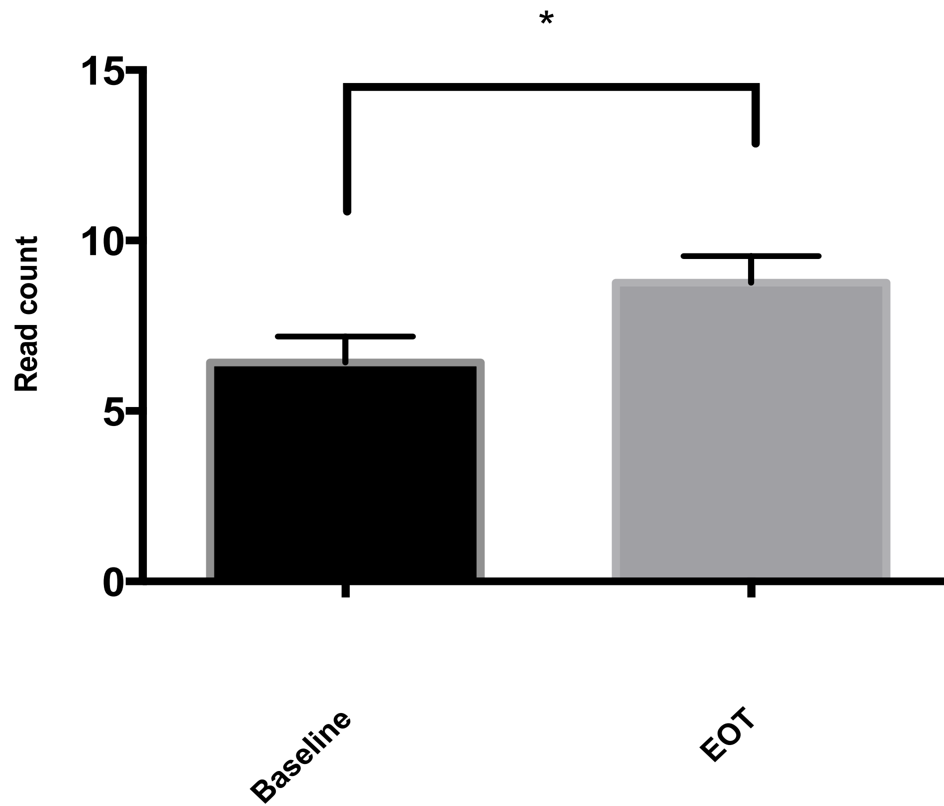


Figure 4.4 MiR-130a expression in bipolar depression

Whole blood microRNA read counts in patients with bipolar depression, n=4. Data expressed as means with standard deviations (SD). *BH Adjusted p-value = 0.040.

4.3.3.4 Differential expression in psychotic and non-psychotic depression

Psychotic depression is a diagnostic subtype of MDD in DSM-IV but some have argued that psychotic depression is distinct from other forms of depression and should be treated as such in upcoming diagnostic classifications (Ostergaard et al., 2012). We therefore looked post-hoc for differences in microRNA expression levels in unipolar psychotic (n=3) and non-psychotic participants (n=9). In psychotic patients 920 microRNA species were identified. The top 100 most expressed microRNAs are displayed in the heatmap below (Figure 4.5). Three microRNAs were differentially expressed in the psychotic subgroup, see Table 4.7 and Figure 4.6-4.7 below. In the non-psychotic group 1166 microRNA species were identified but no differentially expressed microRNAs were identified.

Name	Log_2FC	LFCSE	Stat	Adjusted p-value	Ensembl Transcript ID
miR-942	2.2840	0.5266	4.3370	0.0066	ENST00000401111
miR-106a	-2.1782	0.5242	-4.1550	0.0010	ENST00000384870
miR-126	-2.2344	0.5138	-4.3490	0.0066	NST00000362291

Table 4.7 Differentially expressed microRNA in psychotic patients

P-values adjusted with Benjamini Hochberg correction. Log_2FC: Log2 Fold Change - the level of differential expression, expressed in log2 notation; LFCSE: logFoldChange Standard Error; Stat: Wald Statistic; padj: Benjamini Hochberg (BH) adjusted p-value. n=3

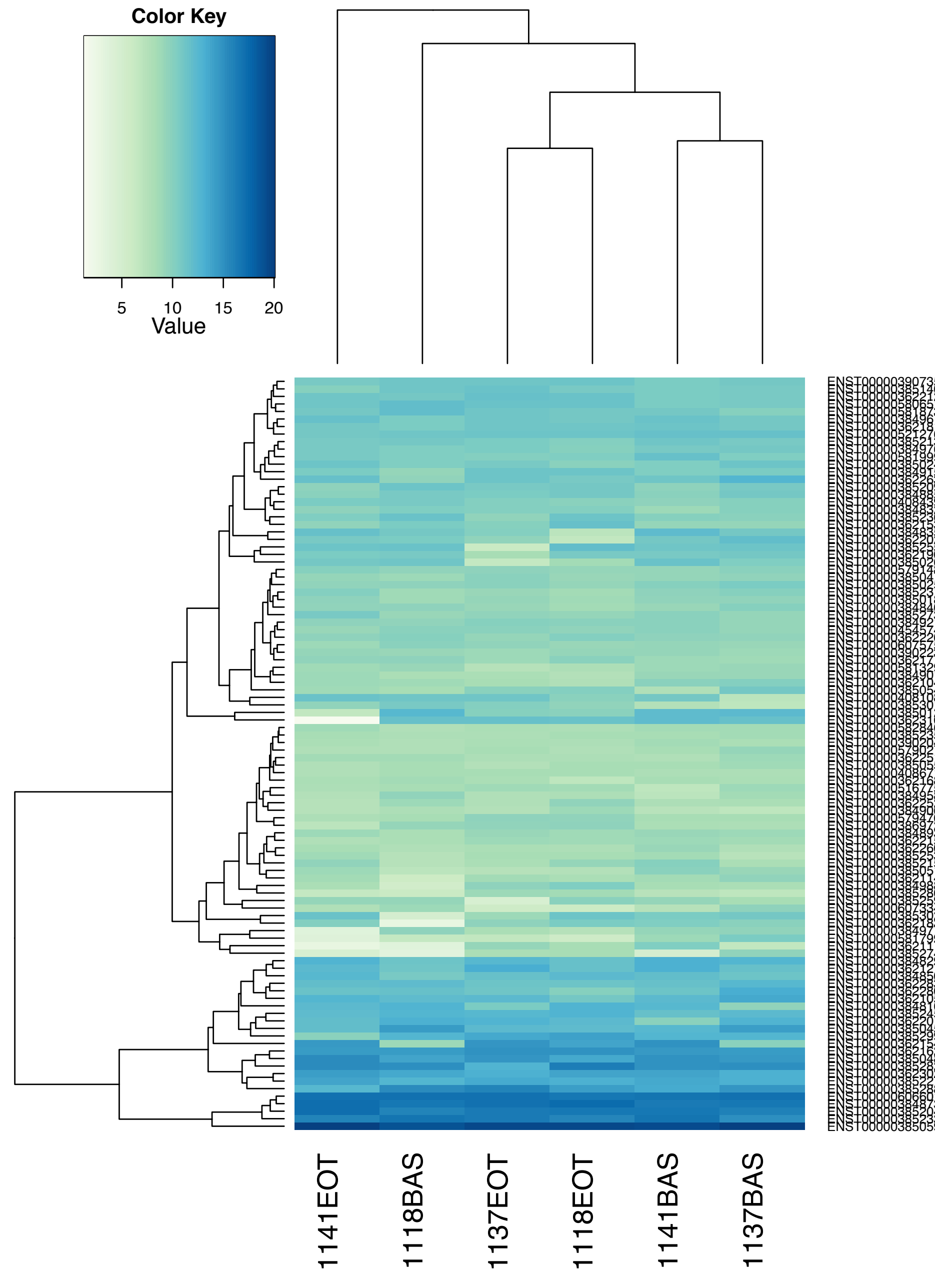


Figure 4.5 Heatmap of microRNA distribution - psychosis

Heatmap of 100 most expressed microRNAs in patients with psychotic depression. Patient trial ID number time of blood collection (BAS or EOT) are on x-axis. MicroRNA ENSEMBL transcript IDs are on the right y-axis. Dark blue indicates high levels, green intermediate and white low expression levels. n=3.

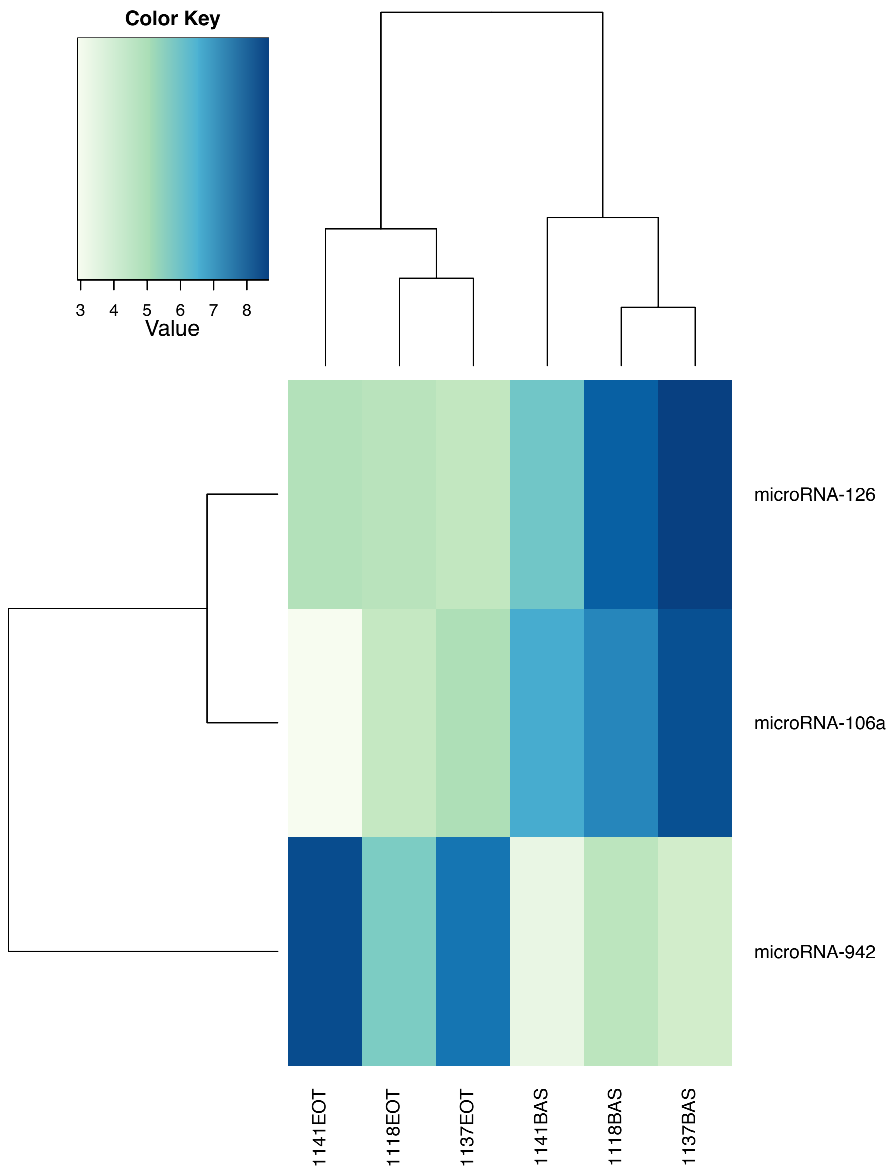


Figure 4.6 Heatmap of differentially expressed microRNAs

Heatmap of differentially expressed microRNAs in patients with psychotic depression. Patient trial ID number and time of blood collection (BAS or EOT) are on x-axis. MicroRNA ENSEMBL transcript IDs are on the right y-axis. Dark blue indicates high levels, green intermediate and white low expression levels. As seen, miR-126 and miR-106a were downregulated after ECT and mir-942 was upregulated. n=3.

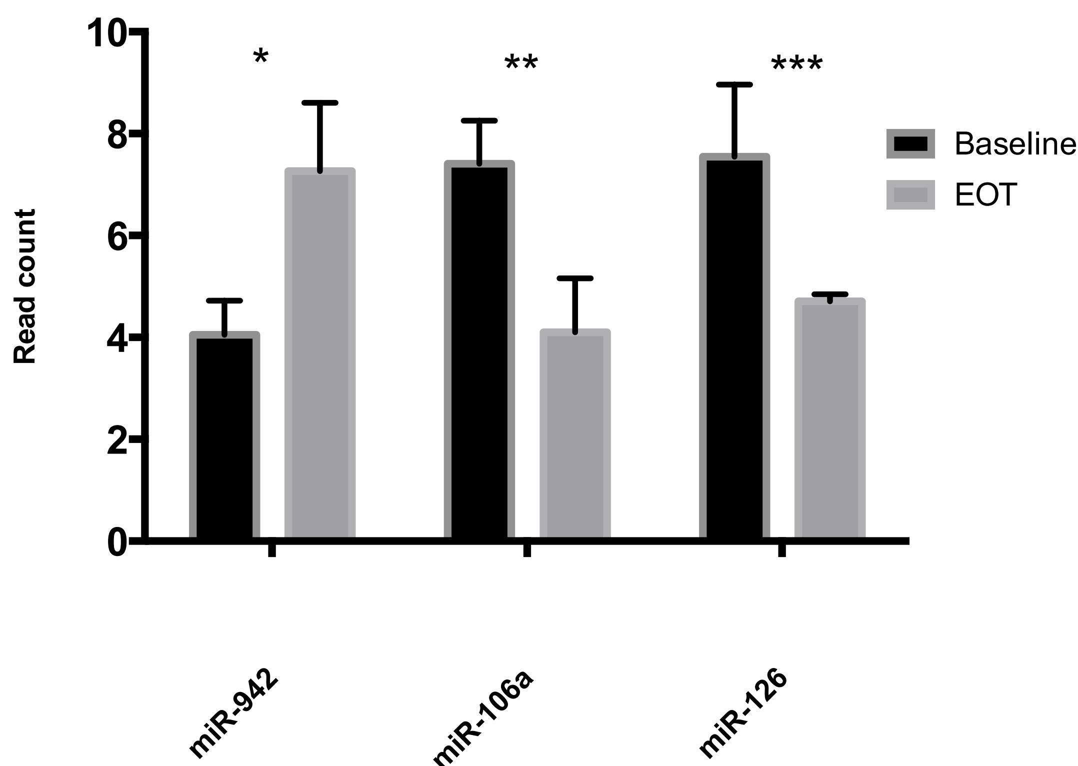


Figure 4.7 Differentially expressed microRNAs in psychotic depression.

Whole blood microRNA read counts in patients with psychotic depression, n=3. Data expressed as means with standard deviations (SD). BH Adjusted p-value = *=0.0066, **=0.0010, ***=0.0066

4.3.3.5 Differential expression by electrode placement

As electrode placement can affect both the effectiveness as well as cognitive side-effects of ECT this was included as a potential post-hoc moderator of microRNA expression. In the bilateral ECT group (n=6) 1057 microRNA species were identified. In the unilateral group (n=10) 1124 microRNA species were identified. There were no statistically significant differences in microRNA expression in these subgroups.

4.3.3.6 Impact of medication on differential expression

To explore whether differences observed in the post-hoc analysis could be due to medications, rather than diagnostic subtypes, groups were created based on their medications including those on/not on antipsychotics, and those on/not on lithium as these have been identified as having a potential effect on microRNA expression (Kolshus et al., 2014). Only one person was prescribed sodium valproate so this was not analysed. As all participants were on antidepressants this was also not analysed. There were no statistically significant differences in microRNA expression from baseline to EOT based on being prescribed antipsychotics (n=11) or lithium (n=6). At baseline, those prescribed antipsychotics irrespective of diagnostic group or subtype had a lower mean read count but this was not statistically significant (see Figure 4.8 below).

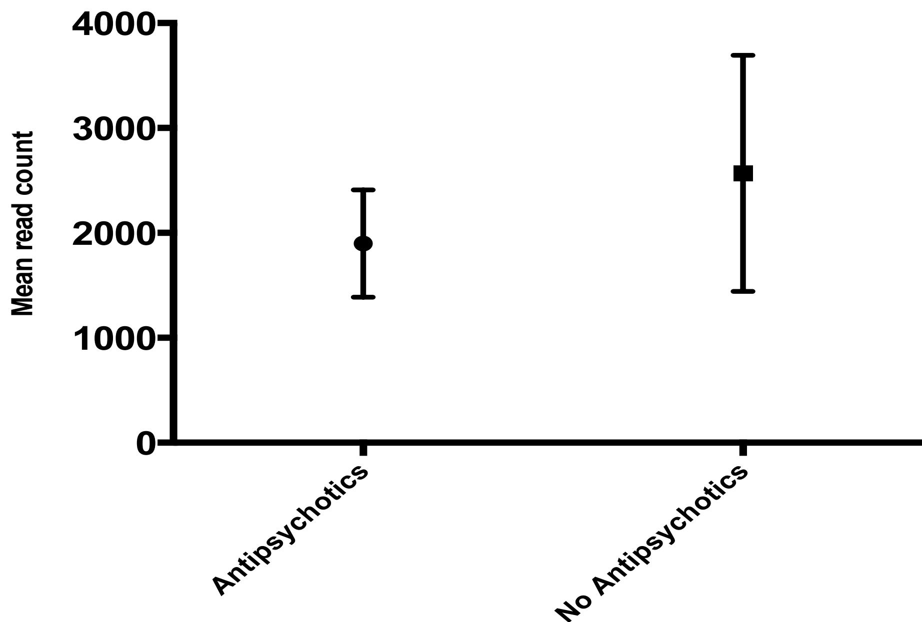


Figure 4.8 Antipsychotic medications and microRNA read count I
Mean baseline microRNA read count by antipsychotic prescription. There was no statistically significant differences between those prescribed antipsychotics and those who were not at baseline. Data are means and 95% confidence intervals, n=16 (antipsychotics n=11, no antipsychotics n=5), p=0.162

When this analysis was limited to those with unipolar depression only, the results remained statistically non-significant.

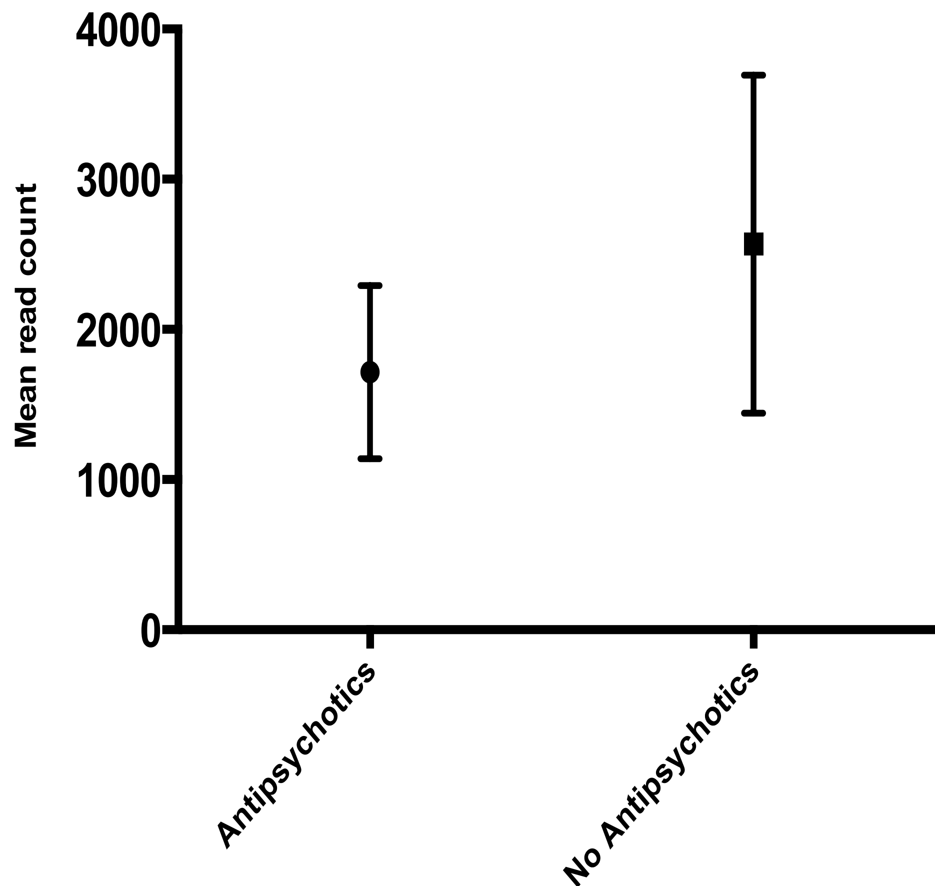


Figure 4.9 Antipsychotic medications and microRNA read count II
Mean baseline microRNA read count by antipsychotic prescription in unipolar depression. There was a stronger trend toward antipsychotics having a lower baseline mean read count, but this did still not reach statistical significance. Data are presented as means with 95 % confidence intervals n=12 (antipsychotics n=7, no antipsychotics n=5), p=0.073

With regard to lithium, there were no statistically significant differences between mean baseline levels of microRNAs based on its prescription (see Figure 4.10 below).

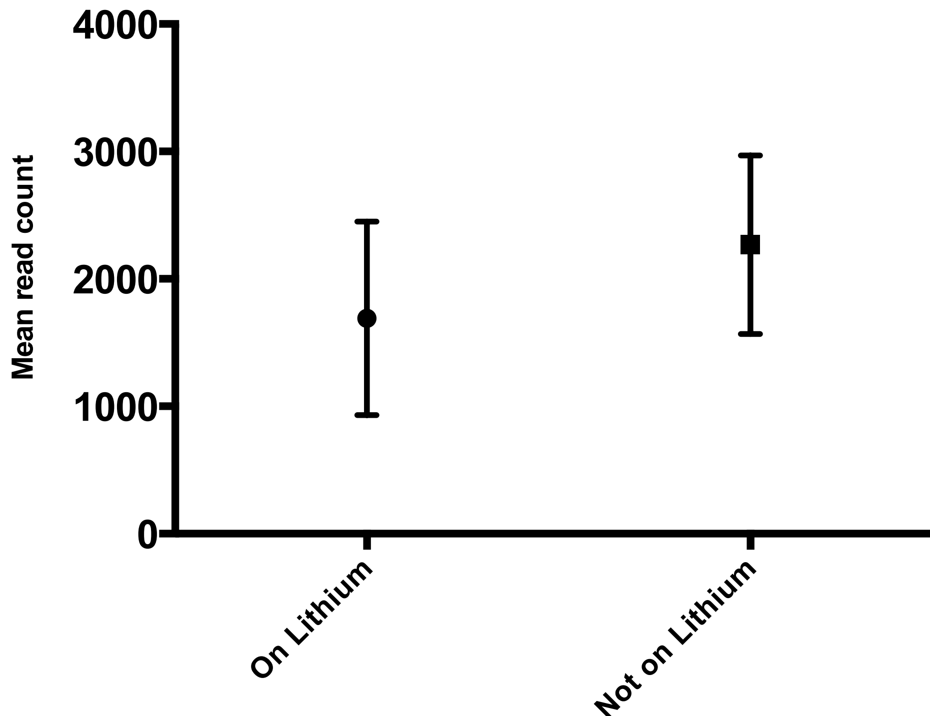


Figure 4.10 Lithium and microRNA read counts
Mean baseline microRNA read count by lithium prescription. There was no statistically significant differences between those prescribed lithium and those who were not at baseline. Data are presented as means and 95% confidence intervals, n=16 (lithium n=6, not on lithium=10), p=0.143.

4.3.4 qRT-PCR confirmation

As described above, the deep sequencing run identified four microRNAs that were differentially expressed in whole blood between baseline and EOT following ECT in certain subgroups. Three microRNAs (miR-126, miR-106a and miR-942) were differentially expressed in three patients with psychotic depression. One microRNA (miR-130a) was differentially expressed in patients with bipolar depression (n=4). qRT-PCR was used to confirm these results. I analysed both mature main strands as well as complementary strands where applicable (i.e. both -3p and -5p strands).

4.3.4.1 Identification of endogenous control

The comparative C_T method was used to quantify relative microRNA expression. This requires an endogenous control against which the microRNA of interest is compared. To this end, three commercially available endogenous controls (RPL 21, RNU6B and RNU49) and two microRNAs commonly expressed in the deep sequencing study that changed minimally from baseline to EOT (miR-1249 and miR-766) were tested. The microRNAs from the deep sequencing experiment were included due to recent criticisms of the commercially available controls (Xiang et al., 2014). The five candidate controls were analysed using RefFinder. Of the five candidates, miR-1249 had the best stability (Figure 4.11) and was therefore used as the endogenous control in the confirmation qRT-PCR study.

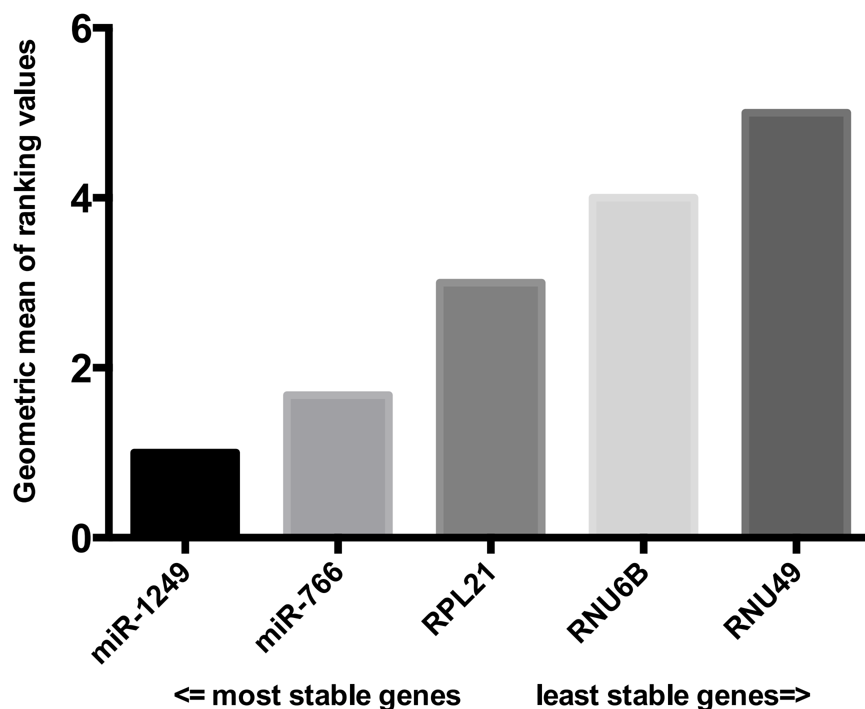


Figure 4.11 Endogenous control comprehensive gene stability

Of the five candidates for endogenous control, miR-1249 was the most stable candidate across a comprehensive range of programs such as geNorm, Normfinder, BestKeeper, and the comparative Ct method.

4.3.4.2 qRT-PCR: Total group analysis

qRT-PCR was carried out on the microRNAs identified in deep sequencing on the same cohort. In the group as a whole, as in deep sequencing, there was no statistically significant differences in microRNA levels from baseline to EOT (see Figure 4.12 below) using paired t-tests and a significance level of 0.05.

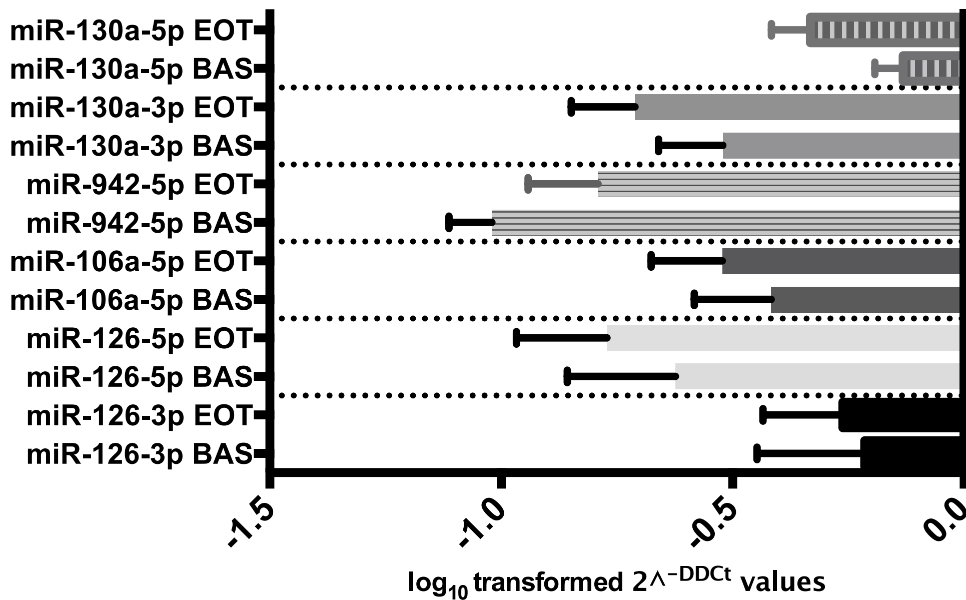


Figure 4.12 qRT-PCR confirmation - whole group analysis

qRT-PCR confirmation of microRNA gene expression before (BAS) and after (EOT) ECT. There were no statistically significant differences in any of the eight microRNAs tested. Two microRNAs (miR-106a-3p and miR-942-3p) failed to amplify. Data are presented as means and SEM, n=16.

4.3.4.3 RT-PCR: bipolar depression

Deep sequencing identified miR-130a levels as being significantly increased following ECT for patients with bipolar depression. qRT-PCR was used to confirm both miR-130a-3p and miR-130a-5p as these two microRNAs share the same hairpin structure. Levels of both microRNA species increased following ECT, but only miR-130-5p reached statistical significance. In line with the deep sequencing results there were no significant changes in miR-130a-3p/5p levels in unipolar depressed subjects.

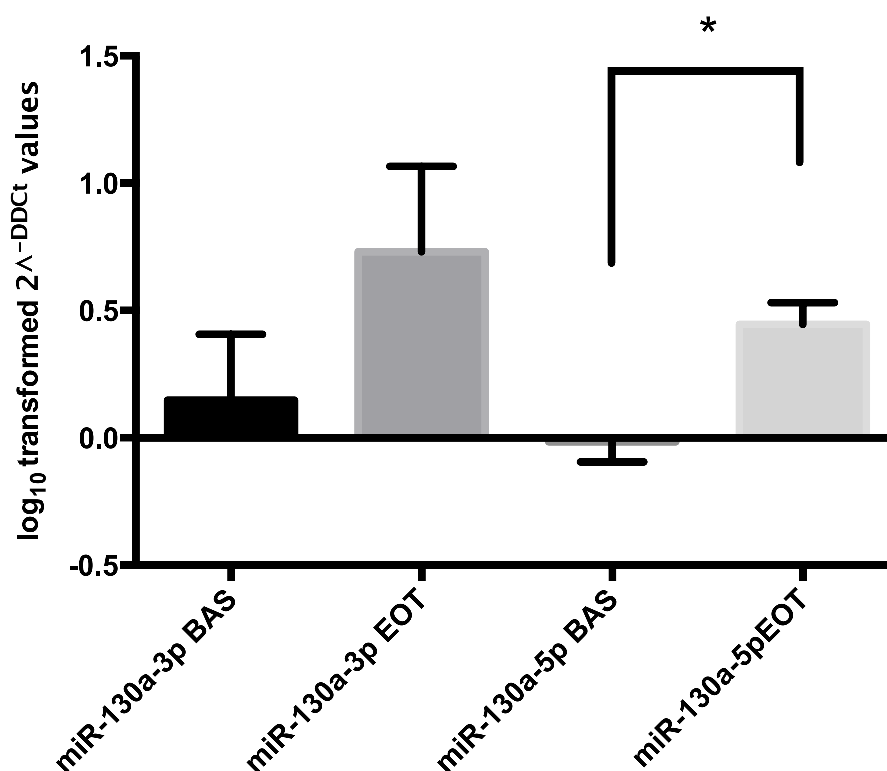


Figure 4.13 qRT-PCR confirmation of miR-130a-3p/5p

There was a statistically significant increase in miR-130a-5p levels following ECT (t=-5.87, p=0.010). There was also an increase in miR-130a-3p levels but this did not reach statistical significance (t=-1.61, p=0.206). Data are presented as means and SEM, n=4, Shapiro-Wilk test of normality p=0.808

4.3.4.4 qRT-PCR: psychotic depression

Deep sequencing identified three potential candidate microRNAs that were differentially expressed following ECT. MiR-942, miR-106a-5p and miR-126-3p/5p levels following ECT were analysed using qRT-PCR. Of the four microRNAs, three were confirmed, although one of the three (miR-942) was in a different direction than observed in the deep sequencing study. In line with the deep sequencing results there were no significant changes in the levels of these microRNAs in non-psychotic depressed subjects.

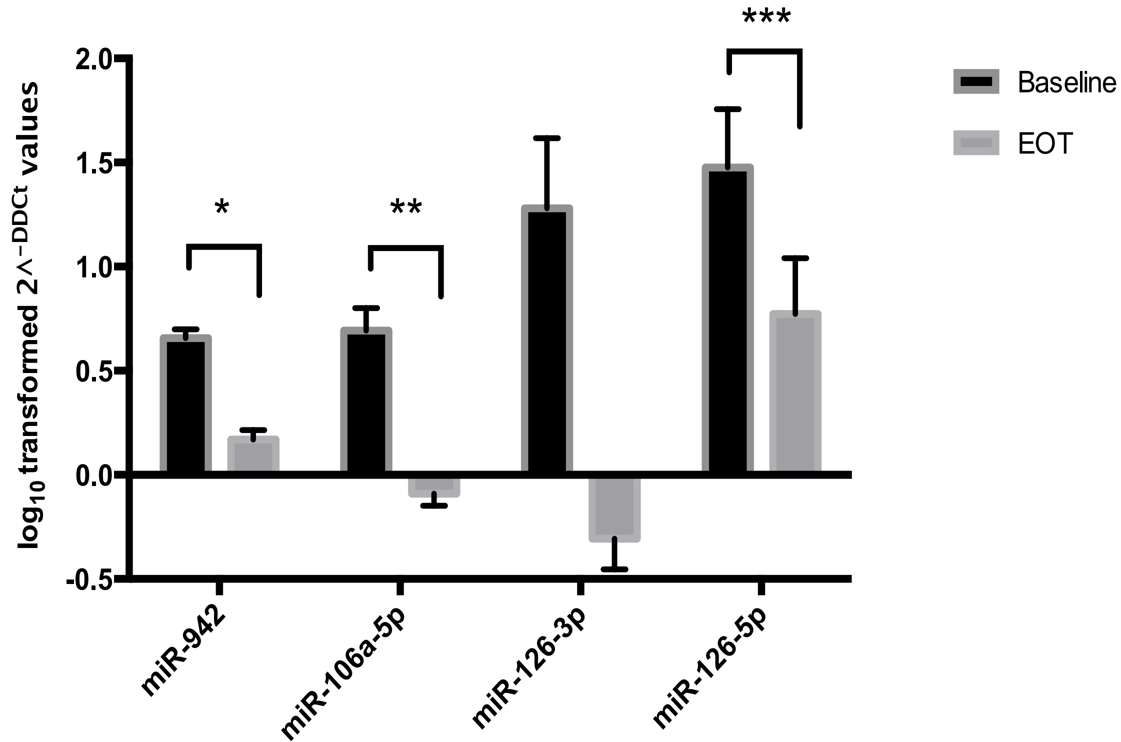


Figure 4.14 qRT-PCR analysis of microRNAs in psychotic depression

There was a statistically significant decrease in miR-942 ($t=6.41$, $p=0.023$*), miR-106a-5p ($t=4.76$, $p=0.041$**) and miRNA-126-5p ($t=12.07$, $p=0.007$***). There was also a decrease in miR-126-3p levels also which nearly reached statistical significance ($t=3.44$, $p=0.075$). Data are presented as means and SEM, n=3. Shapiro-Wilk tests of normality all >0.05.

4.4 Discussion

4.4.1 Deep sequencing study - introduction

To date, this is the first deep sequencing study of microRNA expression in peripheral blood of patients with depression and also the first study of microRNA changes following ECT. I analysed the entire microRNAome in whole blood of sixteen patients with major depression who responded well to ECT. Other profiling studies in depression have used a microarray approach (Bocchio-Chiavetto et al., 2013, Smalheiser et al., 2012, Garbett et al., 2015, Wan et al., 2015, Belzeaux et al., 2012, Smalheiser et al., 2014a). When planning the study, the only three studies at the time that had investigated microRNA levels in depression had identified between eight to thirty microRNAs using a microarray approach (Smalheiser et al., 2012, Bocchio-Chiavetto et al., 2013, Belzeaux et al., 2012). These studies were either comparisons of depressed subjects and controls, or before and after antidepressant treatment. These studies included ten to eighteen depressed patients. It was therefore expected that this deep sequencing study would find at least a similar amount of differentially expressed microRNAs, as deep sequencing is able to identify and quantify all microRNAs present in a sample. Also, our subjects were moderately to severely depressed at baseline, but had all responded well to ECT, the most effective antidepressant treatment available. Any changes were therefore expected to be more pronounced in this sample. It was therefore surprising that there was no evidence of a significant change in microRNA levels in peripheral blood following a successful course of ECT in major depression in the group as a whole.

An important factor in the validity of any profiling study is the quality of the samples used for the study. The samples included in this study were of good purity and integrity and poor sample quality should therefore not be an issue.

4.4.2 Deep sequencing study – the peripheral blood microRNAome

Deep sequencing has particular advantages in terms of describing the complement of expressed microRNAs. This is partly due to the dynamic range of microRNAs themselves, which can range from tens of thousands to a few molecules per cell. Also, as microRNAs are short molecules that often differ only by a single nucleotide, distinguishing similar forms can be difficult using a primer approach. MicroRNAs also have varying melting temperatures, which mean the optimal reaction settings for one microRNA maybe sub-optimal for another using qRT-PCR (Pritchard et al., 2012).

From a technical point of view, deep sequencing appeared to be successful with almost 1300 microRNA precursor species identified when mapped against the microRNA database maintained by ENSEMBL (Cunningham et al., 2015). Seventy-three per cent of the non-coding transcripts consisted of microRNAs. Most of these precursor microRNAs will have -3p and -5p mature microRNAs. Some also represent clustered microRNAs where more than two microRNAs share the same primary transcript (e.g. the miR-144 family). MiRBase currently contains 1881 precursor microRNAs (Griffith-Jones, 2015).

As numerous microRNAs are tissue-specific, we would not expect to see all microRNAs expressed in blood. Whole blood contains microRNAs from various sources, including leucocytes, red blood cells, platelets and plasma. Previous studies using deep sequencing have found 907 mature microRNAs in leucocytes (Vaz et al., 2010), 375 mature microRNAs in plasma (Brenu et al., 2014), and 197 mature microRNAs in red blood cells (Azzouzi et al., 2015). One recent study examined whole blood as well as comparing the various blood compartments and identified a total of 943 mature microRNAs in whole blood with considerable overlap between compartments (Cheng et al., 2014). This study therefore at least matched previous deep sequencing studies in terms of identifying microRNAs in blood. There was some variability in average read counts across samples (Figure 4.2) but this is in line with variability seen in other deep sequencing studies (Schee et al., 2013). With regard to the microRNAs that had the most mappable reads (highest abundance) there was considerable overlap

between the top ten most expressed microRNAs and other profiling studies that reported this data (Figure 4.15 below). Most profiling studies report on differentially expressed microRNAs, but two have included those with highest abundance in blood (Milagro et al., 2013, Wang et al., 2012).

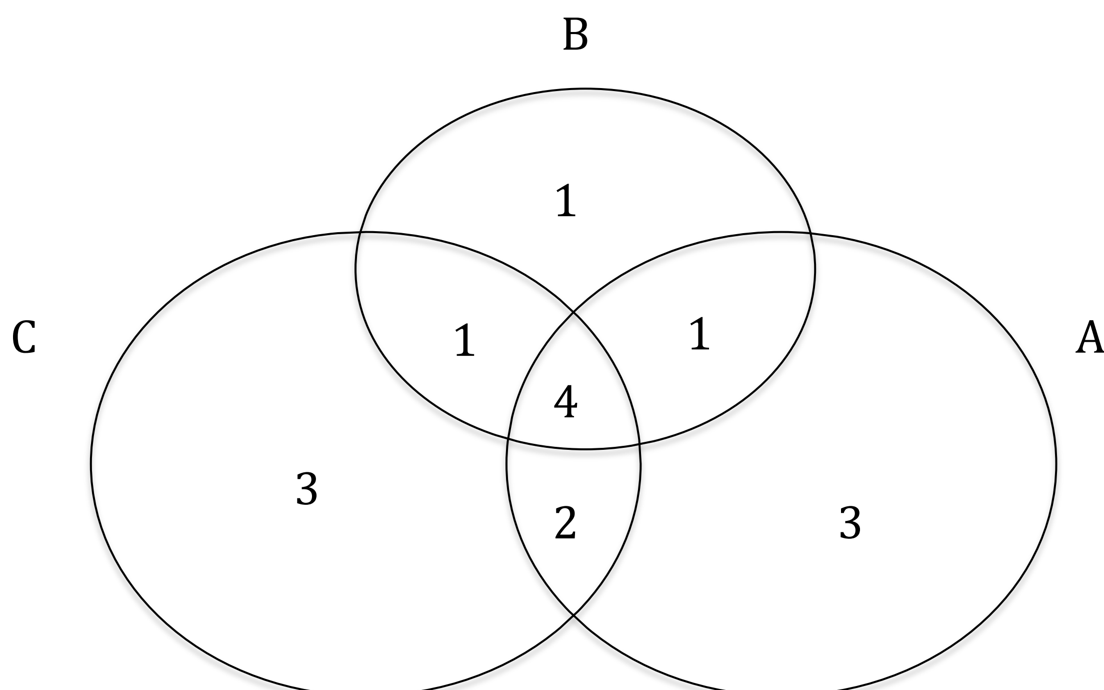


Figure 4.15 Overlap of studies of microRNAs in blood
Venn diagram of overlap of most commonly expressed microRNAs in blood. Data from two published studies A (Milagro et al., 2013); B (Wang et al., 2012); and C: this study.

4.4.3 Deep sequencing study – comparison to literature on microRNA expression in depression

Compared to other studies identified in the literature the results were somewhat disappointing. Thorough systematic reviews of the area have been recently performed (Kolshus et al., 2014, Ryan et al., 2016) but no similar negative findings were identified although this may indicate publication bias. Seven previous studies have profiled microRNA expression in depression (Table 4.8). Of these, none had the depth of interrogation provided by deep sequencing in this study to identify microRNA targets.

2588 microRNAs have been identified in the human genome and this study had the ability to potentially detect all these, whereas the previous studies screened from 10 to 1008 microRNAs. The majority of the other studies found differences in microRNA expression when comparing depressed patients to controls, whereas this study compared microRNA levels before and after treatment. Of the two other studies that compared microRNA levels before and after antidepressant treatment, one used the SSRI escitalopram in ten drug-naïve patients who responded to treatment (Bocchio-Chiavetto et al., 2013). 30 microRNAs were identified. In the second study, nine treatment responders gave blood for microRNA analysis (Belzeaux et al., 2012). Two of the patients underwent ECT during the trial period. Various antidepressants and psychotropics were used, in line with the patients described in this thesis. Eight microRNAs were identified, although many of them had a very low level of fold change. A further limitation in the second study was that the authors did not correct for multiple testing which is essential when dealing with the large datasets derived from a microarray approach. Of note, there was also no overlap between the microRNAs identified in these two studies, and the results were also neither confirmed using a different technique nor validated in a different cohort. Although both arrays screened for over 700 microRNAs, the studies only detected 367-385 microRNAs, much fewer than the number identified in our study. Of note, all the microRNAs identified as having altered levels in the two studies named above were identified by deep sequencing in this study but no significantly altered levels were found. Also, both studies only included patients with unipolar depression.

Year/ Study ID(1-7)	Comparison	Tissue/ Platform	N	Number of microRNAs detected /screened	Number microRNAs with s.s. changes
2012[1]	Controls	PFC/ Microarray	18/ 17	196/367	21
2012[2]	Before/After	Whole blood/ Microarray	10/ 10	385/750	30
2012[3]	a.Controls b.Before/After	PBMC/ Microarray	9/9	243/768	a.14 b.8
2014[4]	Controls	PFC/ Microarray	15/ 15	232/377	2
2014[5]	Controls	Fibroblasts/ Microarray and qRT-PCR	16/ 16	561/1008	38
2014[6]	Controls	PBMC/ Microarray and qRT-PCR	3/3	n.a./723	26 (9 confirmed)
2015[7]	Controls	CSF & serum/ Microarray and qRT-PCR	28/ 37	179	CSF:16 Serum:4

Table 4.8 MicroRNA profiling studies in depression
CSF: Cerebrospinal fluid; N= Number of subjects (for studies using controls, depressed subjects come first); n.a: not available; PBMC: Peripheral blood mononuclear cells; PFC: Prefrontal cortex; qRT-PCR= Quantitative reverse transcriptase polymerase chain reaction; s.s.: statistically significant; Studies: [1](Smalheiser et al., 2012); [2](Bocchio-Chiavetto et al., 2013); [3](Belzeaux et al., 2012); [4](Smalheiser et al., 2014a); [5](Garbett et al., 2015); [6](Fan et al., 2014); [7](Wan et al., 2015)

More studies have compared differential expression between healthy controls and those with depression than before/after treatment. Six out of the seven studies used such an approach. Two examined post-mortem brains (prefrontal cortex - PFC) (Smalheiser et al., 2012, Smalheiser et al.,

2014a), two used peripheral blood mononuclear cells (PBMCs) (Belzeaux et al., 2012, Fan et al., 2014), one used fibroblasts (Garbett et al., 2015) and one used cerebrospinal fluid (CSF) and serum (Wan et al., 2015). All but one excluded patients with bipolar depression. The one study that included bipolar subjects found a marked overlap between bipolar subjects and schizophrenia subjects (Smalheiser et al., 2014a). In bipolar disorder, nine microRNAs were differentially expressed in a comparison of bipolar subjects and controls, but only two were differentially expressed in depression with no overlap between unipolar and bipolar subjects. This, together with other studies comparing microRNA levels in schizophrenia, bipolar disorder and depression indicate that bipolar affective disorder appears to have different molecular mechanisms, including microRNA profiles and should be analysed separately from unipolar depression (Kolshus et al., 2014).

Only one study employed a deep sequencing approach, and this was limited to a subsample of schizophrenia subjects only (Smalheiser et al., 2014a). It is interesting to note that in this study, deep sequencing gave a slightly different picture than microarray in more than 20% of cases in terms of identifying the correct strand of a microRNA. This makes it important to follow up both -3p and -5p strands of microRNAs identified in deep sequencing.

Out of 138 microRNAs identified from profiling studies in depression across seven studies, only twenty-three microRNAs have been identified in at least two studies, and only one - miR-494 - was identified in more (three studies). These discrepancies between studies can be explained to some extent by some comparing pre- and post-treatment levels whereas others compared depressed patients with controls. It could also be explained by the heterogeneity of tissues used. Whether peripheral sources of microRNAs reflect brain levels is still not clear (Gallego et al., 2012, Gladkevich et al., 2004, Dwivedi, 2014). One recent study, which first measured microRNA levels in CSF and then went on to measure microRNA levels in the serum of the same patients, is helpful in this regard (Wan et al., 2015). The authors found that when six depressed patients were compared to healthy controls 16 microRNAs were differentially expressed in depressed patients. In the serum of the same patients, only four were significantly altered. This indicates that there is some correlation between central and peripheral

levels of microRNAs. However, given the impracticalities of central biomarkers, if even some of the signal from the brain is transmitted to the periphery, this may be of use from a biomarker perspective.

Further limitations to the current literature comparing depressed patients to controls include lack of confirmation or validation of the findings, with only three (Garbett et al., 2015, Fan et al., 2014, Wan et al., 2015) out of the six studies carrying out these steps. This means the results from the other studies should be viewed as preliminary only. More concerning is the apparent lack of correction for multiple testing in many of the studies. Given the large number of statistical tests in these profiling studies, typically involving hundreds to thousands of comparisons, some form of correction for multiple testing should be a minimum to avoid a surplus of false positives. Disappointingly, four out of the seven studies listed in Table 4.8 made no attempt at correcting their false discovery rate (Smalheiser et al., 2014a, Garbett et al., 2015, Fan et al., 2014, Wan et al., 2015). To highlight the importance of this, if no adjustment for multiple testing had been made to the present deep sequencing study, 31 microRNAs would have been identified with an unadjusted p-value of less than 0.05, the threshold used in the above studies. Seen in this light, it is perhaps not so disappointing that no clear differences in microRNA levels were seen in the group as whole following correction for multiple testing.

Such differences in methodological, statistical and bioinformatic approaches may be as important in differences due to patient sub-types, medications and other possible confounders (Kolshus et al., 2014, Pritchard et al., 2012, Issler and Chen, 2015). It should also be noted that different authors, having analysed the same set of samples from brain banks have come up with different results, with some microRNAs being identified as statistically significant in one study and not others (Smalheiser et al., 2014a).

4.4.4 Deep sequencing study – post hoc analysis

As discussed, methodological differences may explain the large differences in the microRNA depression literature. On the other hand, differences in diagnostic subtypes and treatments may also play a part. Although no

significant changes in microRNA levels were observed in the group as a whole, when the 16 patients were split into smaller groups significant differences did emerge in groups based on polarity of depression and presence of psychotic depression. No differences were found based on being prescribed antipsychotics or lithium alone. There were no differences based on ECT electrode placement.

In patients with bipolar depression, miR-130a precursor was significantly increased (log_2fold change of 1.75) following ECT. This microRNA appears to be vertebrate-specific (Griffith-Jones, 2015), and was previously identified as being differentially expressed in the prefrontal cortex of depressed subjects (Smalheiser et al., 2012). The miR-130a precursor produces mature miR-130a-3p and miR-130a-5p. Of note, miR-130a-3p targets amyloid beta precursor protein (*APP*) transcripts (Hebert et al., 2009). APP has been implicated in post-synaptic signalling at the serotonergic synapse, where it is has neuroprotective effects, see Figure 4.16 below (Lezoualc'h and Robert, 2003).

In psychotic depression, three microRNA precursors were differentially expressed following ECT: miR-106a, miR-126 and miR-942. The first two were downregulated by a log_2fold change of -2.18 and -2.23 respectively. miR-942 was upregulated by a log_2fold change of 2.29. Although miR-106a has not been implicated in depression previously, miR-106b, which comes from the same microRNA family as miR-106a, was differentially expressed in patients responding to escitalopram treatment (Bocchio-Chiavetto et al., 2013). In another study, miR-106a-5p was increased in the PFC of those with schizophrenia, but not in depressed subjects (Smalheiser et al., 2014a). miRNA-126-3p/5p are only expressed in endothelial cells and are involved in angiogenesis (Wang et al., 2008). miR-126-3p has been shown to be increased in the brains of mice following stress (Rinaldi et al., 2010). Neither form of miR-942 has previously been indicated in depression.

All microRNAs identified as differentially expressed in bipolar and psychotic patients using deep sequencing are expressed in the brain as well as peripheral blood and are therefore worthwhile targets for further exploration. As a first step the microRNAs identified were confirmed in the same study sample using qRT-PCR.

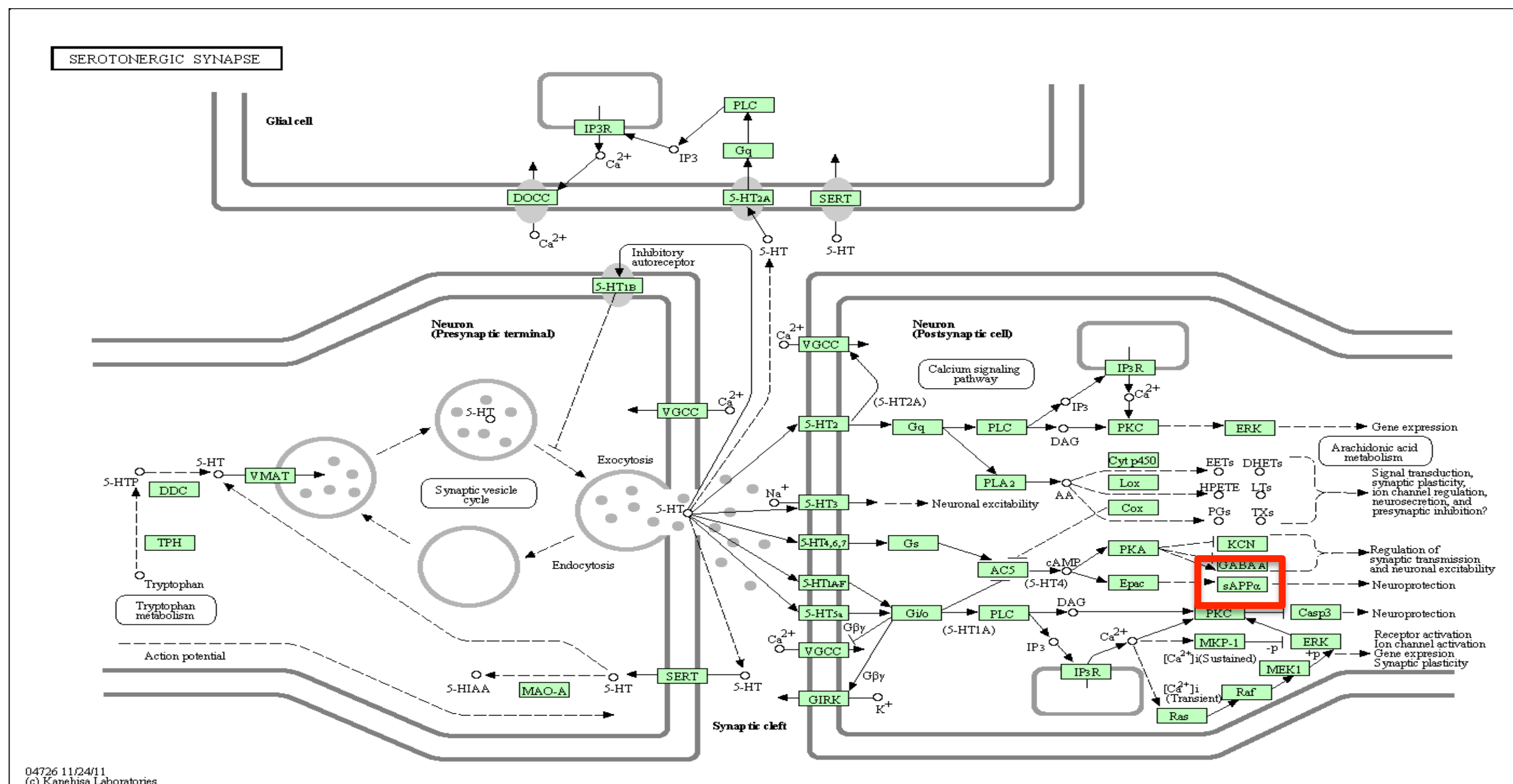


Figure 4.16 KEGG pathway of serotonergic synapse (Kanehisa and Goto, 2000)

Visualised using DIANA Pathway Viewer (Vlachos et al., 2012). miR-130a-3p target (*APP)* highlighted with red box.

4.4.5 PCR confirmation of deep sequencing results

A limitation of several of the profiling studies listed in Table 4.8 was the lack of confirmation of results using a different technological approach to strengthen their claims. Using qRT-PCR, the levels of the mature forms of the four precursor microRNAs identified from deep sequencing was established. Two microRNAs, miR-106a-3p and miR-942-3p were not detected in enough amounts to amplify. This was not altogether surprising as these microRNAs have many fewer validated targets compared to their -5p counterparts, and it is therefore likely that the -5p form is the main biologically active form of these two microRNAs (Chou et al., 2016).

Different endogenous controls in PCR experiments can lead to different results and even some commonly used commercially available controls have been shown to be differentially expressed in depressed cohorts (Liu et al., 2014, Smalheiser et al., 2014a). In the present study a range of potential endogenous controls were tested, including both commercially available controls and microRNAs that appeared stable in our sample. Using this empirical process, miR-1249 (which was not a commercially available control) was identified as being the most stable and suitable in this cohort.

qRT-PCR confirmed the findings from deep sequencing, indicating that miR-130a-5p was significantly increased in bipolar subjects, and levels of miR-130-3p neared significance. With regard to psychotic depression the levels of miR-942, miR-106a-5p and miR-126-5p were significantly decreased following ECT. The levels of miR-126-3p neared significance. Of note, the levels of miR-942, which was increased in deep sequencing was decreased in the qRT-PCR analyses. As in deep sequencing, there was no statistically significant differences in the group as a whole for any of the microRNAs.

4.4.6 Summary

The initial findings of this study suggested that levels of microRNAs in whole blood do not change significantly following a course of ECT for depression. This was in contrast to a limited number of existing clinical studies, with

similar sample size, that had found significant changes in microRNA levels, either before or after treatment or between depressed patients and controls. The current literature however is highly heterogeneous both in terms of methodology and results, and many failed to make basic statistical corrections for multiple testing. If a similar approach had been taken in this study many microRNAs indeed would have been earmarked as differentially expressed, but would likely have arisen purely by chance.

It may also be the case that microRNAs do play an important role in the molecular pathology of depression but that peripheral microRNA levels do not adequately reflect changes that are occurring in the brain. Most other studies have also compared depressed subjects to controls rather than the before/after treatment approach, and it may be that the role of microRNAs is that of a 'trait' rather than a 'state' marker. A further possibility is that the present study was not adequately powered despite using the best knowledge available at the time to guide numbers for the a priori analysis. It is unlikely that deep sequencing failed in terms of capturing the whole range of microRNAs because we identified more microRNAs in the blood of depressed patients than any other previous study. One interpretation was that the results were limited by confounding 'noise' clouding the picture. Post-hoc analysis, based on evidence from the literature on clinical and pathophysiological differences in subgroups of depression, indicated several microRNAs that were differentially expressed following correction for multiple testing. These were separately confirmed with qRT-PCR. The next step in the investigation of these microRNAs is to validate them in a separate, similar but larger set of patients and to compare expression levels to healthy controls.

Chapter 5
Results

5 Validation study of microRNA changes in peripheral blood following electroconvulsive therapy

5.1 Introduction

MicroRNAs are gaining attention as potential regulators of molecular mechanisms involved in depression and other psychiatric disorders. These short (typically 21-23 bases) ribonucleic acid (RNA) molecules do not code for proteins themselves, but rather can regulate gene expression through binding to messenger RNA (mRNA) transcripts, typically preventing their translation or direct degradation. One microRNA can regulate hundreds of genes, and this makes them an attractive target in what are assumed to be polygenic disorders, like depression, where no single gene has been established as having a large effect (O'Connor et al., 2012, Kolshus et al., 2014). Adverse life events and stress are key risk factors for depression and microRNAs represent one of the epigenetic mechanisms through which gene-environment interactions can occur (Issler and Chen, 2015).

Pre-clinical work has implicated microRNAs in the regulation of neuroplastic mechanisms, the hypothalamic-pituitary-adrenal (HPA)-axis control and the therapeutic actions of several antidepressants (Kolshus et al., 2014, Ryan et al., 2016). Although the number of clinical studies of microRNAs in depression is growing, this is lagging behind other areas in medicine, such as hepatology, where phase II clinical trials of microRNA-targeting medications are underway (Janssen et al., 2013). The findings to date from clinical studies of microRNAs in depression remain inconsistent, with little overlap between studies. These differences may be attributable to differences in patients, sample tissue, platform approach, study design, endogenous controls, medications and statistical or bioinformatic approach (Pritchard et al., 2012). Of major concern in many studies is the lack of correction for multiple testing as well as confirmation and validation of identified targets, with probable ensuing high numbers of false positives.

5.1.1 Aims of the study

Chapter Four described the findings from a deep sequencing profiling discovery study of microRNA expression in peripheral blood before and after electroconvulsive therapy (ECT) for major depressive disorder. Although no significant differences in microRNA levels in the group (n=16) as a whole were observed, sub-group analysis identified one microRNA precursor in patients with bipolar depression and three microRNA precursors in patients with psychotic depression following correction for multiple testing. To confirm these findings, quantitative real time polymerase chain reaction (qRT-PCR) of the eight mature isoforms derived from these four precursor microRNAs was carried out on the same samples. Four mature microRNAs remained significant and two neared significance. Two mature microRNAs were not expressed at levels above detection. As the numbers in these post-hoc sub-groups were low they may not have been adequately powered to detect a statistically significant difference in the two mature microRNAs that neared significance. The aim of the present study was therefore to validate the six candidate microRNAs identified from deep sequencing in a larger set of depressed patients before and after ECT as well as comparing the levels of these microRNAs in healthy control subjects. The underlying hypothesis was that levels of circulating microRNAs are significantly altered in psychotic (miR-106a, miR-126 and miR-942) and bipolar (miR-130a) depression treated with ECT. I also hypothesised that measuring levels of microRNAs will i) differentiate those with depression from healthy controls and ii) differentiate between responders and non-responders.

5.2 Methods

5.2.1 Participants and assessment tools

This study included 38 patients with moderate to severe depression enrolled in the EFFECT-Dep Trial, described in detail in Chapter two (see section 2.4.1). The presence or absence of a major depressive disorder, including melancholic and psychotic subtypes, was confirmed by administering the mood disorder component of the research version of the Structured Clinical Interview for DSM-IV Axis 1 Disorders (First et al., 2002). Depression

severity at baseline (BAS) and end of treatment (EOT) was measured using the 24-item version of the Hamilton Depression Rating Scale (HDRS-24) (Beckham and Leber, 1985). Exclusion criteria included substance abuse in the last six months; premorbid existing cognitive impairment; other Axis I disorder; ECT in the past 6 months; inability to consent; current inflammatory, infectious, or haematological disorder. Responders were defined as those who at end of treatment achieved a ≥60% decrease in HDRS from baseline and score ≤16. A remitter was defined as those who at end of treatment in addition to a ≥60% decrease in HDRS from baseline scored ≤10 on two occasions separated by one week.
36 healthy controls were also recruited (see section 2.4.2). Exclusion criteria for healthy controls included any history of any psychiatric disorder. Depression severity was assessed with HDRS-24.

5.2.2 Blood sampling and microRNA extraction

Blood sampling and microRNA extraction and quality analysis were performed as described in detail in Chapter Two (see sections 2.3.1-2.3.3). Briefly, fasting blood samples were taken at baseline and EOT for patients treated with ECT using the PaxGene© system (Qiagen Inc., USA) to collect whole blood. For healthy controls one set of fasting blood samples was taken. MicroRNA was extracted using PaxGene© microRNA kits according to the manufacturer's instructions (Qiagen Inc., USA). Quality analysis was carried out on the NanoDrop© 1000 UV-Vis Spectrophotometer (Fisher Scientific, UK) and the Bioanalyzer© 2100 (Agilent Technologies, Ireland) to establish the purity and integrity of RNA respectively.

5.2.3 PCR

Validation of candidate microRNAs derived from deep sequencing was carried out using stem-loop reverse transcription and qRT-PCR as described in detail in Chapter Two (see section 2.3.5-2.3.7). Stem-loop primers (see Table 5.1)(Applied Biosystems, UK) and a TaqMan® microRNA reverse transcription kit (Applied Biosystems, UK) were used according to the manufacturer's instructions.

MicroRNA	Stem-loop primer sequence
hsa-miR-942-3p	AUUAGGAGAGUAUCUUCUCUGUUUUGGCCAUGUGUG UACUCACAGCCCCUCACACAUGGCCGAAACAGAGAAG UUACUUUCCUAAU
hsa-miR-126-3p	CGCUGGCGACGGGACAUUAUUACUUUUGGUACGCGC UGUGACACUUCAAACUCGUACCGUGAGUAAUAAUGC GCCGUCCACGGCA
hsa-miR-126-5p	CGCUGGCGACGGGACAUUAUUACUUUUGGUACGCGC UGUGACACUUCAAACUCGUACCGUGAGUAAUAAUGC GCCGUCCACGGCA
hsa-miR-130a-3p	UGCUGCUGGCCAGAGCUCUUUUCACAUUGUGCUACU GUCUGCACCUGUCACUAGCAGUGCAAUGUUAAAAGG GCAUUGGCCGUGUAGUG
hsa-miR-130a-5p	UGCUGCUGGCCAGAGCUCUUUUCACAUUGUGCUACU GUCUGCACCUGUCACUAGCAGUGCAAUGUUAAAAGG GCAUUGGCCGUGUAGUG
hsa-miR-106a-5p	CCUUGGCCAUGUAAAAGUGCUUACAGUGCAGGUAGC UUUUUGAGAUCUACUGCAAUGUAAGCACUUCUUACA UUACCAUGG
hsa-miR-1249-3p	GGGAGGAGGGAGGAGAUGGGCCAAGUUCCCUCUGGC UGGAACGCCCUUCCCCCCCUUCUUCACCUG

Table 5.1 Stem-loop primers for PCR

Candidate microRNAs for validation are in white boxes. Endogenous control is in the grey box.

qRT-PCR was carried out using TaqMan® microRNA assays (see Table 5.2) on a StepOnePlus™ instrument (Applied Biosystems, UK) according to the manufacturer's instructions.

MicroRNA	Primer sequence
hsa-miR-942-3p	CACAUGGCCGAAACAGAGAAGU
hsa-miR-126-3p	UCGUACCGUGAGUAAUAAUGCG
hsa-miR-126-5p	CAUUAUUACUUUUGGUACGCG
hsa-miR-130a-3p	CAGUGCAAUGUUAAAAGGGCAU
hsa-miR-130a-5p	UUCACAUUGUGCUACUGUCUGC
hsa-miR-106a-5p	AAAAGUGCUUACAGUGCAGGUAG
hsa-miR-106a-3p	CUGCAAUGUAAGCACUUCUUAC
hsa-miR-1249-3p	ACGCCCUUCCCCCCCUUCUUCA

Table 5.2 TaqMan microRNA/control assay primers
Candidate microRNAs for validation are in white boxes. Endogenous control is in the grey box.

The comparative C_T method was used to assess gene expression for all qRT-PCR analyses with normalisation to an endogenous control to minimise quantification errors. The comparative C_T method compares the C_T values of one target gene to another (e.g. an internal control). A range of different candidates for endogenous control (see section 4.3.4.1) was explored using RefFinder, a web-based tool for evaluating candidate reference genes (Xie et al., 2011). Based on this work, miR-1249-3p was chosen as it showed the best stability between samples in the deep sequencing cohort.

5.2.4 Ethics

Written informed consent was obtained from all participants and ethical approval was granted from the local Research Ethics Committee in St. Patrick's University Hospital.

5.2.5 Data analysis

Data were analysed using SPSS version 22.0 (IBM Corporation, NY),R version 3.1.3 (R Foundation for Statistical Computing, 2014) and GraphPad Prism 6 (GraphPad Software, CA). Data are presented as means with standard deviation (SD) or number per group (% of group) where appropriate unless otherwise specified. Categorical data were tested using chi-square test (χ^2) unless otherwise specified. Fisher's Exact Test was used when numbers were low (n<5 in a cell in the 2x2 table). All data were tested for normality and log-transformed where appropriate. Data with normal distributions were analysed using student's t-test or univariate analysis of variance (ANOVA). Non-parametric data were analysed with the Wilcoxon-Signed Rank test for paired comparisons or the Mann-Whitney U test for non-paired comparisons. A p-value of less than 0.05 was considered statistically significant. Changes over time were investigated with a mixed design ANOVA / General Linear Model (GLM) (repeated-measures) with time (baseline / end of treatment) and responder status (yes/no) as factors. For correlational analysis, data were analysed with Pearson's product-moment correlation coefficient (Pearson's r) to test relationships between microRNA levels and clinical outcomes. Linear regression was used to examine the impact of potential confounders that differed between patients and healthy controls.

Previous studies of changes in microRNA levels following antidepressant treatment have found statistically significant changes using nine to ten patients (Belzeaux et al., 2012, Bocchio-Chiavetto et al., 2013). The latter also included a post-hoc power analysis showing they had over 80% power with ten patients to detect a two-fold change in microRNA levels. For the analysis of before and after treatment, our deep sequencing and earlier PCR confirmation studies had similar effect sizes and standard deviations that indicate that between six to ten subjects would be sufficient to have similar power as above, based on two-tailed paired t-tests. For the comparison between depressed patients and healthy controls sample sizes of 34 were required in each group to have power as above based on independent samples t-tests.

5.2.6 Bioinformatic analysis

Any microRNAs that were validated underwent a bioinformatic analysis to explore potential gene targets. Two main approaches to microRNA target analysis exist; predicted target analysis and validated target analysis. Predicted target analysis is based purely on in-silico analysis of potential mRNA targets for a given microRNA. This can run into hundreds or thousands of targets because of microRNAs' unique ability to bind not just to perfectly complementary targets but also partially complementary targets. Although the predicted targets with perfect complementarity are straightforward, predicting these partially complementary targets is complex.

Several software packages with slight variations in their algorithms to predict such partially complementary targets are available, such as miRanda, PicTar, TargetScan and DIANA-microT (Witkos et al., 2011). However, due to the variation in results between these packages it is often necessary to investigate a given microRNA in several of these packages, as the degree of overlap is not consistent. It has also recently been shown that microRNA-mRNA interactions can often occur with minimal interaction between the seed region (Helwak et al., 2013). Seed region interaction forms a key basis for the majority of the predicted target algorithms, thus adding further doubt on their validity. The high number of returned targets is also liable to include many false positives.

Experimentally validated microRNA target analysis offers a higher level of evidence. There are a number of methods that can be used in experimental validation of microRNA-mRNA interaction. Methods that provide strong evidence include reporter (luciferase) assays, Northern or Western blotting and qRT-PCR. Weaker evidence is provided by methods such as microarray, deep sequencing, stable isotope labelling with amino acids in cell culture (SILAC) proteomic analysis or immunoprecipitation (Hsu et al., 2014). As for predicted target analysis, a number of validated target databases are currently available. A recent review indicated that two of these, miRTarBase (Chou et al., 2016) and TarBase 7.0 (Vlachos et al., 2015) were the most comprehensive and reliable, although not without limitations (Ji Diana Lee et al., 2015). As these databases have to be

manually curated, the quality of the database depends on the methods used to investigate and verify published studies. The results from the TarBase 7.0 and miRTarBase databases were therefore chosen to produce lists of experimentally validated targets, including separate lists based on the strength of evidence (high confidence vs. lower confidence).

The potential functions and process of the experimentally validated gene targets of validated microRNAs were then explored using Gene Ontology (GO) terms. The Gene Ontology project is aimed at creating a set of consistent structured language or vocabulary (ontology) to describe gene products (Harris et al., 2004). Gene Ontology terms were queried using the latest GO Consortium database (Gene Ontology Consortium, 2015).

The GO project describes gene products in three domains; Molecular Function, Biological Process and Cellular Component. Molecular Function refers to elemental activities, including binding or transporting at a molecular level. It does not refer to the location or final function of the activity but the task or ability of the molecule. A molecule may have many functions, and their loose levels of hierarchy with 'parent' terms being more general than 'child terms'. As an example, the Molecular Function terms that maps to serotonin binding is included in Figure 5.1 below.

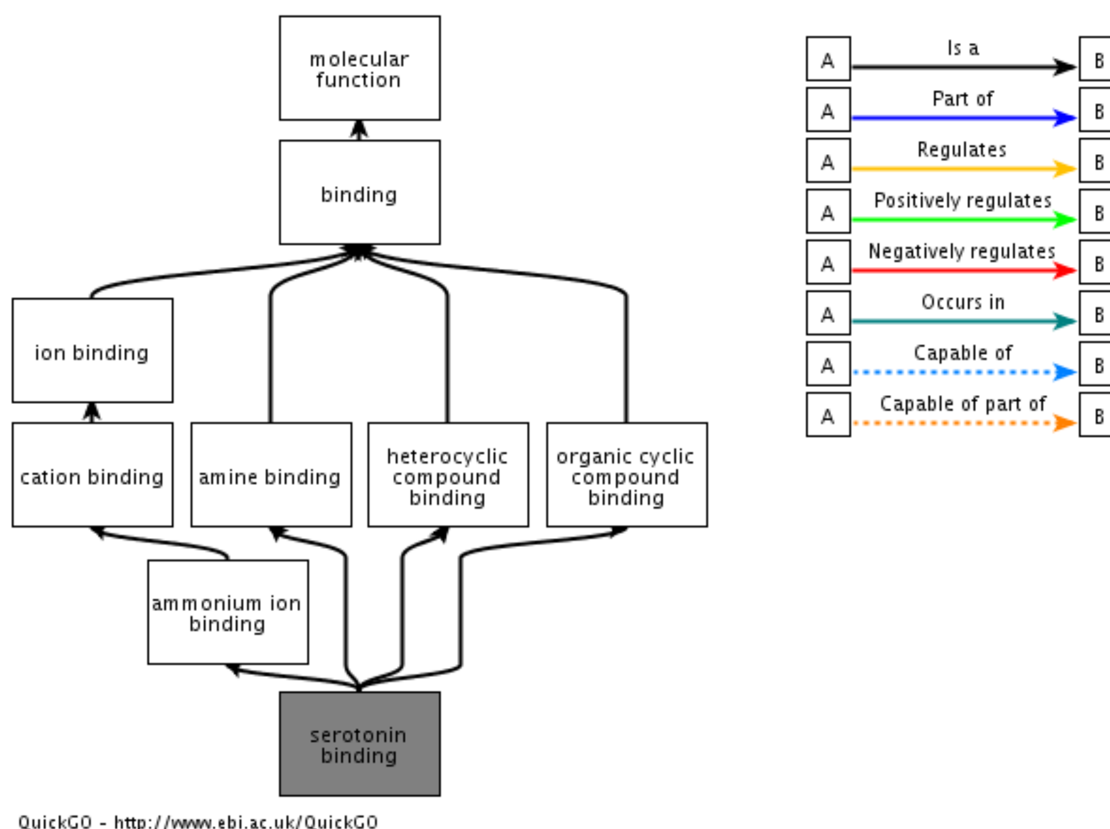


Figure 5.1 GO hierarchy - molecular function

GO hierarchy for the Molecular Function 'serotonin binding' (for explanatory purposes). All the boxes in the diagram are examples of molecular functions. The grey box ('serotonin binding') is the most specific (or 'child') term in this hierarchy. Serotonin binding could also be classified under its various 'parent' terms higher up the hierarchy, although these become progressively less specific. For example, 'serotonin binding' maps on to the term 'amine binding' or further up the hierarchy under the rather non-specific term 'binding'. As an example, querying the GO molecular function of the serotonin receptor gene would return hits to all the molecular functions listed in the hierarchy above. The arrows in the hierarchy are colour-coded as listed in the legend. In this hierarchy, all arrows are black, indicating there are no regulatory relationships involved. Source: (Huntley et al., 2015).

Biological process refers to a recognised series of events or molecular functions with a defined beginning and end. As for Molecular Function, this term can refer to collections of processes that may form a hierarchy, going

from specific to general. An example of the type of terms that make up biological process for VEGF receptor activity is included in Figure 5.2 below.

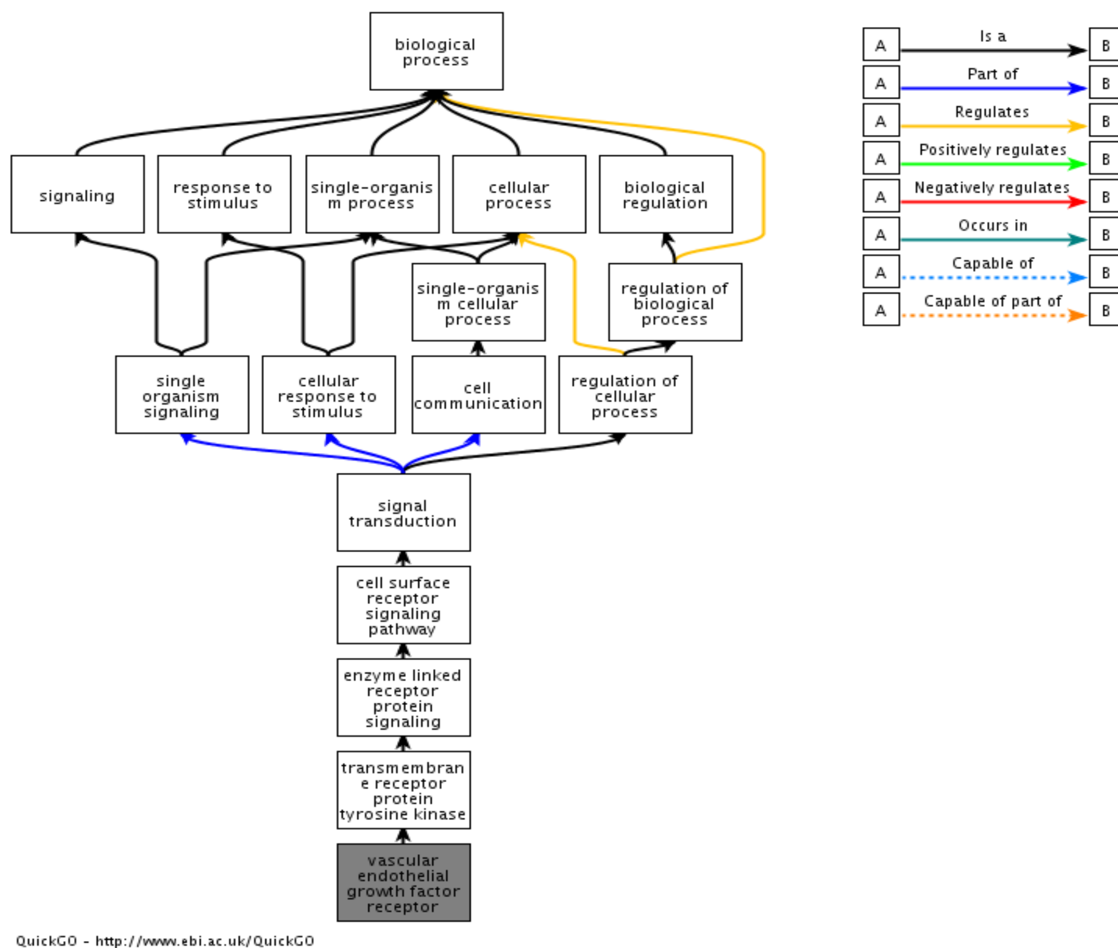


Figure 5.2 GO hierarchy for the Biological Process VEGF receptor

VEGF receptor is the most specific or 'child' term in this hierarchy. It is an example of a 'transmembrane receptor protein tyrosine kinase' (its direct 'parent' term in the hierarchy), which in turn is a type of 'enzyme linked receptor protein signalling' and so on. The process / boxes at the top of the hierarchy are relatively non-specific, and thousands of genes would map on to this level, whereas only a handful may map on to the shaded box. The colour of the arrows indicates whether there are any regulatory relationships between the biological processes. In this example, the yellow arrows indicate a regulatory relationship. The blue and black arrows indicate that one function either 'is a' or is a 'part of' another biological process, without any regulatory relationship.

The final GO category is Cellular Component, which refers to the cellular location, such as mitochondrion or cytoplasm, of gene products.

An initial exploration of the GO terms of the three experimentally validated genes targeted by both microRNAs was carried out using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) Gene List Analysis Tool (Mi et al., 2013). Subsequent analysis included entering lists of high and low confidence experimentally validated gene targets of miR-126-3p or miR-106a-5p into The Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2007). The DAVID algorithm analyses gene sets for statistical over-representation. Does the set of genes map on to sets of GO terms more frequently than you would expect by chance? The set of GO terms returned by such an analysis would give an indication of what role this set of genes may play rather than attempting to analyse the individual GO terms for each gene which becomes impractical once the number of genes increases. The DAVID algorithm selects GO terms based on the Expression Analysis Systematic Explorer (EASE) tool (Hosack et al., 2003). EASE is a conservative variant of the Fisher Exact test as outlined below in Table 5.3 below.

	Hypothetical target gene list	Human Genome
In Biological Process	3	40
Not in Biological Process	234	29960

Table 5.3 Statistical over-representation (EASE analysis)
Hypothetical EASE statistical over-representation analysis (for explanatory purposes). To examine whether a list of target genes are over-represented in a biological process a 2x2 table is drawn up. In the first column, the target genes are divided into those that are involved in the biological process ('In Biological Process') and those that are not ('Not in Biological Process'). A hypothetical microRNA may return a list of 238 validated target genes, 4 of which may be involved in the given biological process, and 234 that are not. The number of genes involved in the process is subtracted by 1 for a more conservative approach (the reduction has a bigger effect if only a few genes are involved as in the example above, whereas it would have a minimal impact if the number of genes involved were large, which would be less likely to occur by chance). In our hypothetical example, this would leave 3 genes in the 'In Biological Process' row. In column 2, the gene numbers are based on the whole human genome, with the first row including all the genes that are involved in the biological process (in this hypothetical case, 40 genes). The second row includes the remainder of the genes in the human genome that are 'Not in Biological Process'. A modified Fisher Exact Test is then run. If the p-value is <0.05 following Bonferroni correction for multiple testing the set of gene targets are considered to be statistically over-represented in the sample compared to random chance.

The final GO analysis includes a pathway analysis using Protein ANalysis THrough Evolutionary Relationships (PANTHER). PANTHER pathways are manually curated pathways using four classes of data; pathway, molecule, reaction and location. Pathway class is an overview of the concept and scope of the pathway and is similar to what is available in textbooks. The molecule class represents classes of molecules (such as proteins, genes, RNA, other small molecules) that have a given mechanistic role in a pathway and is linked to the PANTHER protein library (Mi et al., 2013). The third class is the reaction class, which refers to the biochemical relationships between the pathway molecules. The final class refers to the cellular or tissue location of the components of the pathway.

5.3 Results

5.3.1 Participant details

Demographic and baseline clinical characteristics of the 37 EFFECT-Dep Trial patients and the 34 healthy controls are listed in Table 5.4 below. The two groups were matched in terms of gender and age. Healthy controls were more likely to have completed tertiary education compared to depressed patients (67.6 vs. 29.7%). They also consumed more units of alcohol compared to the EFFECT-Dep Trial patients ($U=3.137$, $p=0.002$). Unsurprisingly, the depressed groups had higher scores on HDRS-24 at baseline compared to healthy controls ($t=23.43$, $p<0.001$). Patients enrolled in the EFFECT-Dep Trial were more likely to be smokers compared to healthy controls (54.1 vs. 24.4%).

Variable	EFFECT-Dep Group (n=37)	Healthy Controls (n=34)	Formal test
Age, range	53.2 (12.8) 32-81	54.7 (13.7) 26-77	t=-0.483 p=0.630
Gender, female n (%)	28 (75.7)	24 (70.6)	χ^2=0.234 p=0.789
Education completed, n (%)			
Primary	8 (21.6)	5 (14.7)	χ^2=10.820
Secondary	18 (48.6)	6 (21.4)	p=0.004
Tertiary	11 (29.7)	23 (67.6)	
Alcohol consumption, median units per week, (range)	2 (0-20)	7 (0-24)	U=3.137 p=0.002
Smoker (Yes/No)	20/17	6/28	χ^2=10.119 p=0.003
HDRS Baseline	29.5(5.7)	3.4(3.1)	t=23.43 p=<0.001
Episode duration, median weeks, (range)	20 (4-84)	n/a	n/a
Treatment-resistant*, n (%)	26(70.3)	n/a	n/a
Previous ECT, n (%)	11(28.9)	n/a	n/a
Psychotic subtype, n (%)	7(18.4)	n/a	n/a
Melancholic subtype, n (%)	28 (73.7)	n/a	n/a
Bipolar depression, n (%)	10 (26.3)	n/a	n/a
Medications, n (%) taking			
SSRI	8 (21.6)	n/a	
SNRI	17 (45.9)		

TCA	10 (27.0)	
Mirtazapine	10 (27.0)	
Lithium	18 (48.6)	
Antipsychotics	23 (62.2)	
BZD	19 (51.4)	
Pregabalin	7 (18.9)	
Hypnotic	20 (54.1)	
Valproate	4 (10.8)	
Trazodone	6 (16.2)	
Buproprion	3 (8.1)	

Table 5.4 Demographic and clinical details of participants

Details of EFFECT-Dep Trial patients and healthy controls. BZD: Benzodiazepines; HDRS: 24-item Hamilton Depression Rating Scale; n/a: not applicable; SNRI: Serotonin and noradrenaline reuptake inhibitor; SSRI: Selective serotonin reuptake inhibitor; TCA: Tricyclic antidepressants; U=Standardised Mann-Whitney test; *Treatment resistance measured using Antidepressant Treatment History Form (ATHF).

5.3.2 MicroRNA quality assessment

The purity of RNA in the samples was assessed by measuring the absorbance ratio at 260/280 (A260/280) nm on the NanoDrop© 1000 UV-Vis Spectrophotometer. A minimally accepted threshold of 1.8 was set for A260/280 values (Farrell, 2010). Three samples had an absorbance ratio below this threshold of 1.8 and were removed from all analysis, including description of baseline characteristics. Of the remaining samples, the mean A260/280 was 2.1 with a standard deviation of 0.19. With regard to RNA integrity, all samples had a RIN value above 5.8. The mean RIN value was 8.3 with a standard deviation of 1.5. This indicates that the purity and integrity of RNA in the samples were of good quality and suitable for PCR analysis.

5.3.3 qRT-PCR Validation

5.3.3.1 Total ECT group analysis

In Chapter four, microRNAs that were differentially expressed in EFFECT-Dep Trial patients with psychotic (miR-106a, miR-126a and miR-942) or bipolar (miR-130a) depression were identified. These changes were not seen in the depressed group as a whole (n=16). As part of validating these findings, changes in the levels of these microRNAs following ECT were investigated in a different and larger group of EFFECT-Dep Trial patients. In the group as a whole, there were no statistically significant differences in microRNA levels from baseline to EOT using paired t-tests and a significance level of 0.05.

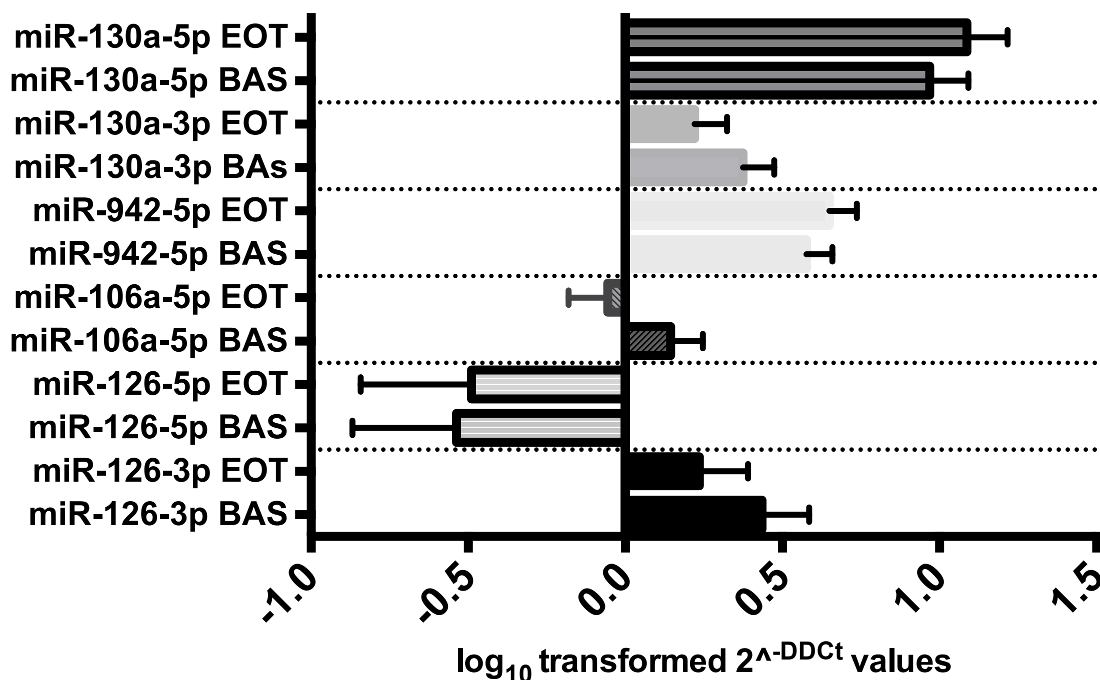


Figure 5.3 Whole group microRNA qRT-PCR validation

qRT-PCR validation of microRNA gene expression before (BAS) and after (EOT) ECT. There were no statistically significant differences in any of the six microRNAs. MiR-126-5p was detected in low levels and failed to amplify in numerous replicates and was therefore not included in the analysis of control subjects. Data are presented as means and SEM, n=37

5.3.3.2 qRT-PCR: Bipolar depression

Previous results identified elevated miR-130a levels following ECT in patients with bipolar depression. qRT-PCR was used to validate both miR-130a-3p and miR-130a-5p in those with bipolar depression (n=10) as these two microRNAs share the same hairpin structure. Levels of miR-130a-3p decreased slightly following ECT whereas levels of miR-130a-5p increased. Neither change however, was statistically significant. In line with the previous deep sequencing results, there were no significant changes in miR-130a levels in unipolar depressed subjects (n=27). There were no differences in baseline levels of miR-130a-3p between unipolar and bipolar patients (t=-0.189, p=0.851) or between psychotic and non-psychotic patients (t=-1.723, p=0.095). Neither were there any differences in the baseline levels of mir-130a-5p between unipolar and bipolar patients (t=0.760, p=0.453) or between psychotic and non-psychotic patients (t=-1.071, p=0.292).

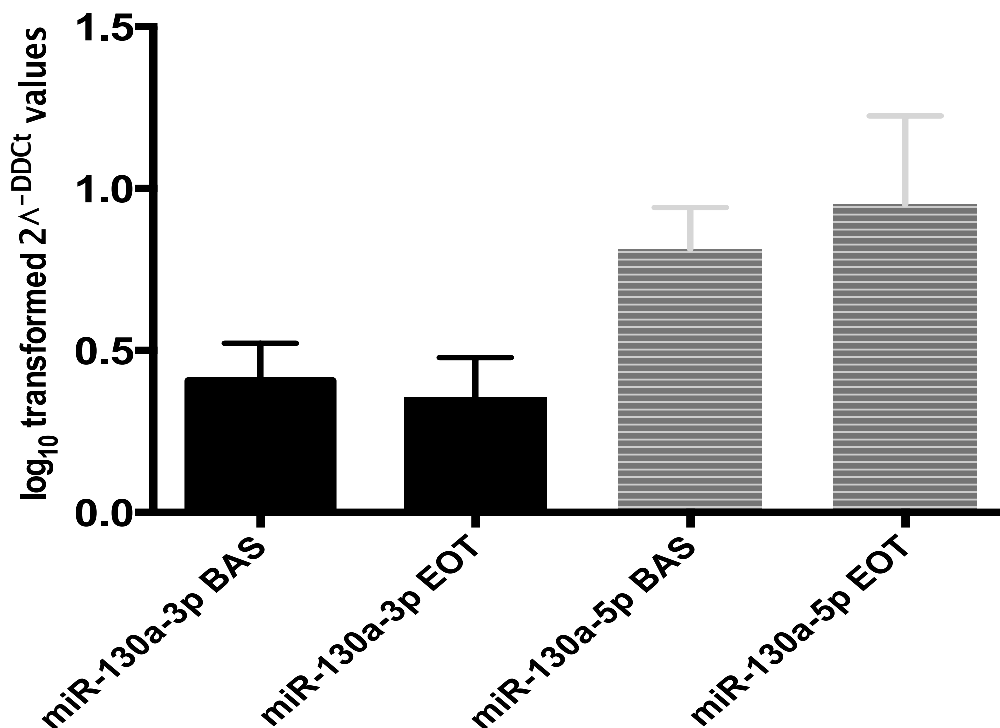


Figure 5.4 qRT-PCR validation of miR-130a-3p/5p

There was a small, non-significant, increase in miR-130a-5p levels following ECT (t=-0.89, p=0.41), and a small, non-significant decrease in miR-130a-3p (t=0.263, p=0.802). Data are presented as means and SEM, n=10.

5.3.3.3 qRT-PCR: Psychotic depression

Previous results identified four potential candidate microRNAs for validation in psychotic depression; miR-942-5p, miR-106a-5p and miR-126-3p/5p. These were validated here in a different subgroup of patients also undergoing ECT (n=7). Of the four candidates, two (miR-106a-5p and miR-126-3p) showed a statistically significant reduction in peripheral blood following ECT, in line with previous results. This however, did not hold true for miR-942-5p or miR-126-5p were the changes were non-significant. In line with the deep sequencing results there were no significant changes in the levels of these microRNAs in non-psychotic depressed subjects (n=30).

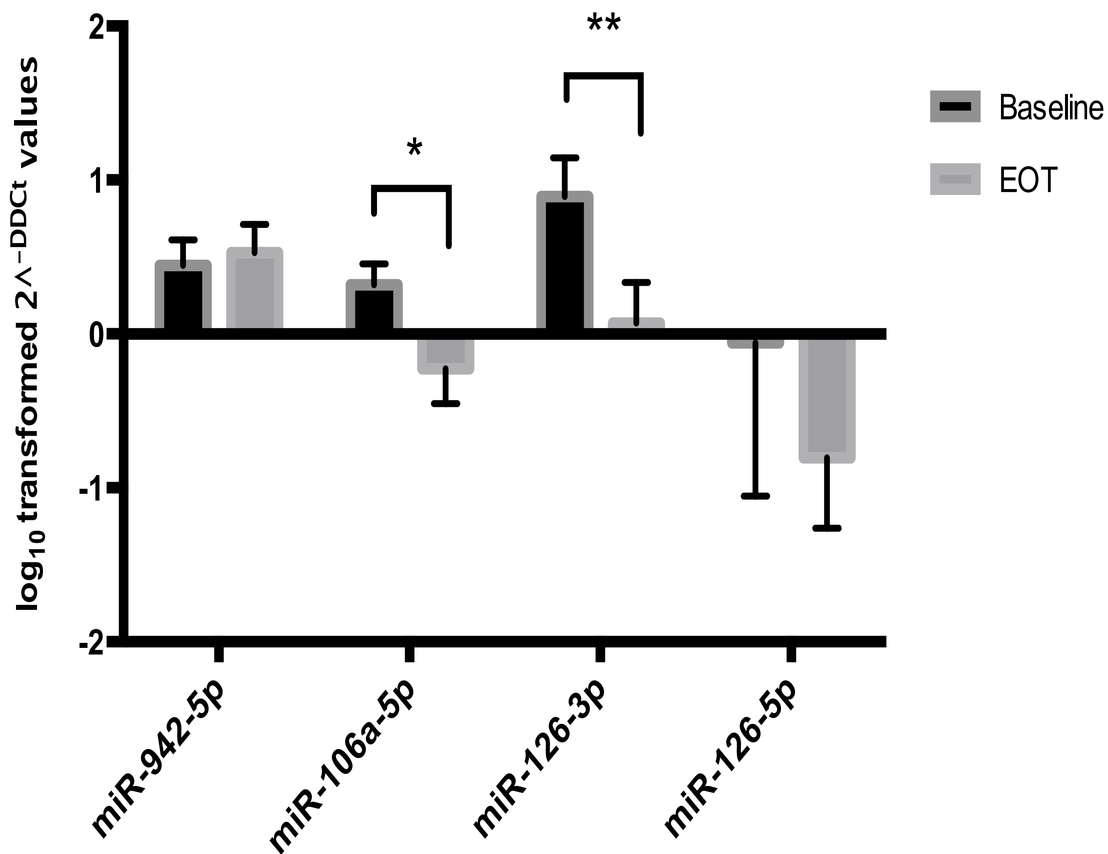


Figure 5.5 qRT-PCR validation in psychotic depression

qRT-PCR validation of microRNAs in psychotic depression. There was a statistically significant decrease in miR-106a-5p (t=2.868, p=0.029*) and miR-126-3p (t=3.041, p=0.023**). Changes in miR-942-5p and miR-126-5p were non-significant. Data are presented as means and SEM, n=7.

5.3.4 Comparison between patients and healthy controls

To test the hypothesis that microRNA levels in peripheral blood have the potential to differentiate between depressed patients and healthy controls, a comparison between baseline microRNA levels in these groups were carried out. Baseline levels of miR-130a-3p ($t=3.816$, $p=<0.001$) and miR-130a-5p ($t=6.63$, $p=<0.001$) were significantly lower in healthy controls. There were no significant differences in the other microRNAs at baseline (miR-126-3p: $t=1.578$, $p=0.120$; miR-106a-5p: $t=0.138$, $p=0.891$; miR-942-5p: $t=0.519$, $p=0.606$). As patients and controls differed in terms of alcohol consumption, correlational analyses were carried out to test for potential significant relationships between alcohol and baseline microRNA levels. There were no significant correlations between baseline microRNA levels and alcohol consumption levels.

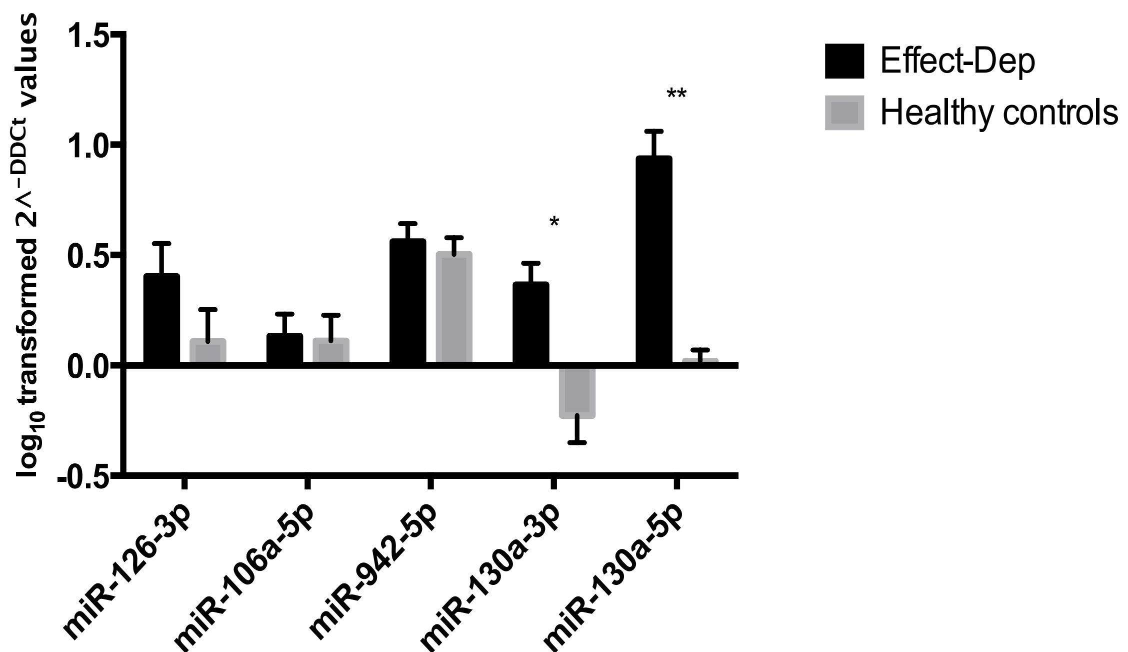


Figure 5.6 Comparison of depressed patients and healthy controls

Comparison between baseline values of microRNAs in depressed patients and healthy controls. Significant differences were seen for miR-130a-3p ($t=3.816$, $p=<0.001$*) and miR-130a-5p ($t=6.63$, $p=<0.001$**). Data are presented as means and SEM, n=37 (Effect-Dep Trial) and 34 (healthy controls), false discovery rate set at 1%.

5.3.5 Baseline differences between responders and non-responders

Responders were defined as those who at end of treatment achieved a ≥60% decrease in HDRS-24 from baseline and a final score ≤16. The number of responders in the Effect-Dep Trial validation group was relatively low, n=14 (36.8%), as those with the best response had been selected for the earlier deep sequencing experiment. There were no significant differences in baseline HDRS (F=0.065, p=0.80), age (F=3.502, p=0.067) or gender (χ^2 =2.331, p=0.147) between responders and non-responders. Baseline levels of the microRNAs under investigation were then compared between responders and non-responders. No significant differences were found between these groups in the cohort as a whole.

MicroRNA	Responder	Non-responder	F value	p-value
miR-126-3p	0.347	0.476	0.156	0.696
miR-106a-5p	0.211	0.113	0.191	0.665
miR-942-5p	0.621	0.556	0.130	0.721
miR-130a-3p	0.303	0.409	0.242	0.626
miR-130a-5p	1.122	0.896	0.724	0.401

Table 5.5 Baseline microRNA level by response status

MicroRNA expression levels at baseline in responder and non-responders. Data are presented as $\log_{10}$ transformed $2^{\wedge\text{-DDCt}}$ values for responders and non-responders with corresponding F and p-values (ANOVA). n=37 (14 responders/23 non-responders).

5.3.5.1 Change in microRNA levels in responders and non-responders

No significant changes in microRNA levels were seen in the group as a whole between baseline and end of treatment. No baseline differences were observed between responders and non-responders either. There was also no difference in microRNA levels from baseline to end of treatment based on responder status.

MicroRNA	Time effect		Time x response	
	F value	p-value	F value	p-value
miR-126-3p	0.731	0.401	0.192	0.666
miR-106a-5p	1.765	0.197	0.163	0.691
miR-942-5p	0.108	0.746	0.002	0.967
miR-130a-3p	1.645	0.212	0.179	0.676
miR-130a-5p	0.000	0.991	0.436	0.516

Table 5.6 MicroRNA changes over time by response status

MicroRNA expression change and relationship with treatment response. GLM of time effect and time x response effect with respective F and p-values. n=37 (14 responders/23 non-responders).

MicroRNA levels and scores on the HDRS-24 at baseline were significantly correlated for miR-130a-3p (Pearson's r=0.451, p<0.001) but not for miR-130a-5p (Pearson's r=-0.097, p=0.629). No significant changes in miR-130a-3p/5p levels were observed following ECT treatment.

5.3.6 Psychotic subgroup analysis

Two microRNAs (miR-126-3p and miR-106a-5p) were validated as having significantly reduced levels in peripheral blood of patients with psychotic depression following a course of ECT. These changes were not seen in the patient group as a whole. Also, in the group as a whole, these microRNAs did not differentiate between depressed patients and healthy controls at baseline, nor did they predict treatment response. Although our healthy controls matched the depressed group at baseline, the patients with psychotic depression had a higher proportion of female gender (6/7) and therefore each patient in the psychotic depression group was therefore closely matched to three healthy controls for comparison. With this new gender matching, there were no significant differences between the psychotic depression group and healthy controls in age (t=0.246, p=0.807), smoking (χ^2 =1.587, p=0.318, (Fisher's Exact Test)), or alcohol consumption (U=-0.775, p=0.571). The EFFECT-Dep Trial patients in

general came from a lower socio-economic group than the healthy controls (χ^2=10.793, p=0.029).

5.3.6.1 MiR-126-3p

A comparison between baseline levels of miR-126-3p in those with psychotic depression (n=7) and matched healthy controls (n=21) showed significantly higher levels in psychotic patients (t=3.015, p=0.006). However, at end of treatment, miR-126-3p levels in patients no longer differed from healthy controls. A linear regression model of baseline miR-126-3p levels adjusting for socio-economic group indicated no statistically significant relationship between socio-economic group and microRNA levels (B=0.078, p=0.472).

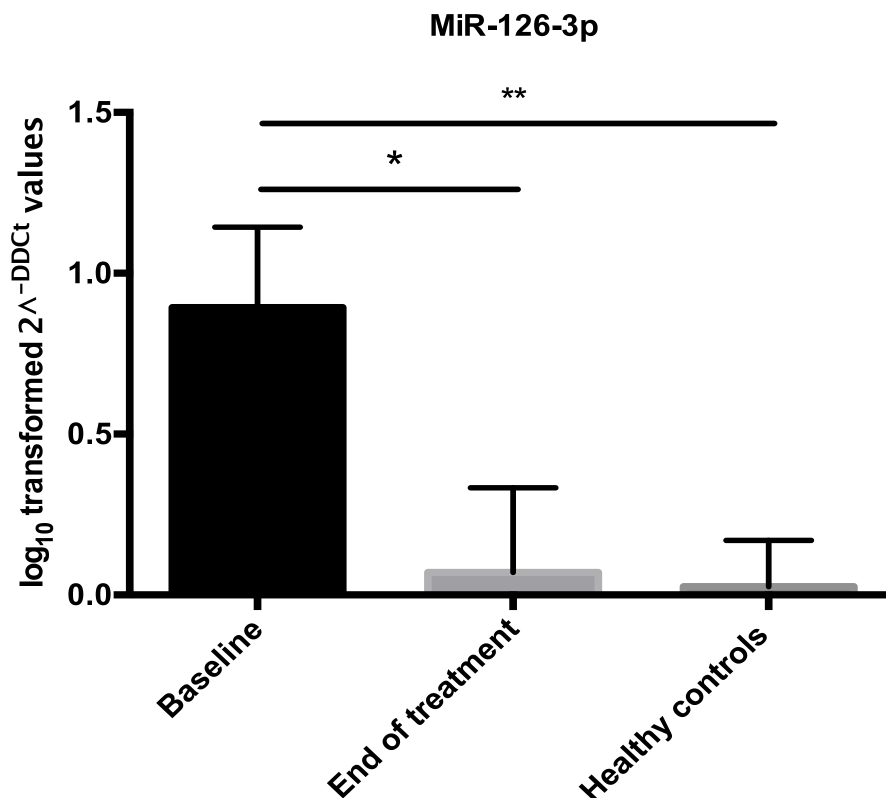


Figure 5.7 MiR-126-3p expression in psychotic depression

Comparison of miR-126-3p levels in psychotic depression versus controls. Baseline levels of miR-126-3p in psychotic depression were significantly higher than both healthy controls and for end of treatment levels in the same patients. Data are presented as means and SEM, n=28 (7 patients, 21 controls). *: p=0.023; **: p=0.006.

5.3.6.2 MiR-106a-5p

The same approach used for miR-126-3p was used for miR-106a-5p. A similar picture emerged, with those with psychotic depression having significantly higher miR-106a-5p levels at baseline compared to controls, ($t=2.598$, $p=0.025$) which then normalised at the end of treatment. A linear regression model of baseline miR-106a-5p levels adjusting for socio-economic group indicated no statistically significant relationship between socio-economic group and microRNA levels ($B=0.074$, $p=0.374$).

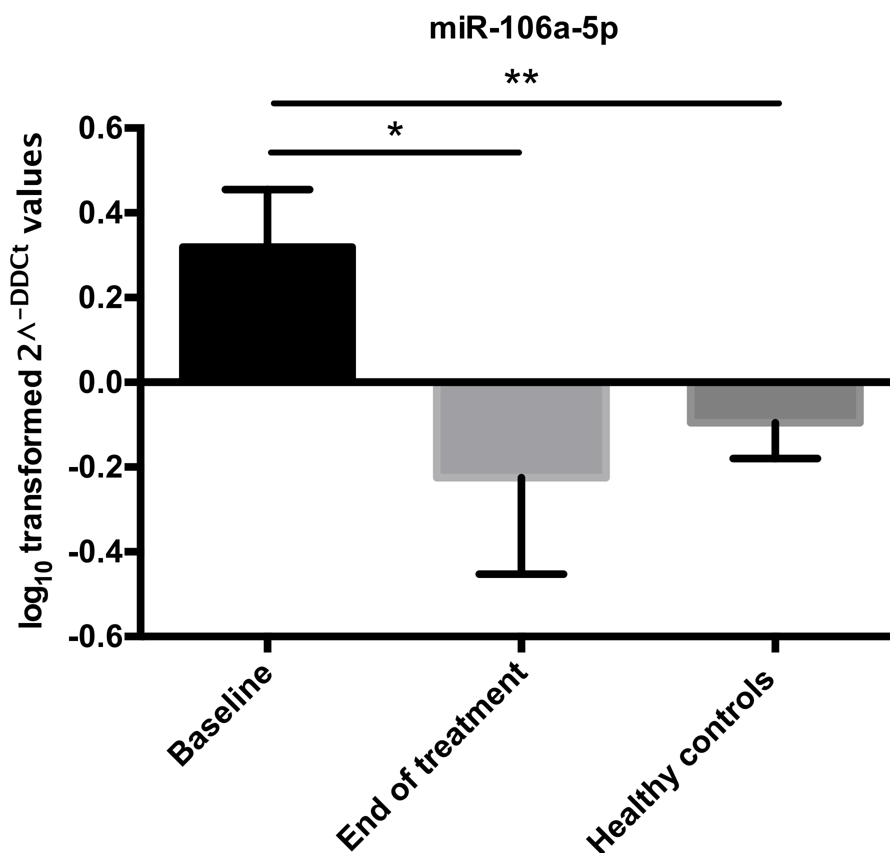


Figure 5.8 MiR-106a-5p expression in psychotic depression

Comparison of miR-106a-5p levels in psychotic depression versus controls. Baseline levels of miR-106a-5p in psychotic depression were significantly higher than end of treatment levels in the same patients (*: $p=0.029$) and compared to healthy controls (**: $p=0.025$). There was no significant difference between end of treatment levels and those of healthy controls. Data are presented as means and SEM, n=28 (7 patients, 21 controls).

5.3.6.3 Responders versus non-responders

As described for the whole-group analysis previously, a GLM with time (baseline and end of treatment) and responder status (yes/no) was run using patients with psychotic depression for both miR-126-3p and miR-106a-5p. As expected from previous results there was a significant difference for time, however, there was no significant effect of responder status on the change in these microRNA levels.

MicroRNA	Time effect		Time x response	
	F value	p-value	F value	p-value
miR-126-3p	7.454	0.041	0.036	0.857
miR-106a-5p	7.779	0.038	0.415	0.548

Table 5.7 Relationship between response and psychosis
MicroRNA expression change and relationship with treatment response in psychotic depression. GLM of time effect and time x response effect with respective F and p-values. n=7 (3 responders/4 non-responders).

5.3.6.4 Correlation between baseline levels of microRNAs and change in depression scores

To assess whether miR-126-3p or miR-106a-5p baseline levels could predict changes in depression, the change in HDRS-24 score was correlated with baseline levels of these two microRNAs. No significant correlation (as measured by Pearson's Correlation) was found with either microRNA (miR-126-3p r=-.157, p=0.71; miR-106a-5p r=-.061, p=0.89). This remained true when patients with psychotic depression were analysed separately (miR-126-3p r=-0.048, p=0.92, miR-106a-5p r=-0.139, p=0.78).

5.3.6.5 Role of antipsychotic medication

Initial results point to the differential expression of two microRNAs in psychotic depression. All patients with psychotic depression were prescribed antipsychotics. To explore whether these effects could be due to antipsychotic medications rather than the presence of psychosis per se,

those who were prescribed antipsychotics (n=23) and those who were not (n=14) were compared to each other. There were no significant differences in the expression of miR-126-3p or miR-106a-5p on the basis of being prescribed an antipsychotic at any stage (baseline or end of treatment).

MicroRNA	Antipsychotic Yes	Antipsychotic No	F value	p-value
miR-126-3p BAS	0.463	0.263	0.001	0.993
miR-126-3p EOT	0.369	-0.205	0.577	0.454
miR-106a-5p BAS	0.222	0.075	0.036	0.852
miR-106a-5p EOT	0.066	-0.297	1.532	0.227

Table 5.8 MicroRNA expression levels and antipsychotics

Data are presented as mean $\log_{10}$ transformed 2^{-DDCt} values based on antipsychotic prescription with corresponding F and p-values (ANOVA). BAS: Baseline; EOT: End of treatment. n=37

5.3.7 MicroRNA gene targets

Target analysis using miRTarBase 6.0 (Chou et al., 2016) and TarBase 7.0 (Vlachos et al., 2015) identified 1609 genes that have been experimentally validated as targets of either miR-126-3p or miR-106a-5p. Validation experiments are typically conducted in non-neural systems. There has been an immense increase in the number of microRNA targets that have been validated in recent years, with an over ten-fold increase from 2013 to 2015. This increase has largely been driven by next generation sequencing experiments that would be considered to have a weaker evidence base than reporter assays or blotting techniques (Hsu et al., 2014). Analysing this number of target genes requires powerful bioinformatic techniques. To narrow the focus a list of target genes that were either i) validated using strong evidence (such as reporter assay or blotting) or ii) validated with more than one form of weaker evidence (microarray, next generation sequencing) in both databases were included. For miR-106a-5p this left 38 genes meeting criteria. These are listed in Table 5.9 below.

Gene	Gene description	Relationship with depression
E2F1	E2 Transcription Factor 1	Decreased in primate DLPFC following oestradiol treatment (Wang et al., 2004); plays part in adult neurogenesis in mice (Cooper-Kuhn et al., 2002)
***CDKN1A* (*p21*)**	Cyclin-dependent kinase inhibitor 1A	Associated with regulation of neurogenesis (Pechnick et al., 2011); differentially expressed in psychotic depression (Epp et al., 2013)
HIPK3	Homeodomain interacting protein kinase 3	Increased in depressed patients compared to controls (Belzeaux et al., 2012)
MYLIP	Myosin regulatory light chain interacting protein	
RB1	Retinoblastoma 1	
APP	Amyloid beta (A4) precursor protein	Deleted *APP* gene impairs hippocampal synaptic plasticity in mice (Seabrook et al., 1999); implicated in post-synaptic signalling at serotonergic synapse (Lezoualc'h and Robert, 2003).
RUNX1	Runt-related transcription factor 1	
RUNX3	Runt-related transcription factor 3	
ARID4B	AT rich interactive domain 4B	
VEGFA	Vascular endothelial growth factor A	Elevated levels in serum of depressed patients compared to controls (Tseng et al., 2015); implicated in the mechanism of action of ECT and other

		antidepressants (Minelli et al., 2014, Minelli et al., 2011, Warner-Schmidt and Duman, 2008)
IL10	Interleukin 10	Potential target of antidepressants (Dinan et al., 2013); serum levels appear unaffected in depression (Hiles et al., 2012)
FAS	Fas cell surface death receptor	Implicated in neurogenesis (Corsini et al., 2009); polymorphism in *FAS* gene associated with treatment resistant depression (Santos et al., 2015).
TGFBR2	Transforming growth factor, beta receptor II	
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	
PTEN	Phosphatase and tensin homolog	Modulator of synaptic plasticity (Jurado et al., 2010); increased levels found in suicide post-mortem brains (Dwivedi et al., 2010)
SIRPA	Signal-regulatory protein alpha	
CASP7	Caspase 7, apoptosis-related cysteine peptidase	
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	Increased in schizophrenia (Kuzman et al., 2009)
BMP2	Bone morphogenetic protein 2	Regulatory role in neurogenesis (Shou et al., 2000)
KPNA2	Karyopherin alpha 2	
MTF1	Metal-regulatory transcription factor 1	

DCBLD2	Discoidin, CUB and LCCL domain containing 2	SNP identified in GWAS study of major depression (Sullivan et al., 2009)
SIRT1	Sirtuin 1	SNP associated with major depression in two separate GWAS studies (Kishi et al., 2010, Converge consortium, 2015); implicated in neurogenesis (Libert et al., 2008); lower levels of *SIRT1* mRNA in WBC's of depressed patients vs. healthy controls (Abe et al., 2011).
STAT3	Signal transducer and activator of transcription 3	Controls IL-6 regulation of serotonin transporter (Kong et al., 2015); decreased in learned helplessness model of depression (Mingmalairak et al., 2010)
LIMK1	LIM domain kinase 1	Regulates neural dendrite size (Duman and Aghajanian, 2012); decreased in learned helpless model of depression (Nakatani et al., 2004)
CCDN1	Cyclin D1	Upregulated by sertraline / dexamethasone co-administration (Anacker et al., 2011)
ATM	ATM serine/threonine kinase	Required for adult neurogenesis (Allen et al., 2001); associated with schizophrenia (Zhang et al., 2008)
BCL10	B-cell CLL/lymphoma 10	
TIMP2	TIMP metallopeptidase inhibitor 2	Component of neurogenesis signalling cascade (Jaworski and Perez-Martinez, 2006)
MAPK9	Mitogen-activated protein kinase 9	Increased in PFC of suicide victims compared to controls (Choi et al., 2011)

MAPK14	mitogen-activated protein kinase 14	Active in neurodegeneration (Jovicic et al., 2013); inhibitor of VEGF-A (Issbrucker et al., 2003)
FASTK	Fas-activated serine/threonine kinase	
RBL2	Retinoblastoma-like 2	
TP53	Tumour protein p53	Associated with schizophrenia (Ni et al., 2005)
MCL1	Myeloid cell leukaemia 1	Involved in cortical neurogenesis (Arbour et al., 2008)
GCM1	Glial cells missing homolog 1	Regulators of neurogenesis (Mao et al., 2012)

Table 5.9 Experimentally validated target genes of miR-106a-5p
DLPFC: Dorsolateral prefrontal cortex; GWAS: genome-wide association study; PFC: Prefrontal cortex; SNP: single-nucleotide polymorphism; WBC: White blood cells.

This list includes a number of genes of interest in the depression literature. These include genes whose products have been identified as potential biomarkers such as *VEGFA, CDKN1A, HIPK3, IL10* and *MAPK9*, genes that have been implicated in neurogenesis and neuroplasticity such as *CDKN1A, APP, VEGFA, FAS, PTEN, BMP2, LIMK1, ATM, TIMP2, MCL1* and *GCM1* as well as genes identified in GWAS analyses of depression including *DCBLD2* and *SIRT1*.

Applying the same criteria as above to miR-126-3p returned 49 genes (Table 5.10 below).

Gene	Gene description	Relationship with depression
E2F1	E2 Transcription Factor 1	Decreased in primate DLPFC following oestradiol treatment (Wang et al., 2004); plays part in adult neurogenesis in mice (Cooper-Kuhn et al., 2002)
***CDKN1B* (p27kip1)**	Cyclin-dependent kinase inhibitor 1B	Regulator of neurogenesis (Andreu et al., 2015)
SPRED1	Sprouty-related, EVH1 domain containing 1	Enriched in neurogenesis (Phoenix and Temple, 2010); required for hippocampal-dependent learning (Denayer et al., 2008)
PLK2	Polo-like kinase 2	Stabiliser of neural circuits during epileptiform activity (Seeburg and Sheng, 2008)
SLC45A3	Solute carrier family 45, member 3	
CCNE2	Cyclin E2	Downregulated in DRN in depressed patients versus controls (Kerman et al., 2012)
RGS3	Regulator of G-protein signalling 3	Increased in BPAD subjects treated with lithium vs. controls (Willmroth et al., 2002)
TOM1	Target of myb1 membrane trafficking protein	SNP associated with psychosis in BPAD (Potash et al., 2008)
HOXA9	Homeobox A9	
VEGFA	Vascular endothelial growth factor A	Elevated levels in serum of depressed patients compared to controls (Tseng et al., 2015); implicated in the mechanism of

		action of ECT and other antidepressants (Minelli et al., 2014, Minelli et al., 2011, Warner-Schmidt and Duman, 2008)
CRK	V-crk avian sarcoma virus CT10 oncogene homolog	
***PIK3R2* (p85b)**	Phosphoinositide-3-kinase, regulatory subunit 2 (beta)	Reduced expression in PFC and hippocampus of suicide completers (Dwivedi et al., 2008)
VCAM1	Vascular cell adhesion molecule 1	Associated with inflammation and late-life depression (Dimopoulos et al., 2006); involved in synaptic remodelling (Wu and Reddy, 2012)
IRS1	Insulin receptor substrate 1	Plays role in VEGF-A mediated angiogenesis (Jiang et al., 2003)
SOX2	SRY (sex determining region Y)-box 2	Expressed by neural progenitors in adult rodent brain (Komitova and Eriksson, 2004); regulator of adult neurogenesis through chromatin remodelling (Amador-Arjona et al., 2015).
TWF1	Twinfilin actin binding protein 1	
TWF2	Twinfilin actin binding protein 2	
PTPN7	Protein tyrosine phosphatase, non-receptor type 7	Upregulated in monocytes in BPAD (Drexhage et al., 2010)
DNMT1	DNA (cytosine-5-)-methyltransferase 1	State-dependent decreased levels in depression (Higuchi et al., 2011)
KRAS	Kirsten rat sarcoma viral oncogene homolog	Increases VEGF mediated angiogenesis (Matsuo et al., 2009)
IGFBP2	Insulin-like growth factor	Modulator of neuronal plasticity

	binding protein 2, 36kDa	(Jeong et al., 2013); increased by ECS in rodent model (Newton et al., 2003); increases VEGF mediated angiogenesis (Azar et al., 2011).
PITPNC1	phosphatidylinositol transfer protein, cytoplasmic 1	
MERTK	MER proto-oncogene, tyrosine kinase	Plays part in adult hippocampal neurogenesis (Ji et al., 2013)
EGFL7	EGF-like-domain, multiple 7	Promotes VEGF-A signalling (Nikolic et al., 2010); host gene of miR-126 (Meister and Schmidt, 2010)
SLC7A5	Solute carrier family 7 (amino acid transporter light chain, L system), member 5	
PIK3CG	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma	Involved in NMDA regulation of learning in rodent model (Kim et al., 2011)
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha	Regulates VEGF-A mediated angiogenesis (Zhang et al., 2003)
TEK	TEK tyrosine kinase, endothelial	
ADAM9	ADAM metallopeptidase domain 9	Candidate biomarker for delusion phenotype (Kurian et al., 2011)
MMP7	Matrix metallopeptidase 7	Potential neuroprotective role in seizures (Phillips et al., 2014)
CXCL12	Chemokine (C-X-C motif) ligand 12	Modulates serotonin transmission in rodent raphe nucleus (Heinisch and Kirby, 2010)
CXCR4	Chemokine (C-X-C motif) receptor 4	Mediator of adult neurogenesis (Cui et al., 2013)
CRKL	V-crk avian sarcoma	Candidate gene for schizophrenia

	virus CT10 oncogene homolog-like	(Kiehl et al., 2009)
SIRT1	Sirtuin 1	SNP associated with major depression in two separate GWAS studies (Kishi et al., 2010, Converge consortium, 2015); implicated in neurogenesis (Libert et al., 2008); lower levels of SIRT1 mRNA in WBC's of depressed patients vs. healthy controls (Abe et al., 2011).
PGR	Progesterone receptor	
FOXO3	Forkhead box O3	Regulator of synaptogenesis (Maiese et al., 2007)
BCL2	B-cell CLL/lymphoma 2	Associated with treatment resistance in depression (Zhang et al., 2014); neuroprotective in hippocampal neurons (Howard et al., 2002); modulator of V*EGFA* expression (Biroccio et al., 2000)
ADGRE5	Adhesion G protein-coupled receptor E5	
CCNE1	Cyclin E1	
RHOU	Ras homolog family member U	
LRP6	Low density lipoprotein receptor-related protein 6	Regulator of adult neurogenesis (Schafer et al., 2015)
ADM	Adrenomedullin	SNP associated with affective disorders in separate GWAS (Huang et al., 2010) (Savitz et al., 2013)
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells	

	inhibitor, alpha	
ETS1	V-ets avian erythroblastosis virus E26 oncogene homolog 1	
AKT1	v-akt murine thymoma viral oncogene homolog 1	SNP associated with depression (Yang et al., 2012) (Pereira et al., 2014)
MAPK1	mitogen-activated protein kinase 1	Associated with treatment resistance and remission in depression (Calati et al., 2013); mediator of neuroplasticity in rodent model (Ramirez-Rodriguez et al., 2014)
TAB3	TGF-beta activated kinase 1/MAP3K7 binding protein 3	
TCF4	Transcription factor 4	Associated with psychotic phenotype (Wirgenes et al., 2012); downregulated by ECS in rodent brain (Sakaida et al., 2013)
SPP1	secreted phosphoprotein 1	

Table 5.10 Experimentally validate target genes of miR-126-3p

BPAD: Bipolar affective disorder; DLPFC: Dorsolateral prefrontal cortex; DRN: Dorsal raphe nucleus; ECS: Electroconvulsive stimulation; GWAS: genome-wide association study; NMDA: N-methyl-D-aspartate; PFC: Pre-frontal cortex; SNP: single-nucleotide polymorphism

This list also includes a number of genes of interest from the depression literature. Potential biomarker genes include *VEGFA*, *CCNE2*, *RGS3*, *PTPN7*, *ADAM9* and *MAPK1*, several genes implicated in neurogenesis and neuroplasticity such as *CDKN1B*, *SPRED1*, *SOX2*, *IGFBP2*, *CXCR4*, *FOXO3* and *MAPK1*. GWAS analysis has also implicated target genes such as *SIRT1*, *ADM* and *AKT1* in depression and *TOM1* for psychosis. In terms of overlap, three genes are experimentally validated targets of both miR-106a-5p and miR-126-3p: *VEGFA*, *SIRT1* and *E2F1*.

5.3.8 Gene ontology analysis

To further explore the potential biological and molecular function of miR-126-3p and miR-106a-5p their gene targets were queried using the latest GO Consortium database (Gene Ontology Consortium, 2015). First, the three experimentally validated genes that were targeted by both microRNAs were entered into the Panther Gene List Analysis Tool to explore their Gene Ontology classification. The results are listed in Table 5.11 below.

Gene Name	GO-slim Molecular Function	GO-slim Biological Process	Pathway
VEGFA	Growth factor activity	Cell cycle Cell communication Angiogenesis Response to stress	1.Angiogenesis 2.VEGF signalling pathway
SIRT1	Deacetylase activity Nucleic acid binding Chromatin binding	Transcription from RNA polymerase II promoter Cellular process Chromatin organisation	1. p53 pathway
E2F1	Sequence specific DNA binding transcription factor activity	Transcription from RNA polymerase II promoter Cell cycle Cell communication	-

Table 5.11 Shared GO terms for miR-126-3p and miR-106a-5p
Gene Ontology terms for union of intersected target genes of miR-126-3p and miR-106a-5p. Go-slim: PANTHER selected subset of GO terms reflecting high-level view of terms (Harris et al., 2004).

Because of the many functional interactions and partnerships between proteins, the gene products of the same three gene targets (*VEGFA*, *SIRT1*, *E2F1*) were entered into the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, see Figure 5.9 below) (Szklarczyk et al., 2015). The STRING tool is a database of known (experimentally validated) and predicted protein interactions, which can be physical or functional. STRING interactions are derived from a number of sources, including known experimental interactions from primary protein interaction databases, pathway data from manually curated databases, statistical and semantic links using automated text-mining and predicted interactions based on genomic data by a number of algorithms (Szklarczyk et al., 2015).

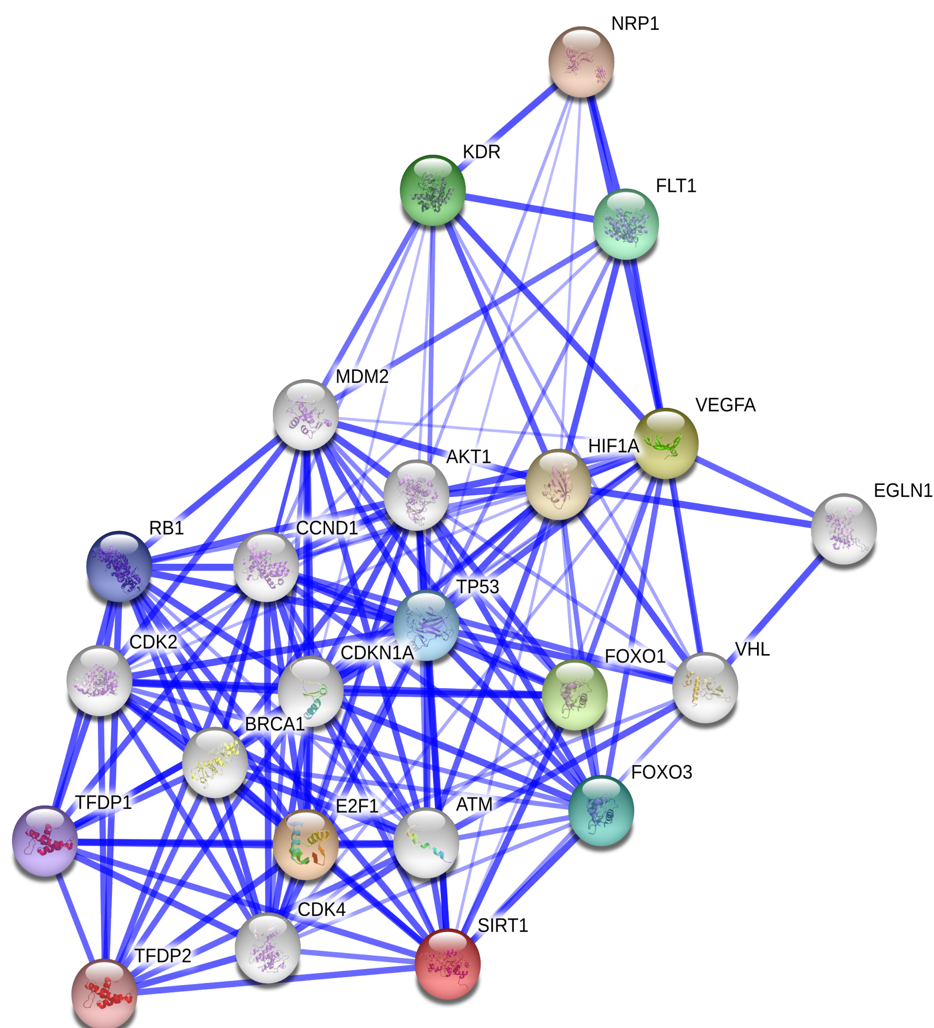


Figure 5.9 STRING interaction network

VEGFA, SIRT1 and E2F1 were entered into STRING. The STRING algorithm then produced a list of the top 20 most closely connected gene products. The thickness of the lines connecting the gene products indicated the confidence of the interaction.

The list of genes returned through this analysis revealed a number of target genes that were among the experimentally validated gene targets of either miR-126-3p or miR-106a-5p described in Section 5.3.7. Indeed, 12 out of the 20 most closely interactive gene products in the STRING network were also identified by validated target analysis. Of these, seven (*AKT1*, *TP53*, *FOXO3*, *CCDN1*, *CDKN1A*, *ATM*) were amongst the high confidence gene targets listed in Tables 5.9 and 5.10. Lower confidence gene targets that overlap with the STRING gene set include *FLT1*, *EGLN1*, *MDM2*, *HIF1A*

and *CDK4*. These interactions indicate the relevance of including all validated gene targets in the search for joint biological and molecular functions. It is the ability of a microRNA to target multiple genes that makes them appealing from a psychiatric point of view, as psychiatric disorders' genetic component is likely from multiple genes of small effect. Two further analyses were therefore made. The first included the high confidence experimentally validated gene targets as listed in Tables 5.9 and 5.10. The second included all experimentally validated gene targets. Both gene target lists were entered separately into the DAVID Functional Classification Tool as this offers a category (GO-FAT) that filters out very broad terms that would otherwise be returned when larger numbers of gene targets are entered (Huang et al., 2007).

5.3.8.1 High confidence gene ontology analysis

The list of high confidence gene targets returned by DAVID of miR-126-3p and miR-106a-5p (validated with techniques such as reporter assay or blotting) included 79 genes. Gene ontology analysis returns results in three domains: biological process, molecular function and cellular component. The DAVID algorithm returns GO terms that are statistically over-represented, i.e. those that are found more frequently on a target list than what would be expected from the background list (known human genes). Only GO terms that remained significant ($p<0.05$) following stringent Bonferroni correction were retained. The specific GO terms found to be significant by DAVID are listed in Tables 5.12-5.14. For a broader overview, a visual representation of 'parent'/broad terms (PANTHER GO-slim) that this gene list are mapped on to can be found in Figures 5.10-5.12. The list of high confidence genes was also entered into the STRING algorithm to visualise the interactions between target genes (see Figure 5.13).

Term	Count	% of Total count	Unadjusted p-value	Fold Enrich-ment	Bonferroni corrected p-value
GO:0042981~regulation of apoptosis	26	32.098	1.31E-12	5.537	2.13E-09
GO:0043067~regulation of programmed cell death	26	32.098	1.63E-12	5.483	2.65E-09
GO:0010941~regulation of cell death	26	32.098	1.77E-12	5.462	2.87E-09
GO:0048545~response to steroid hormone stimulus	15	18.518	3.87E-12	13.378	6.29E-09
GO:0010604~positive regulation of macromolecule metabolic process	26	32.098	5.34E-12	5.195	8.70E-09
GO:0042127~regulation of cell proliferation	25	30.864	6.31E-12	5.439	1.03E-08
GO:0042325~regulation of phosphorylation	20	24.691	1.14E-11	7.349	1.86E-08
GO:0019220~regulation of phosphate metabolic process	20	24.691	2.29E-11	7.061	3.73E-08
GO:0051174~regulation of phosphorus metabolic process	20	24.691	2.29E-11	7.061	3.73E-08
GO:0010628~positive regulation of gene expression	21	25.925	6.34E-11	6.189	1.03E-07
GO:0043549~regulation of kinase activity	17	20.987	1.54E-10	8.154	2.51E-07
GO:0009725~response to hormone stimulus	17	20.987	2.31E-10	7.932	3.77E-07
GO:0051338~regulation of transferase activity	17	20.987	2.82E-10	7.825	4.59E-07
GO:0009991~response to extracellular stimulus	14	17.283	3.39E-10	10.897	5.52E-07

GO:0051325~interphase	11	13.580	4.58E-10	17.770	7.45E-07
GO:0010557~positive regulation of macromolecule biosynthetic process	21	25.925	5.15E-10	5.498	8.39E-07
GO:0051726~regulation of cell cycle	16	19.753	5.33E-10	8.277	8.68E-07
GO:0051384~response to glucocorticoid stimulus	10	12.345	5.80E-10	21.953	9.45E-07
GO:0007242~intracellular signalling cascade	28	34.567	6.51E-10	3.817	1.06E-06
GO:0006468~protein amino acid phosphorylation	21	25.925	7.27E-10	5.391	1.18E-06
GO:0051270~regulation of cell motion	13	16.049	9.63E-10	11.534	1.57E-06
GO:0009719~response to endogenous stimulus	17	20.987	9.76E-10	7.187	1.59E-06
GO:0031328~positive regulation of cellular biosynthetic process	21	25.925	1.15E-09	5.249	1.88E-06
GO:0031667~response to nutrient levels	13	16.049	1.22E-09	11.300	1.98E-06
GO:0031960~response to corticosteroid stimulus	10	12.345	1.27E-09	20.145	2.07E-06
GO:0045893~positive regulation of transcription, DNA-dependent	18	22.222	1.30E-09	6.461	2.12E-06
GO:0043066~negative regulation of apoptosis	16	19.753	1.35E-09	7.739	2.19E-06
GO:0051254~positive regulation of RNA metabolic process	18	22.222	1.48E-09	6.408	2.41E-06
GO:0009891~positive regulation of biosynthetic process	21	25.925	1.48E-09	5.174	2.42E-06
GO:0043069~negative regulation of programmed cell death	16	19.753	1.63E-09	7.631	2.66E-06

GO:0060548~negative regulation of cell death	16	19.753	1.69E-09	7.610	2.76E-06
GO:0045941~positive regulation of transcription	19	23.456	2.28E-09	5.768	3.72E-06
GO:0043065~positive regulation of apoptosis	17	20.987	2.32E-09	6.769	3.78E-06
GO:0043068~positive regulation of programmed cell death	17	20.987	2.56E-09	6.723	4.17E-06
GO:0010942~positive regulation of cell death	17	20.987	2.74E-09	6.692	4.46E-06
GO:0051173~positive regulation of nitrogen compound metabolic process	20	24.691	2.76E-09	5.318	4.50E-06
GO:0010033~response to organic substance	21	25.925	2.80E-09	4.987	4.55E-06
GO:0051094~positive regulation of developmental process	14	17.283	5.90E-09	8.623	9.60E-06
GO:0045597~positive regulation of cell differentiation	13	16.049	6.73E-09	9.721	1.10E-05
GO:0012501~programmed cell death	19	23.456	8.02E-09	5.324	1.31E-05
GO:0045859~regulation of protein kinase activity	15	18.518	8.86E-09	7.445	1.44E-05
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	19	23.456	1.11E-08	5.214	1.81E-05
GO:0016310~phosphorylation	21	25.925	1.64E-08	4.495	2.67E-05
GO:0006793~phosphorus metabolic process	23	28.395	1.64E-08	4.047	2.67E-05
GO:0006796~phosphate metabolic process	23	28.395	1.64E-08	4.047	2.67E-05
GO:0045786~negative regulation of cell cycle	9	11.111	1.94E-08	19.026	3.16E-05
GO:0007243~protein kinase cascade	15	18.518	2.15E-08	6.942	3.49E-05

GO:0000082~G1/S transition of mitotic cell cycle	8	9.876	2.98E-08	24.462	4.85E-05
GO:0006917~induction of apoptosis	14	17.283	3.15E-08	7.491	5.13E-05
GO:0012502~induction of programmed cell death	14	17.283	3.27E-08	7.468	5.32E-05
GO:0006915~apoptosis	18	22.222	4.18E-08	5.120	6.81E-05
GO:0030334~regulation of cell migration	11	13.580	4.29E-08	11.145	6.99E-05
GO:0040008~regulation of growth	14	17.283	6.65E-08	7.030	1.08E-04
GO:0008219~cell death	19	23.456	9.73E-08	4.525	1.58E-04
GO:0016265~death	19	23.456	1.08E-07	4.493	1.76E-04
GO:0040012~regulation of locomotion	11	13.580	1.43E-07	9.810	2.33E-04
GO:0009628~response to abiotic stimulus	14	17.283	1.61E-07	6.514	2.62E-04
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	14	17.283	1.77E-07	6.461	2.88E-04
GO:0003006~reproductive developmental process	12	14.814	2.88E-07	7.843	4.69E-04
GO:0006357~regulation of transcription from RNA polymerase II promoter	18	22.222	6.18E-07	4.239	0.00100580
GO:0008285~negative regulation of cell proliferation	13	16.049	9.67E-07	6.166	0.00157320
GO:0007584~response to nutrient	9	11.111	1.37E-06	11.008	0.00223001
GO:0045792~negative regulation of cell size	8	9.876	1.57E-06	13.837	0.00254902
GO:0001558~regulation of cell growth	10	12.345	1.66E-06	8.826	0.00269125
GO:0033273~response to vitamin	7	8.641	2.09E-06	18.161	0.00339075

GO:0042698~ovulation cycle	7	8.641	2.28E-06	17.890	0.00370671
GO:0048534~hemopoietic or lymphoid organ development	11	13.580	2.31E-06	7.244	0.00375021
GO:0043627~response to oestrogen stimulus	8	9.876	2.33E-06	13.046	0.00378873
GO:0008361~regulation of cell size	10	12.345	2.72E-06	8.312	0.00441501
GO:0045637~regulation of myeloid cell differentiation	7	8.641	2.96E-06	17.124	0.00480117
GO:0032535~regulation of cellular component size	11	13.580	3.34E-06	6.950	0.00542922
GO:0002520~immune system development	11	13.580	3.94E-06	6.824	0.00638624
GO:0051385~response to mineralocorticoid stimulus	5	6.172	4.62E-06	42.810	0.00749168
GO:0007049~cell cycle	17	20.987	7.11E-06	3.751	0.01151552
GO:0030097~hemopoiesis	10	12.345	8.23E-06	7.255	0.01330776
GO:0045137~development of primary sexual characteristics	8	9.876	8.27E-06	10.786	0.01337805
GO:0000278~mitotic cell cycle	12	14.814	8.32E-06	5.553	0.01344569
GO:0006469~negative regulation of protein kinase activity	7	8.641	1.05E-05	13.777	0.01700615
GO:0006355~regulation of transcription, DNA-dependent	26	32.098	1.15E-05	2.511	0.01855949
GO:0033673~negative regulation of kinase activity	7	8.641	1.28E-05	13.318	0.02064454
GO:0000079~regulation of cyclin-dependent protein kinase activity	6	7.407	1.42E-05	19.026	0.02284218
GO:0009416~response to light stimulus	8	9.876	1.43E-05	9.926	0.02294901

GO:0030308~negative regulation of cell growth	7	8.641	1.45E-05	13.029	0.02339630
GO:0051252~regulation of RNA metabolic process	26	32.098	1.70E-05	2.455	0.02732698
GO:0051348~negative regulation of transferase activity	7	8.641	1.86E-05	12.486	0.02977022
GO:0009314~response to radiation	9	11.111	1.92E-05	7.705	0.03075006
GO:0051272~positive regulation of cell motion	7	8.641	2.09E-05	12.231	0.03343288
GO:0009411~response to UV	6	7.407	2.20E-05	17.414	0.03513504
GO:0006916~anti-apoptosis	9	11.111	2.37E-05	7.481	0.03790820
GO:0008284~positive regulation of cell proliferation	12	14.814	2.37E-05	4.963	0.03791943
GO:0022403~cell cycle phase	12	14.814	2.37E-05	4.963	0.03791943
GO:0007548~sex differentiation	8	9.876	2.56E-05	9.072	0.04077437
GO:0022602~ovulation cycle process	6	7.407	2.80E-05	16.571	0.04458760

Table 5.12 GO Biological Process (high confidence list)

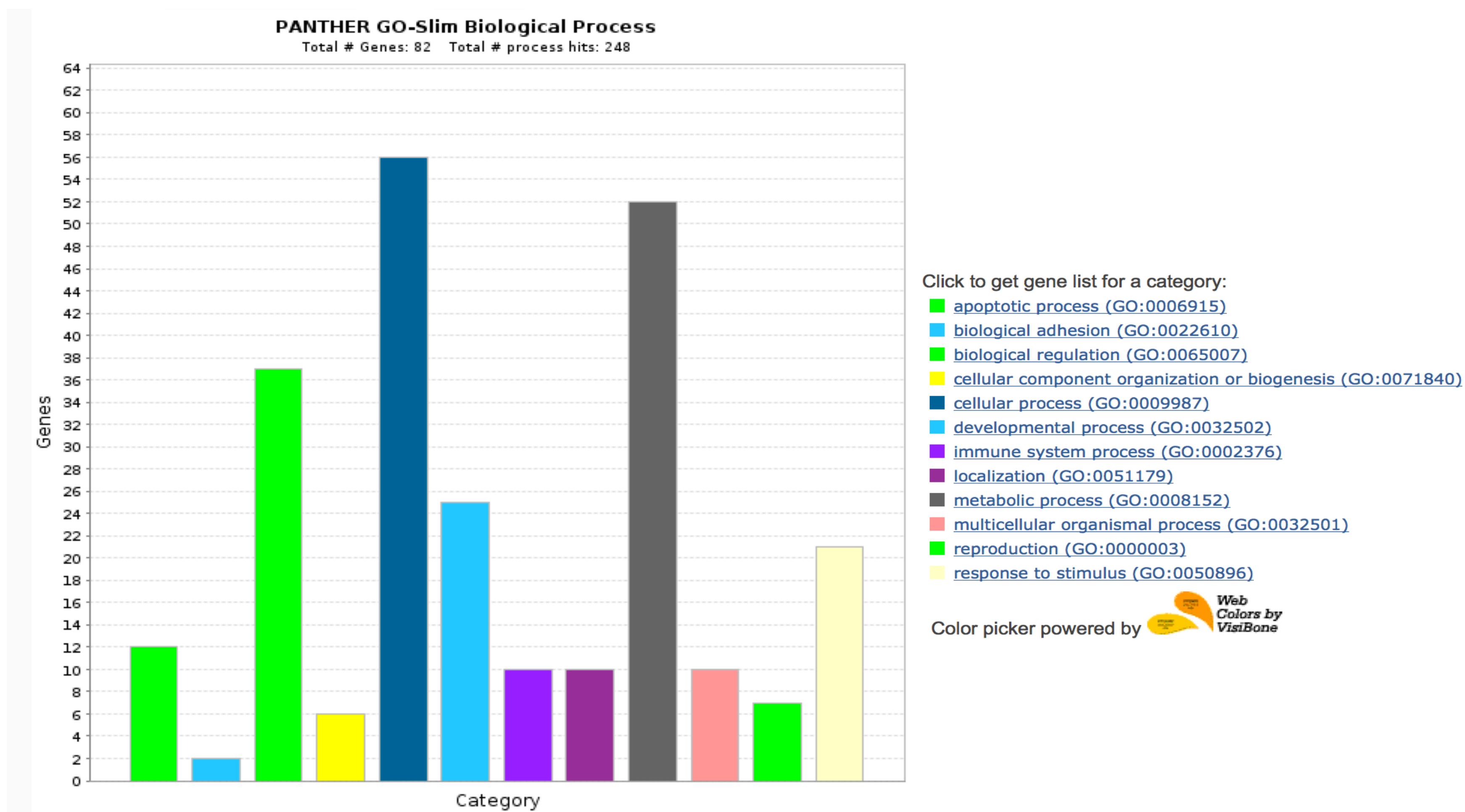


Figure 5.10 PANTHER GO-Slim overview Biological Process

PANTHER GO-Slim overview of most mapped to 'parent' Biological Process terms from high confidence gene list (Table 5.12).

Term	Count	% of Total count	Unadjusted p-value	Fold Enrichment	Bonferroni corrected p-value
GO:0019900~kinase binding	10	12.345	1.06E-06	9.298	2.79E-04
GO:0008134~transcription factor binding	15	18.518	1.61E-06	4.866	4.23E-04
GO:0019899~enzyme binding	15	18.518	2.02E-06	4.773	5.31E-04
GO:0004672~protein kinase activity	16	19.753	2.19E-06	4.394	5.76E-04
GO:0019901~protein kinase binding	8	9.876	2.56E-05	9.058	0.00672103
GO:0016303~1-phosphatidylinositol-3-kinase activity	4	4.938	3.20E-05	60.526	0.00837695
GO:0035004~phosphoinositide 3-kinase activity	4	4.938	4.25E-05	55.482	0.01110637
GO:0016538~cyclin-dependent protein kinase regulator activity	4	4.938	1.28E-04	39.164	0.03322777
GO:0032403~protein complex binding	8	9.876	1.58E-04	6.793	0.040734756

Table 5.13 GO Molecular Function (high confidence list)

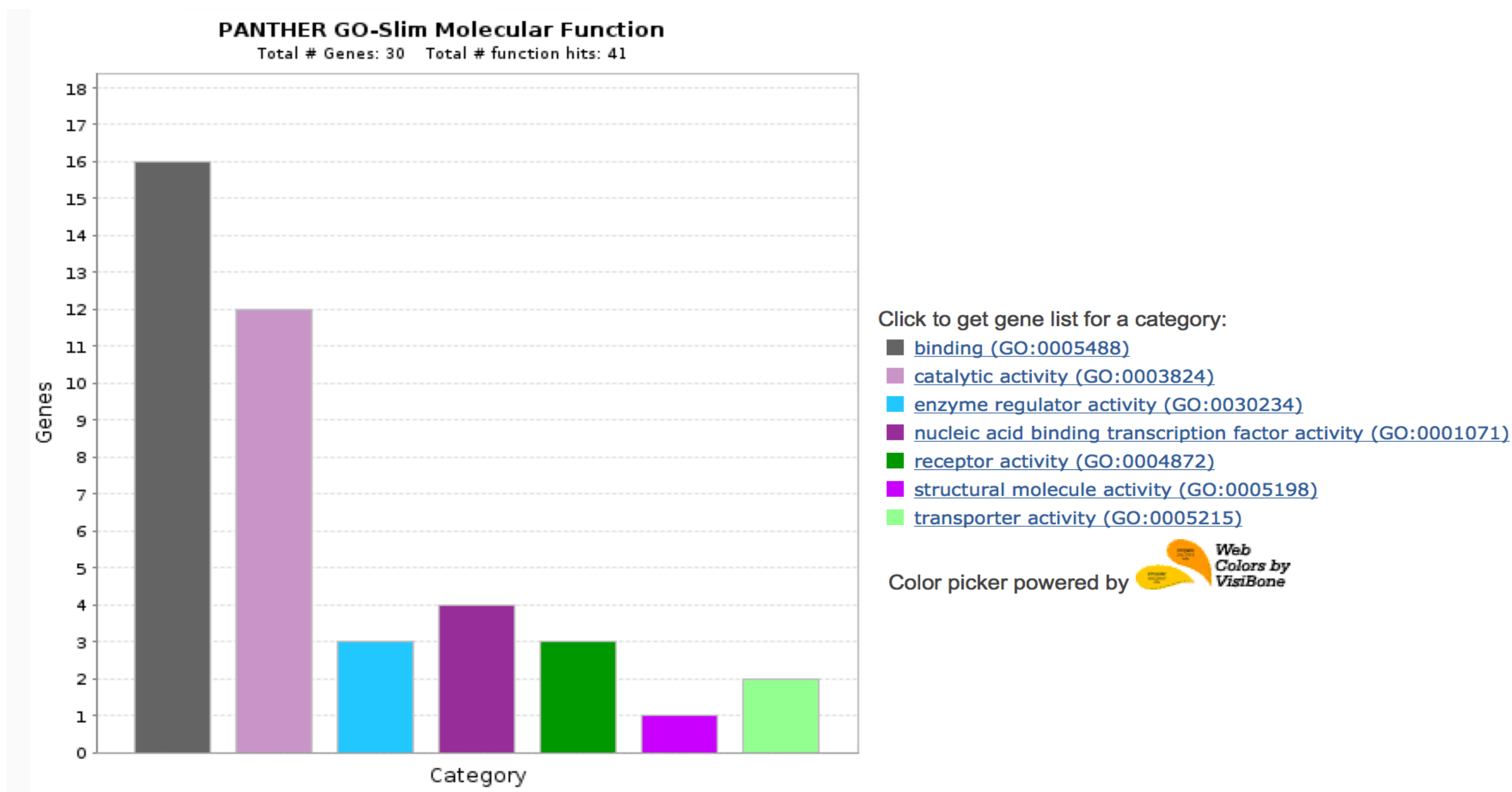


Figure 5.11PANTHER GO-Slim overview Molecular Function

PANTHER GO-Slim of most mapped to 'parent' Molecular Function terms from high confidence gene list (Table 5.13).

Term	Count	% of Total count	Unadjusted p-value	Fold Enrichment	Bonferroni corrected p-value
GO:0005829~cytosol	25	30.864	2.48E-08	3.533	3.67E-06
GO:0005654~nucleoplasm	20	24.691	7.28E-08	4.262	1.08E-05
GO:0031981~nuclear lumen	21	25.925	3.30E-05	2.722	0.004867974
GO:0043233~organelle lumen	23	28.395	8.95E-05	2.375	0.013159667
GO:0005667~transcription factor complex	8	9.876	1.10E-04	7.160	0.016188947
GO:0031974~membrane-enclosed lumen	23	28.395	1.20E-04	2.329	0.017642527

Table 5.14 GO Cellular Component (high confidence list)

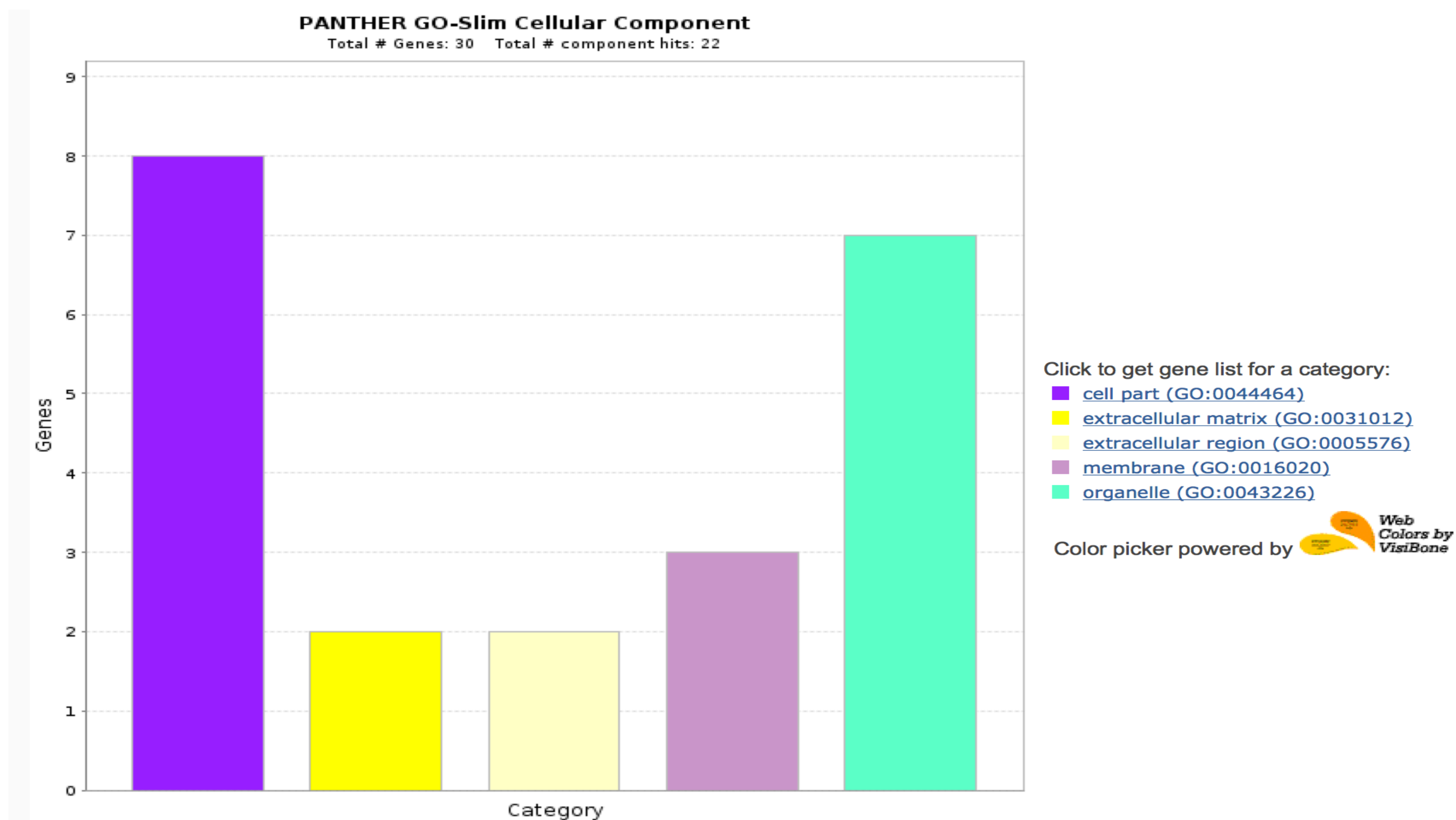


Figure 5.12 PANTHER GO-Slim overview of Cellular Component

PANTHER GO-Slim overview of most mapped to 'parent' Cellular Component terms from high confidence gene list (Table 5.14).

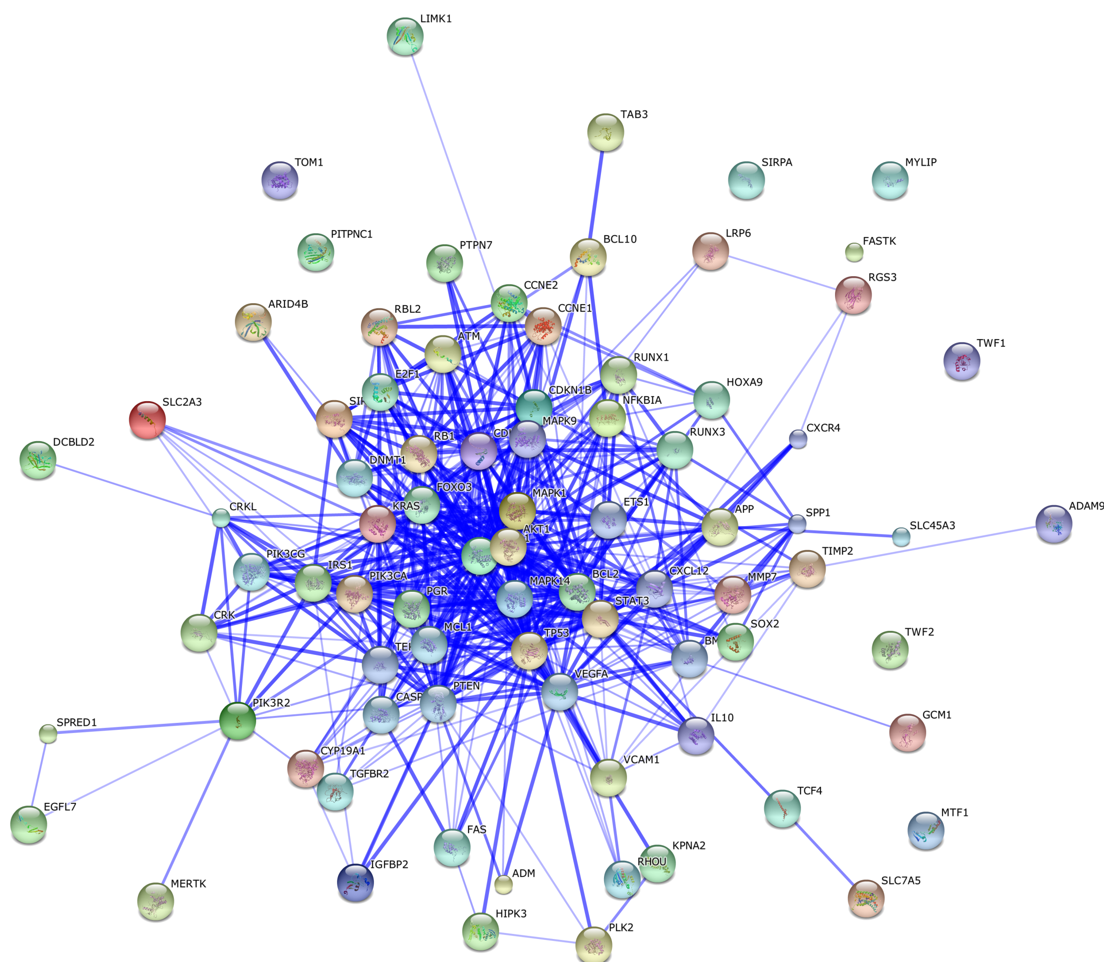


Figure 5.13 STRING interaction network

The input to the analysis was all high-confidence experimentally validated targets of miR-126-3p and miR-106a-5p. The connecting lines are based on the degree of confidence of the interaction with thick lines representing higher confidence.

1.1.1.1 High confidence gene pathway analysis

Although STRING analysis displays the connectivity between gene products, it does not provide further information regarding the potential interaction between genes, biochemical reactions nor cellular location. Pathway analysis integrates these three types of information into one schematic. The list of high confidence target genes was therefore entered into the manually curated PANTHER pathway analysis tool (Mi et al., 2013). Stringent Bonferroni correction was used for multiple testing (see Table 5.15 below)

PANTHER Pathway	# In Human Genome	# In Target List	# Exp.	Fold Change	Bonferroni corrected p-value
p53 pathway feedback loops 2	51	12	0.2	> 5	5.10E-16
Insulin/IGF pathway-protein kinase B signalling cascade	39	7	0.15	> 5	4.20E-08
Hypoxia response via HIF activation	28	5	0.11	> 5	1.70E-05
PI3 kinase pathway	49	8	0.19	> 5	4.44E-09
VEGF signalling pathway	59	9	0.23	> 5	4.43E-10
Cell cycle	22	3	0.09	> 5	1.51E-02
p53 pathway	81	11	0.32	> 5	5.19E-12
Axon guidance mediated by netrin	34	4	0.13	> 5	1.73E-03
Ras Pathway	79	9	0.31	> 5	5.75E-09
Oxidative stress response	27	3	0.11	> 5	2.76E-02
Apoptosis signalling pathway	115	11	0.45	> 5	2.20E-10
Interferon-gamma signalling pathway	32	3	0.13	> 5	4.53E-02
Interleukin signalling pathway	97	9	0.38	> 5	3.44E-08
B cell activation	65	6	0.26	> 5	4.08E-05
CCKR signalling map	169	15	0.67	> 5	4.05E-14
T cell activation	80	7	0.32	> 5	5.64E-06
Angiogenesis	154	13	0.61	> 5	8.51E-12
Alzheimer disease-amyloid secretase pathway	65	5	0.26	> 5	1.02E-03
Toll receptor signalling pathway	56	4	0.22	> 5	1.19E-02
TGF-beta signalling pathway	94	6	0.37	> 5	3.41E-04
Endothelin signalling pathway	87	5	0.34	> 5	4.10E-03
EGF receptor signalling pathway	130	7	0.51	> 5	1.44E-04
PDGF signalling pathway	138	7	0.54	> 5	2.13E-04

FGF signalling pathway	123	6	0.48	> 5	1.56E-03
Parkinson disease	107	5	0.42	> 5	1.09E-02
Integrin signalling pathway	181	8	0.71	> 5	1.01E-04
Inflammation mediated by chemokine and cytokine signalling pathway	245	9	0.97	> 5	9.03E-05
Gonadotropin releasing hormone receptor pathway	225	8	0.89	> 5	5.03E-04

Table 5.15 Statistically over-represented PANTHER pathways

In Human Genome: total number of genes in human genome known to be involved in pathway; # In Target List: Number of genes from target list involved in pathway; # Exp: Number of genes expected from target list to be involved in pathway; Fold change between observed and expected list; P adj: Bonferroni corrected p-values.

5.3.8.3 Lower confidence gene ontology analysis

Experimentally validated gene targets of miR-126-3p and miR-106a-5p include a large number of genes that have been derived from techniques that give lower confidence (e.g. microarray). The list of lower confidence gene targets returned by DAVID included 1527 genes. A list of GO terms that are statistically over-represented (i.e found more frequently on the basis of the target list than what would be expected from the background list (known human genes)) are listed in Tables 5.16-5.18 below. Only terms that passed stringent Bonferroni correction were included.

5.3.8.4 Lower confidence gene pathway analysis

The lower confidence gene target list was, as before, submitted to the PANTHER pathway analysis tool, with Bonferroni correction for multiple testing. Statistically significant pathways are listed in Table 5.19 below.

PANTHER Pathway	# In Human Genome	# In Target List	# Exp.	Fold Change	Bonferroni corrected p-value
p53 pathway feedback loops 2	51	12	0.2	> 5	5.10E-16
Insulin/IGF pathway-protein kinase B signalling cascade	39	7	0.15	> 5	4.20E-08
Hypoxia response via HIF activation	28	5	0.11	> 5	1.70E-05
PI3 kinase pathway	49	8	0.19	> 5	4.44E-09
VEGF signalling pathway	59	9	0.23	> 5	4.43E-10
Cell cycle	22	3	0.09	> 5	1.51E-02
p53 pathway	81	11	0.32	> 5	5.19E-12
Axon guidance mediated by netrin	34	4	0.13	> 5	1.73E-03
Ras Pathway	79	9	0.31	> 5	5.75E-09
Oxidative stress response	27	3	0.11	> 5	2.76E-02
Apoptosis signalling pathway	115	11	0.45	> 5	2.20E-10
Interferon-gamma signalling pathway	32	3	0.13	> 5	4.53E-02
Interleukin signalling pathway	97	9	0.38	> 5	3.44E-08
B cell activation	65	6	0.26	> 5	4.08E-05
CCKR signalling map	169	15	0.67	> 5	4.05E-14
T cell activation	80	7	0.32	> 5	5.64E-06
Angiogenesis	154	13	0.61	> 5	8.51E-12
Alzheimer disease-amyloid secretase pathway	65	5	0.26	> 5	1.02E-03
Toll receptor signalling pathway	56	4	0.22	> 5	1.19E-02
TGF-beta signalling pathway	94	6	0.37	> 5	3.41E-04
Endothelin signalling pathway	87	5	0.34	> 5	4.10E-03
EGF receptor signalling pathway	130	7	0.51	> 5	1.44E-04
PDGF signalling pathway	138	7	0.54	> 5	2.13E-04

GO:0010604~positive regulation of macromolecule metabolic process	117	7.662	9.84E-07	1.558	0.003535049
GO:0016265~death	102	6.679	1.33E-06	1.608	0.004772704
GO:0006350~transcription	243	15.913	1.40E-06	1.320	0.005038259
GO:0045893~positive regulation of transcription, DNA-dependent	74	4.846	1.49E-06	1.771	0.005357142
GO:0010557~positive regulation of macromolecule biosynthetic process	94	6.155	1.55E-06	1.640	0.005576199
GO:0030335~positive regulation of cell migration	24	1.571	1.58E-06	3.078	0.005659793
GO:0031328~positive regulation of cellular biosynthetic process	97	6.352	2.01E-06	1.616	0.007203232
GO:0051254~positive regulation of RNA metabolic process	74	4.846	2.05E-06	1.756	0.007350909
GO:0007265~Ras protein signal transduction	26	1.702	2.88E-06	2.826	0.010298239
GO:0000082~G1/S transition of mitotic cell cycle	18	1.178	3.36E-06	3.669	0.012040477
GO:0009891~positive regulation of biosynthetic process	97	6.352	3.79E-06	1.593	0.01354217
GO:0043066~negative regulation of apoptosis	58	3.798	4.44E-06	1.870	0.015849709
GO:0006357~regulation of transcription from RNA polymerase II promoter	100	6.548	4.96E-06	1.570	0.017703163
GO:0022402~cell cycle process	82	5.370	5.50E-06	1.656	0.019601747
GO:0042981~regulation of apoptosis	108	7.072	5.82E-06	1.533	0.020725894
GO:0042127~regulation of cell proliferation	106	6.941	6.37E-06	1.537	0.022669056
GO:0007178~transmembrane receptor protein serine/threonine kinase signalling pathway	25	1.637	6.74E-06	2.770	0.023979702
GO:0043069~negative regulation of programmed cell death	58	3.798	6.92E-06	1.844	0.024597606
GO:0060548~negative regulation of cell death	58	3.798	7.58E-06	1.839	0.026915805
GO:0007243~protein kinase cascade	59	3.863	8.58E-06	1.820	0.03043278
GO:0043067~regulation of programmed cell death	108	7.072	9.16E-06	1.518	0.03243827
GO:0040017~positive regulation of locomotion	24	1.571	9.18E-06	2.795	0.032509346

GO:0007242~intracellular signalling cascade	154	10.085	9.60E-06	1.399	0.033978642
GO:0010941~regulation of cell death	108	7.072	1.07E-05	1.512	0.037661646
GO:0006355~regulation of transcription, DNA-dependent	205	13.425	1.26E-05	1.319	0.044444193

Table 5.16 GO Biological Process (lower confidence list)

Term	Count	% of Total count	P-Value	Fold Change	Bonferroni corrected p-value
GO:0004672~protein kinase activity	107	7.007	8.99E-13	2.037	9.96E-10
GO:0004674~protein serine/threonine kinase activity	83	5.435	4.38E-12	2.227	4.85E-09
GO:0000166~nucleotide binding	268	17.550	4.84E-09	1.377	5.36E-06
GO:0032555~purine ribonucleotide binding	227	14.865	5.90E-09	1.426	6.54E-06
GO:0032553~ribonucleotide binding	227	14.865	5.90E-09	1.426	6.54E-06
GO:0017076~purine nucleotide binding	234	15.324	1.03E-08	1.407	1.14E-05
GO:0008134~transcription factor binding	84	5.500	1.42E-08	1.889	1.57E-05
GO:0001882~nucleoside binding	199	13.032	7.95E-08	1.424	8.81E-05
GO:0005524~ATP binding	185	12.115	9.34E-08	1.445	1.04E-04
GO:0032559~adenyl ribonucleotide binding	186	12.180	1.53E-07	1.433	1.69E-04
GO:0030554~adenyl nucleotide binding	193	12.639	2.47E-07	1.412	2.74E-04
GO:0001883~purine nucleoside binding	195	12.770	3.01E-07	1.405	3.34E-04
GO:0019899~enzyme binding	78	5.108	2.28E-06	1.721	0.002524374
GO:0046332~SMAD binding	16	1.047	4.10E-06	4.014	0.004533827
GO:0019787~small conjugating protein ligase activity	33	2.161	1.26E-05	2.294	0.013895003
GO:0004842~ubiquitin-protein ligase activity	30	1.964	2.02E-05	2.355	0.022135525

Table 5.17 GO Molecular Function (lower confidence list)

Term	Count	% of Total count	P-Value	Fold Change	Bonferroni corrected p-value
GO:0031974~membrane-enclosed lumen	227	14.865	8.98E-12	1.523	5.32E-09
GO:0043233~organelle lumen	220	14.407	6.74E-11	1.505	4.00E-08
GO:0070013~intracellular organelle lumen	216	14.145	7.19E-11	1.512	4.26E-08
GO:0005654~nucleoplasm	125	8.185	2.07E-10	1.765	1.23E-07
GO:0031981~nuclear lumen	181	11.853	4.58E-10	1.555	2.71E-07
GO:0005829~cytosol	168	11.001	9.68E-10	1.573	5.74E-07
GO:0043228~non-membrane-bounded organelle	274	17.943	2.20E-07	1.314	1.31E-04
GO:0043232~intracellular non-membrane-bounded organelle	274	17.943	2.20E-07	1.314	1.31E-04
GO:0044451~nucleoplasm part	76	4.977	4.43E-06	1.705	0.002621509
GO:0005667~transcription factor complex	36	2.357	2.55E-05	2.135	0.015024513
GO:0005794~Golgi apparatus	103	6.745	5.25E-05	1.471	0.030625643

Table 5.18 GO Cellular Component (lower confidence list)

PANTHER Pathways	# Human Genome	# Target List	# Exp.	Fold Change	P-value (Bonferroni)
Hypoxia response via HIF activation	28	14	2.16	> 5	1.13E-05
p53 pathway feedback loops 2	51	22	3.94	> 5	3.72E-08
Insulin/IGF pathway-protein kinase B signalling cascade	39	16	3.01	> 5	1.94E-05
Cell cycle	22	9	1.7	> 5	1.09E-02
PI3 kinase pathway	49	19	3.79	> 5	3.22E-06
VEGF signalling pathway	59	18	4.56	3.95	2.28E-04
Insulin/IGF pathway-MAPK kinase/MAP kinase cascade	33	10	2.55	3.92	4.92E-02
p53 pathway	81	24	6.26	3.83	7.55E-06
Ras Pathway	79	22	6.11	3.6	7.44E-05
TGF-beta signalling pathway	94	23	7.27	3.17	3.56E-04
Angiogenesis	154	37	11.9	3.11	5.72E-07
Interleukin signalling pathway	97	22	7.5	2.93	1.86E-03
CCKR signalling map	169	36	13.06	2.76	1.73E-05
Apoptosis signalling pathway	115	24	8.89	2.7	2.91E-03
PDGF signalling pathway	138	28	10.67	2.62	1.03E-03
EGF receptor signalling pathway	130	25	10.05	2.49	7.29E-03
Integrin signalling pathway	181	32	13.99	2.29	3.54E-03
Gonadotropin releasing hormone receptor pathway	225	39	17.39	2.24	7.55E-04
Inflammation mediated by chemokine and cytokine signalling pathway	245	37	18.94	1.95	2.15E-02

Table 5.19 PANTHER pathways (lower confidence)

#Human Genome: Genes in human genome involved in pathway; #Target List: Genes from target list involved in pathway; # Expected: Genes expected from target list to be involved in pathway; Fold change between observed and expected list.

The pathways derived from the two alternate gene target lists (Tables 5.15 and 5.19) had considerable overlap. 18/19 pathways returned from the lower confidence list overlapped with the high confidence target list. The only pathway in the lower confidence list that was not shared was the insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade. 18/28 pathways in the high confidence target pathway list were shared by the lower confidence list.

For illustrative purposes, pathway diagrams of 15 out of the 18 shared pathways have been included below. Pathway diagrams were created with the PANTHER pathway tool (Mi et al., 2013). Three pathways, (PI3 kinase pathway, CCKR signalling map and EGF receptor signalling pathway) have not been manually curated yet and are thus not available for visualisation. Gene targets that are part of a given pathway are highlighted in red. Many of the pathways are too complex for each component to be visualised adequately on a single page format, but the diagrams are meant to give an indication of the level of involvement of the target genes in a given pathway. This involvement can be gauged by the number of gene products that are highlighted in red. Large proportions of red boxes indicate higher involvement of target genes of miR-126-3p and miR-106a-5p in the respective pathways.

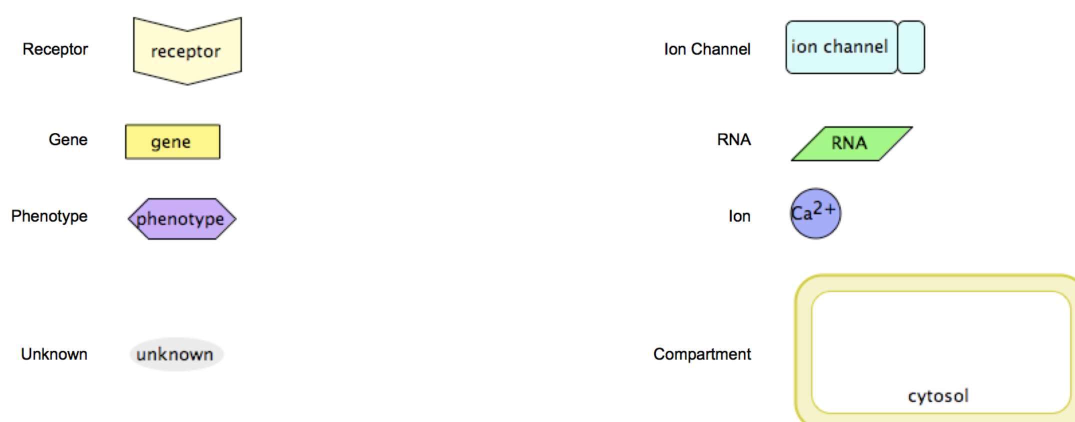


Figure 5.14 PANTHER Pathway diagram components

Apart from gene products (proteins) the pathway diagrams contain references to other molecular components and locations as outlined above.

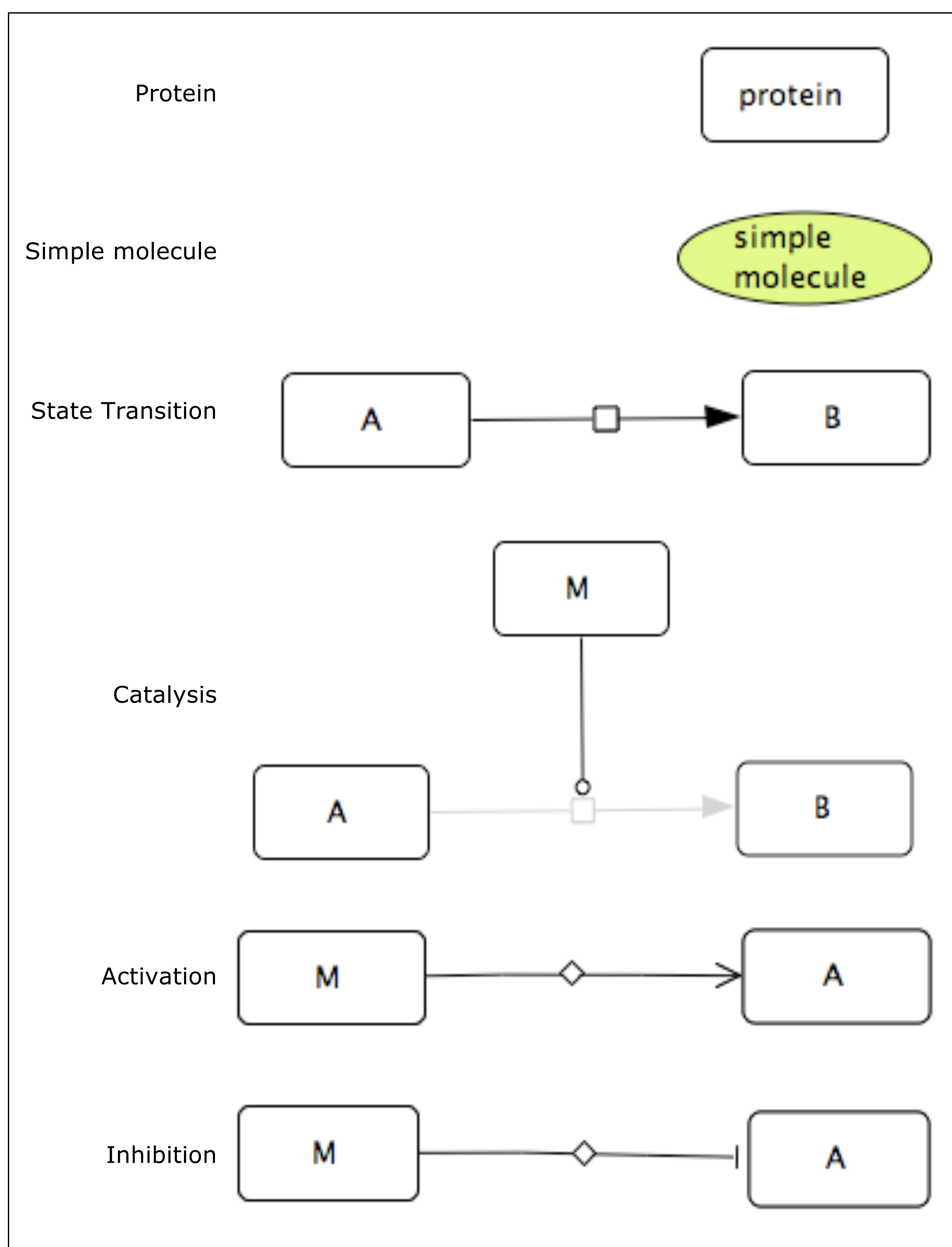


Figure 5.15 PANTHER Pathway diagram interactions

The PANTHER pathway diagrams consist predominantly of protein interactions such as catalysis, activation or inhibition, which have separate symbols (arrows) as outlined above. A protein highlighted in red is a product of miR-126-3p or miR-106a-5p.

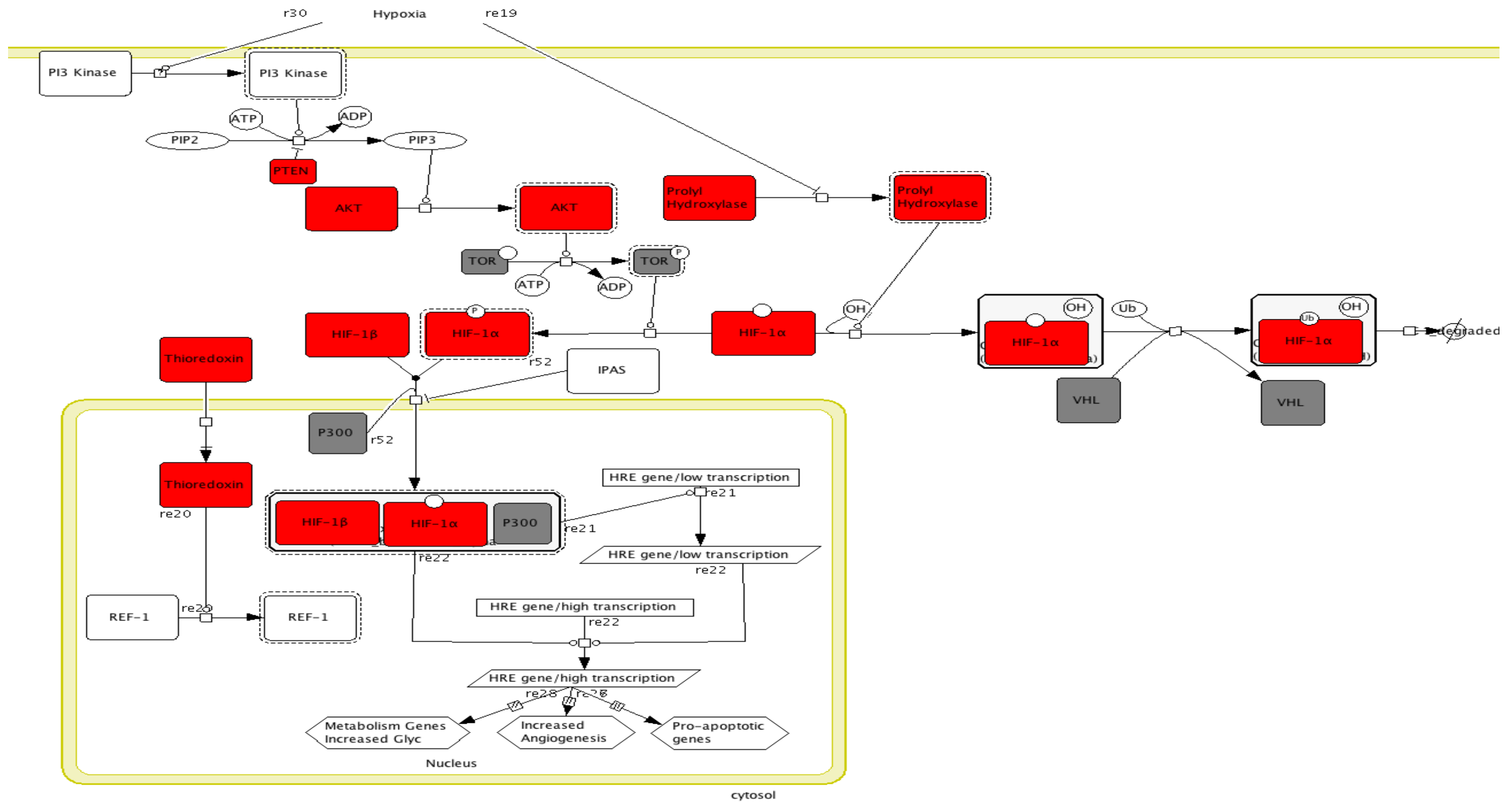


Figure 5.16 Hypoxia response via HIF activation

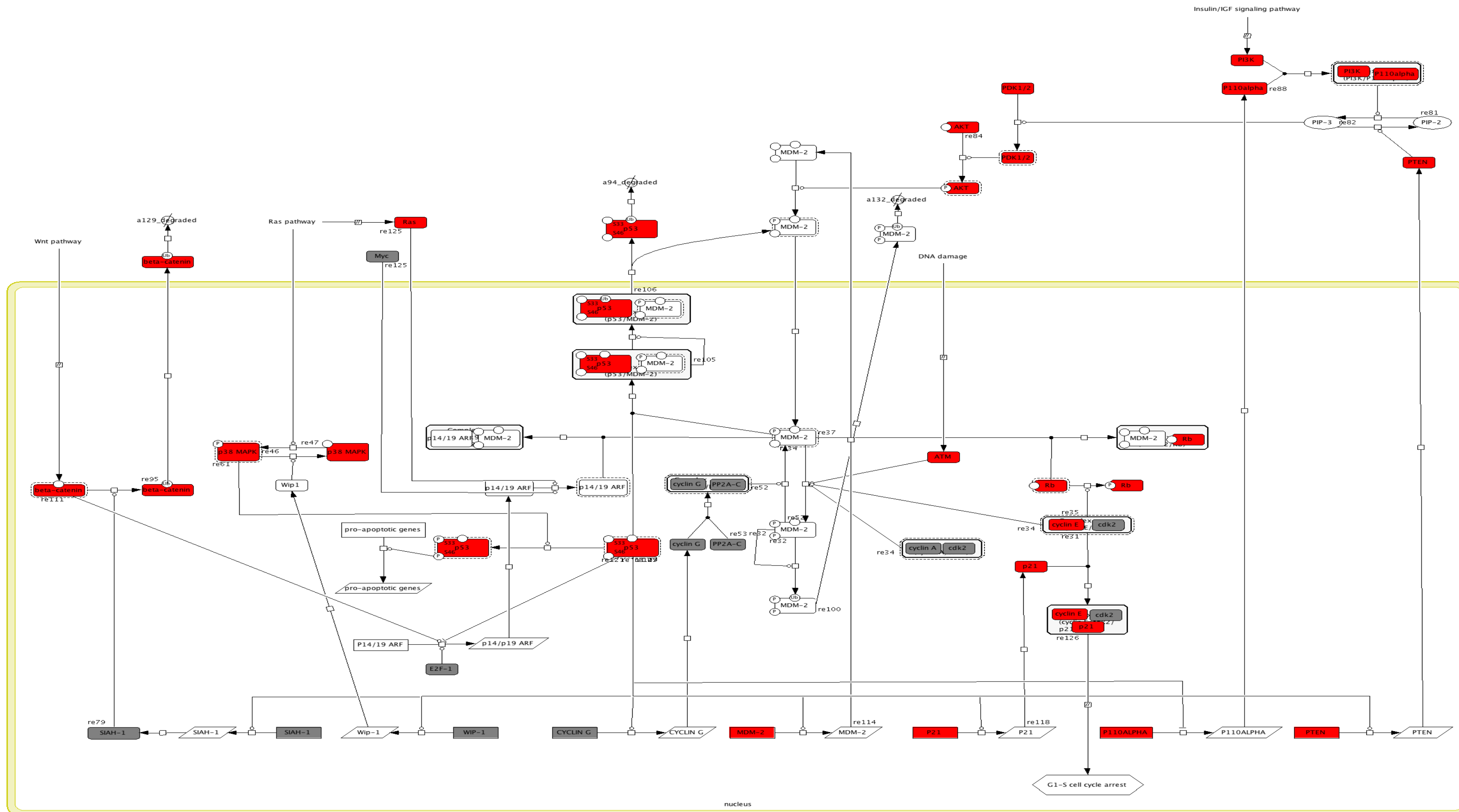


Figure 5.17 p53 pathway feedback loops 2

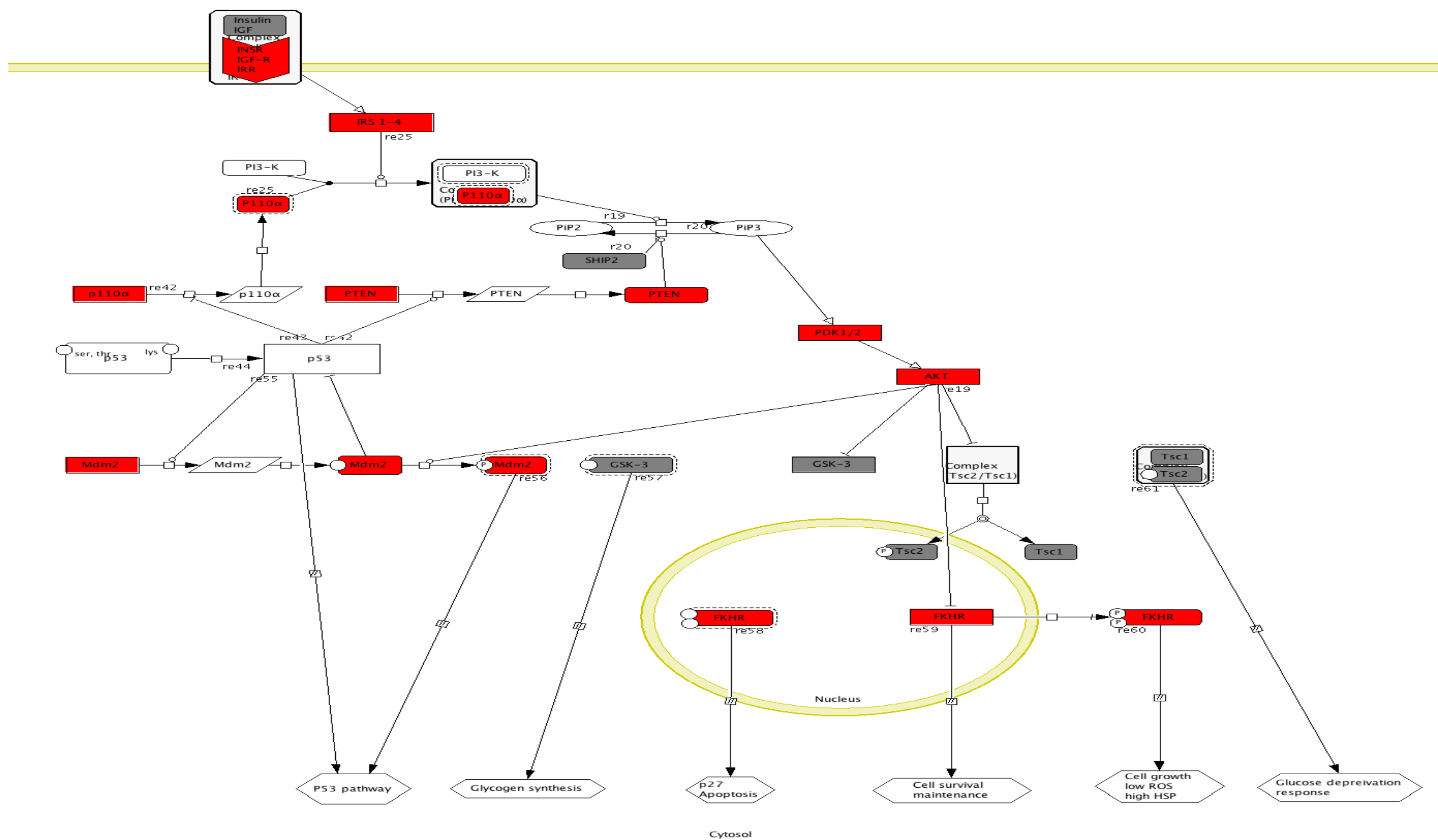


Figure 5.18 Insulin/IGF pathway-protein kinase B signalling cascade

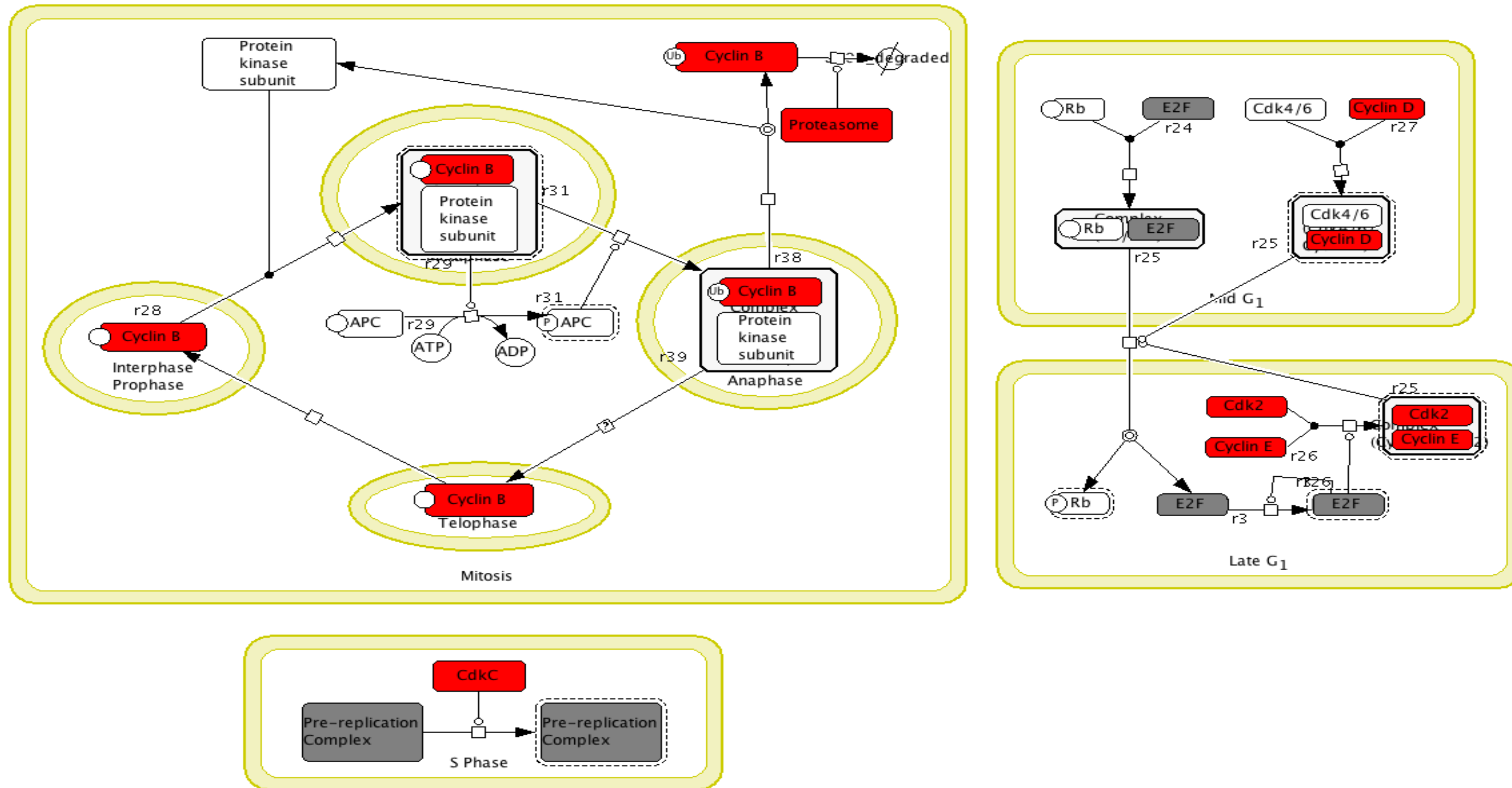


Figure 5.19 Cell cycle

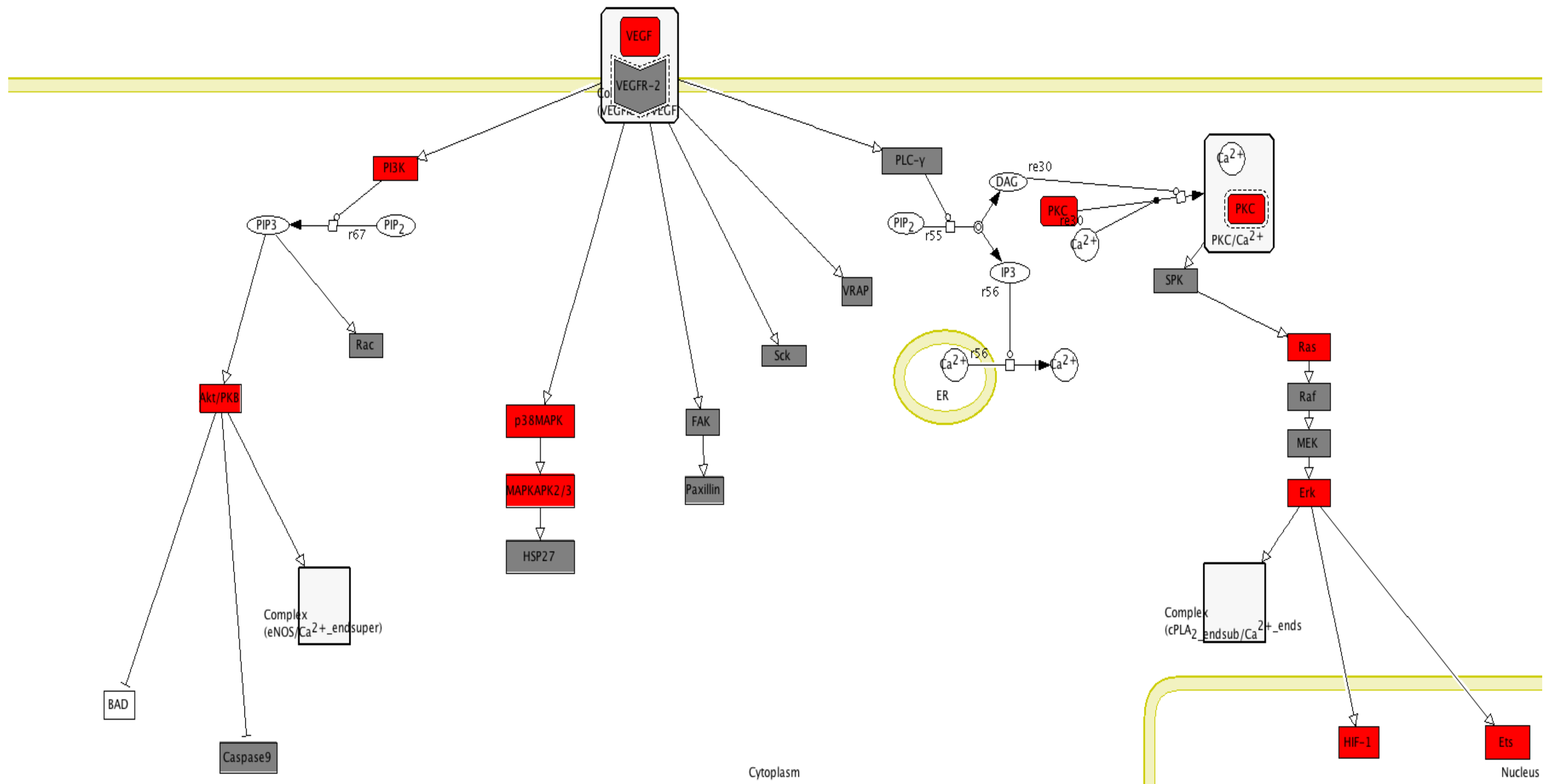


Figure 5.20 VEGF signalling pathway

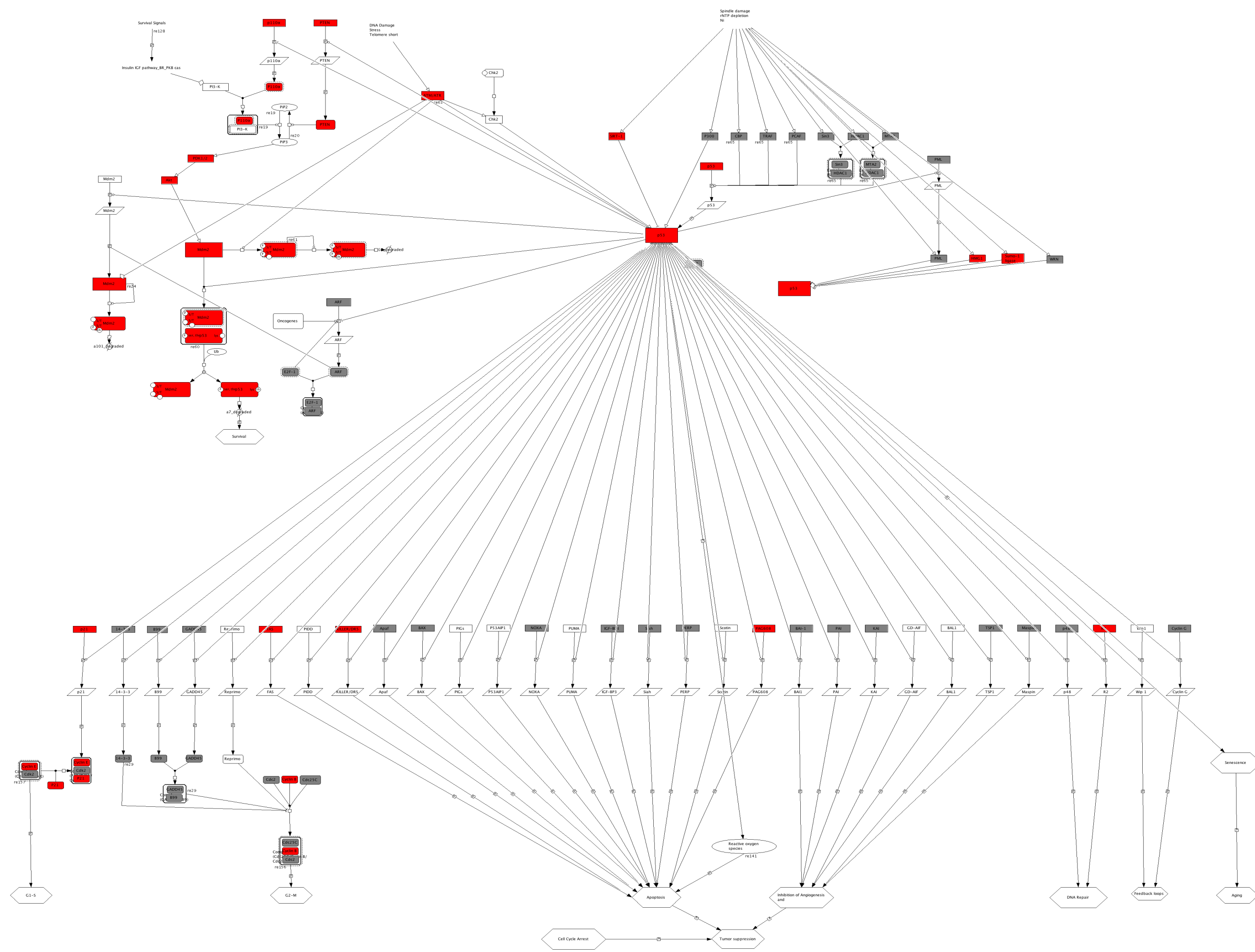


Figure 5.21 p53 pathway

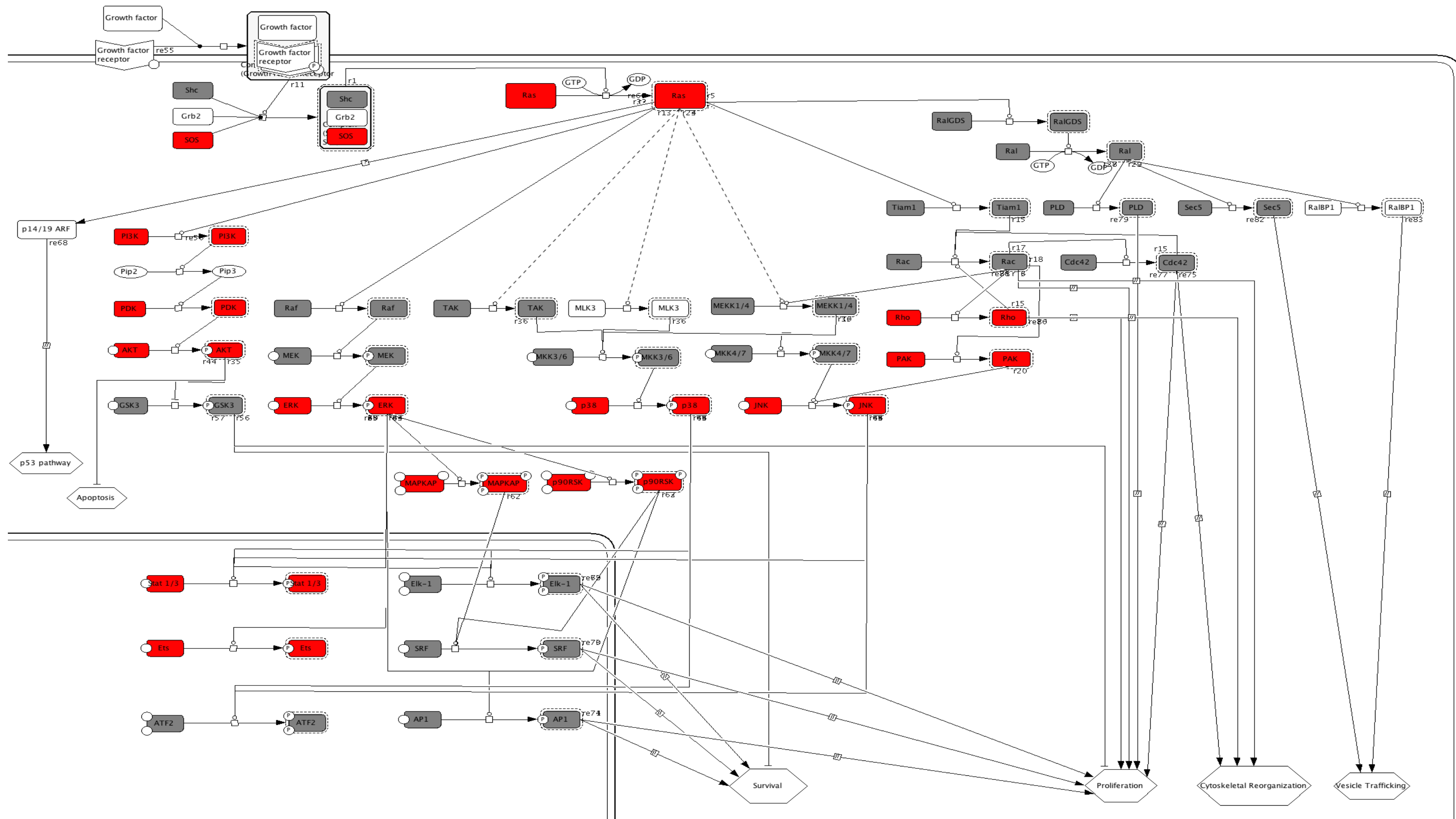


Figure 5.22 Ras pathway

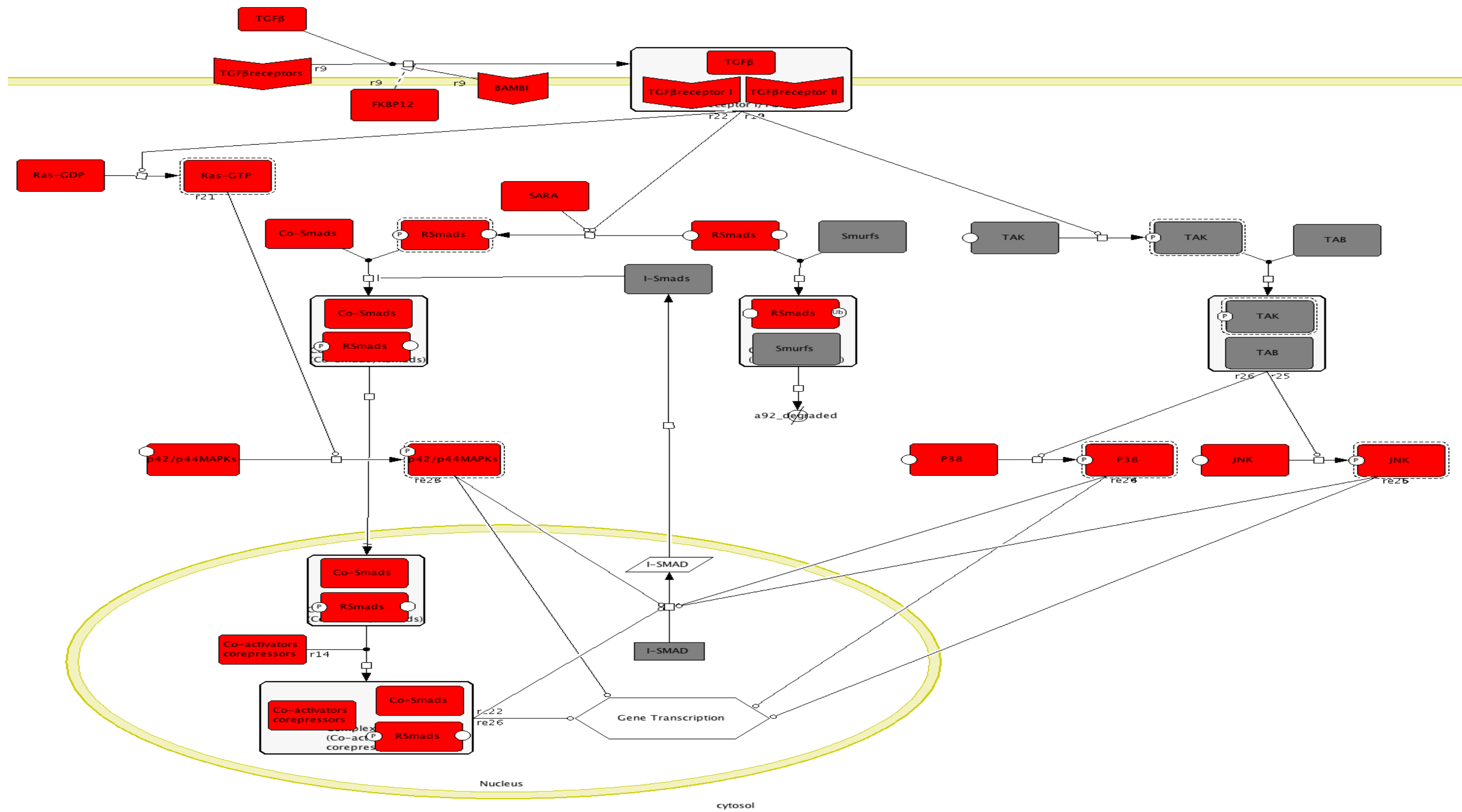


Figure 5.23 TGF-beta signalling pathway

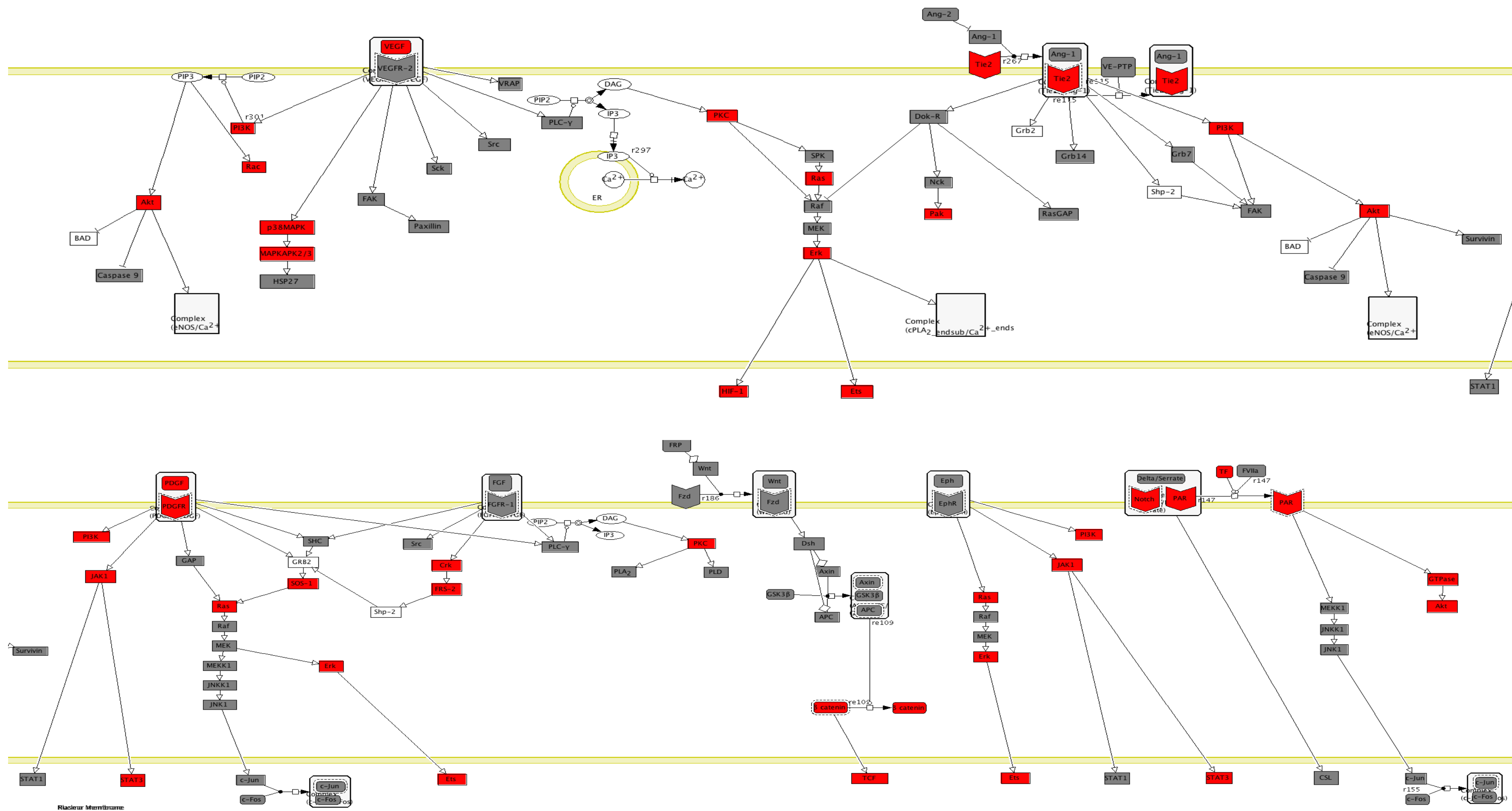


Figure 5.24 Angiogenesis (Split view)

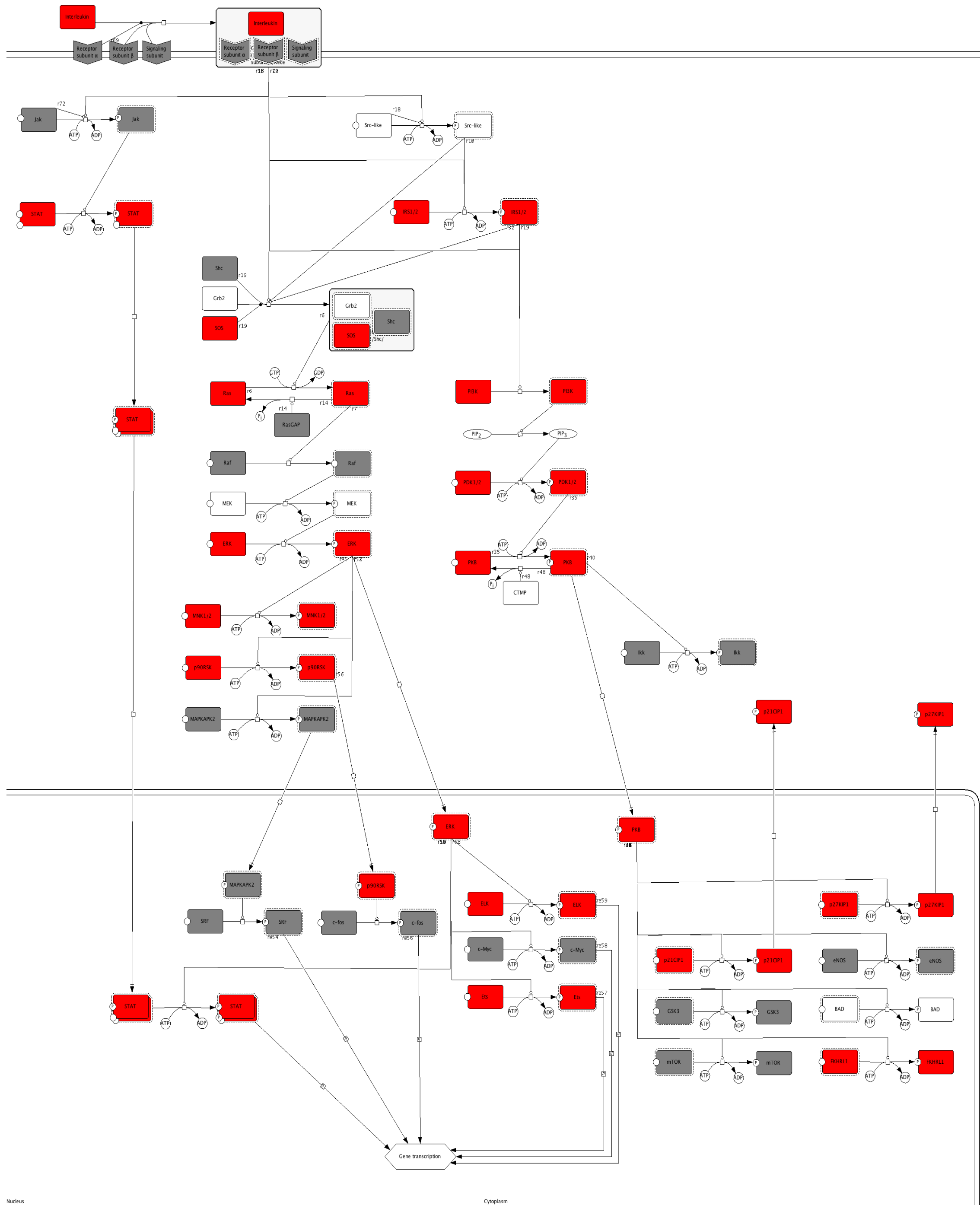


Figure 5.25 Interleukin signalling pathway

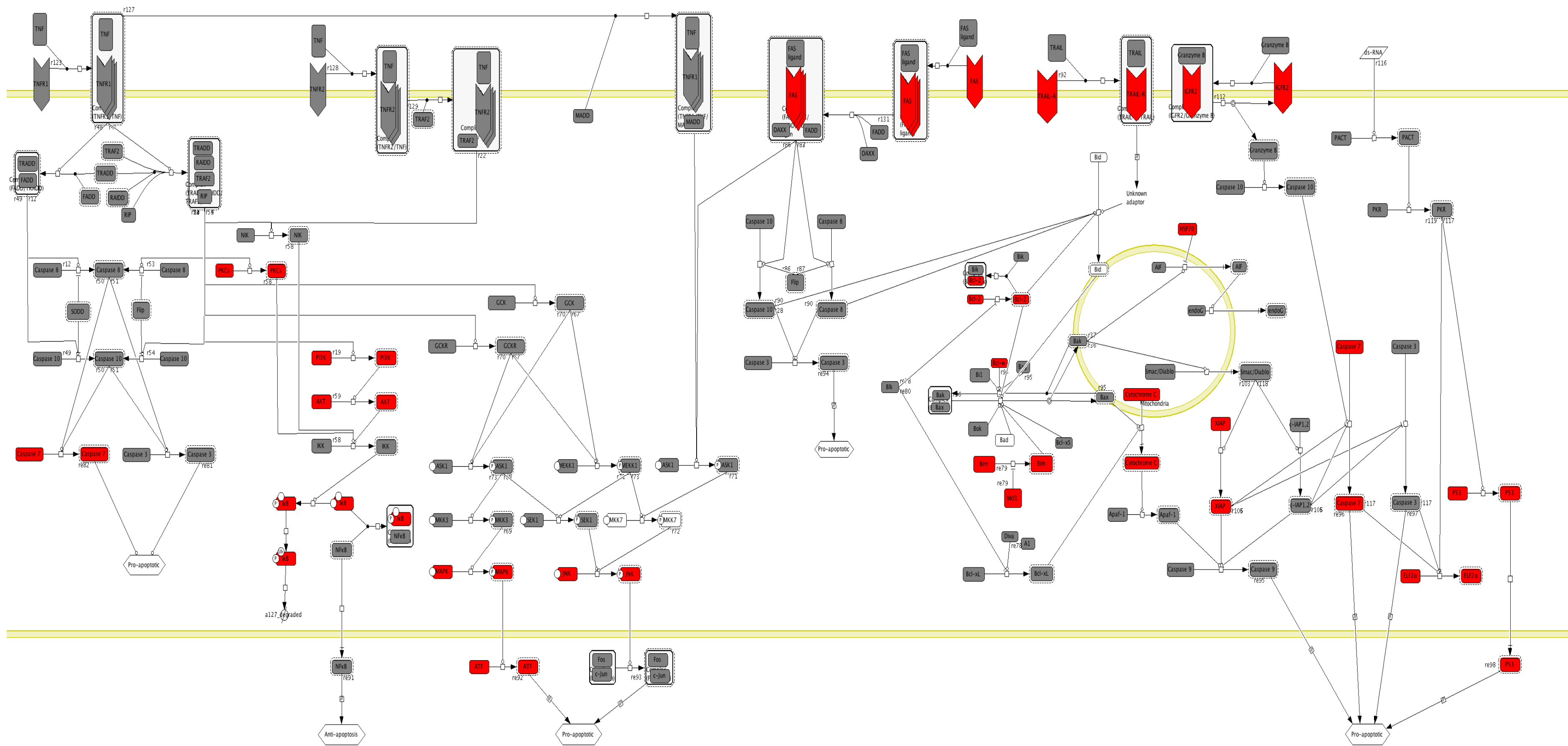


Figure 5.26 Apoptosis signalling pathway

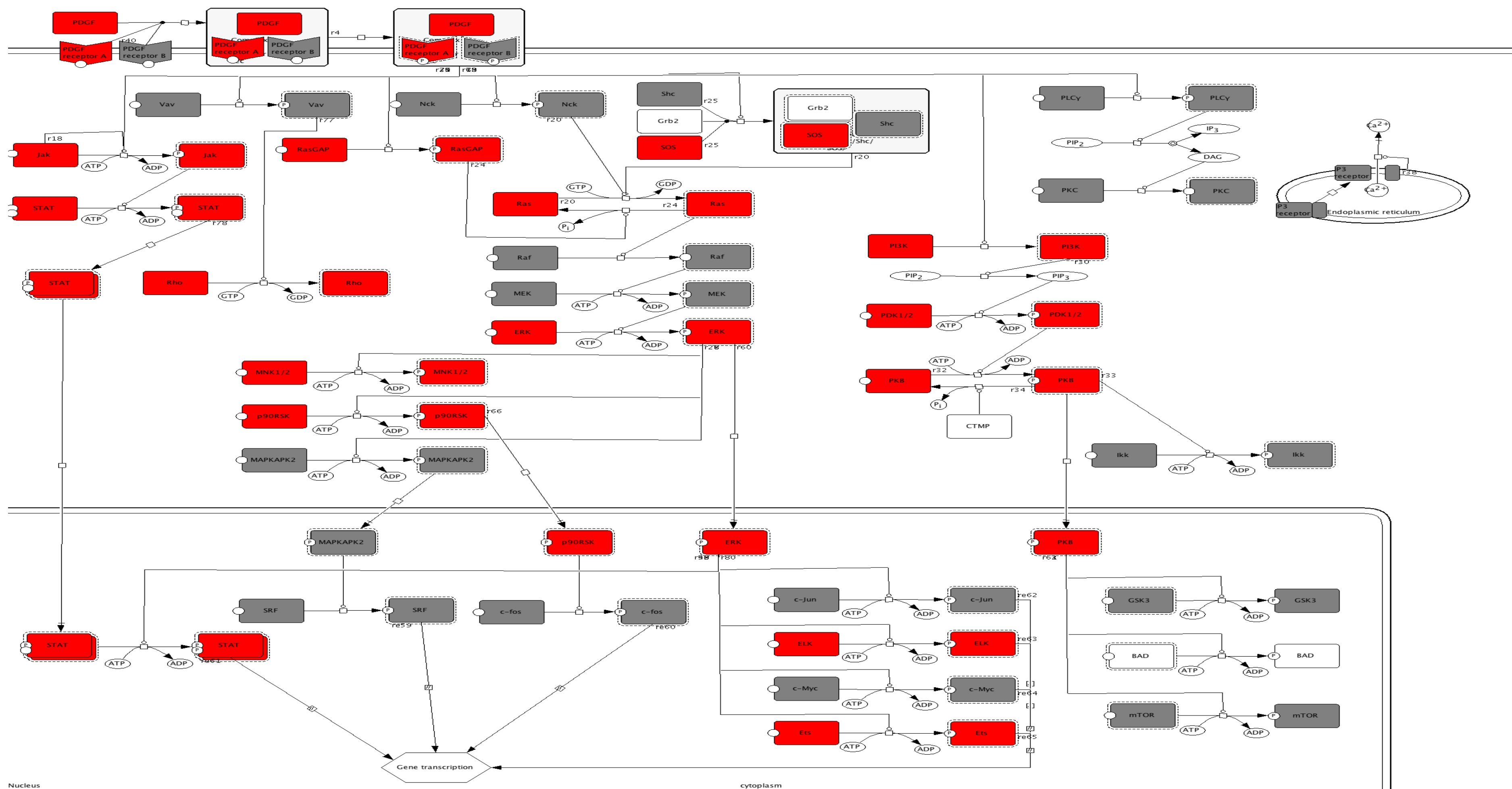


Figure 5.27 PDGF signalling pathway

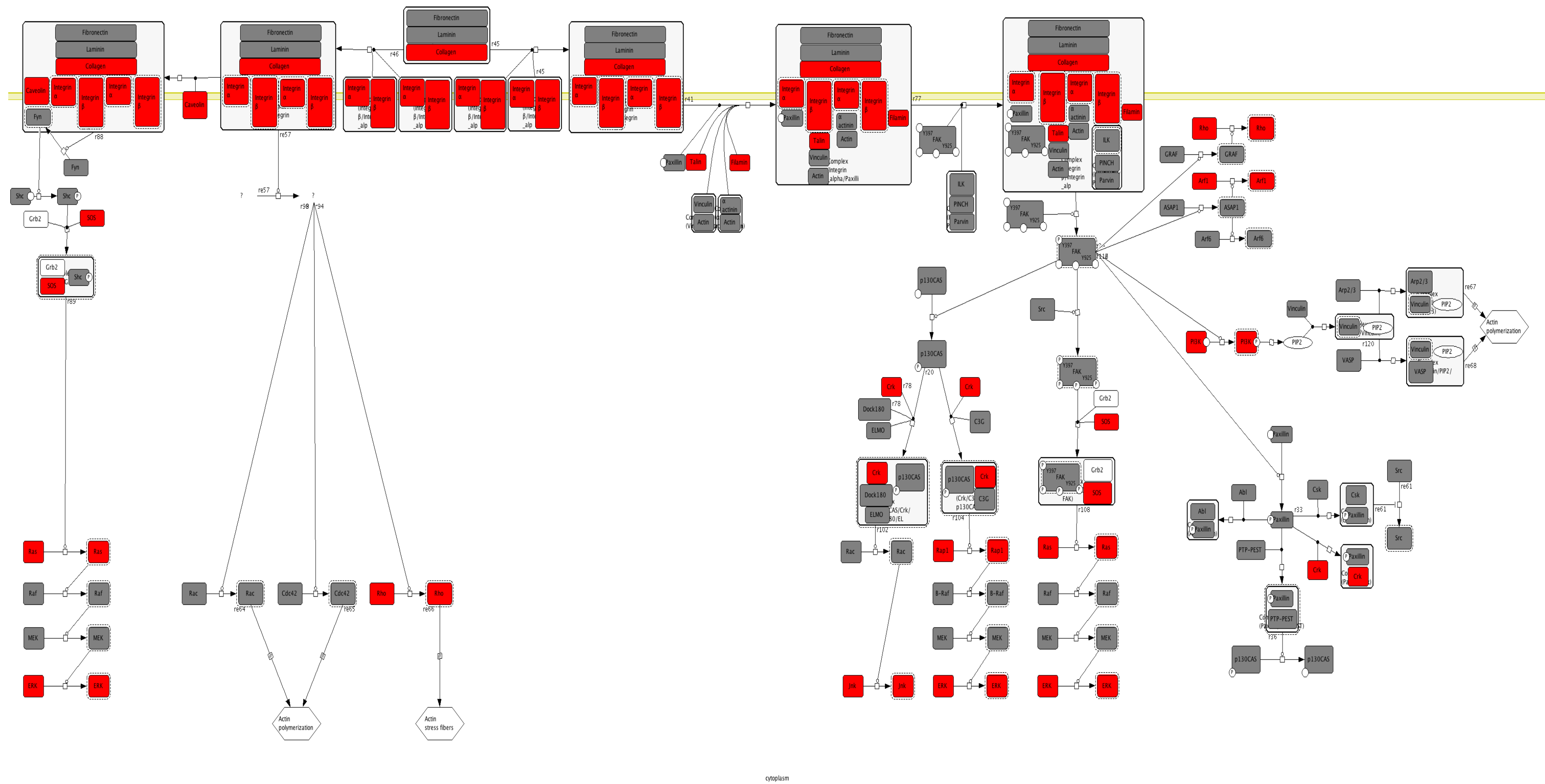


Figure 5.28 Integrin signalling pathway

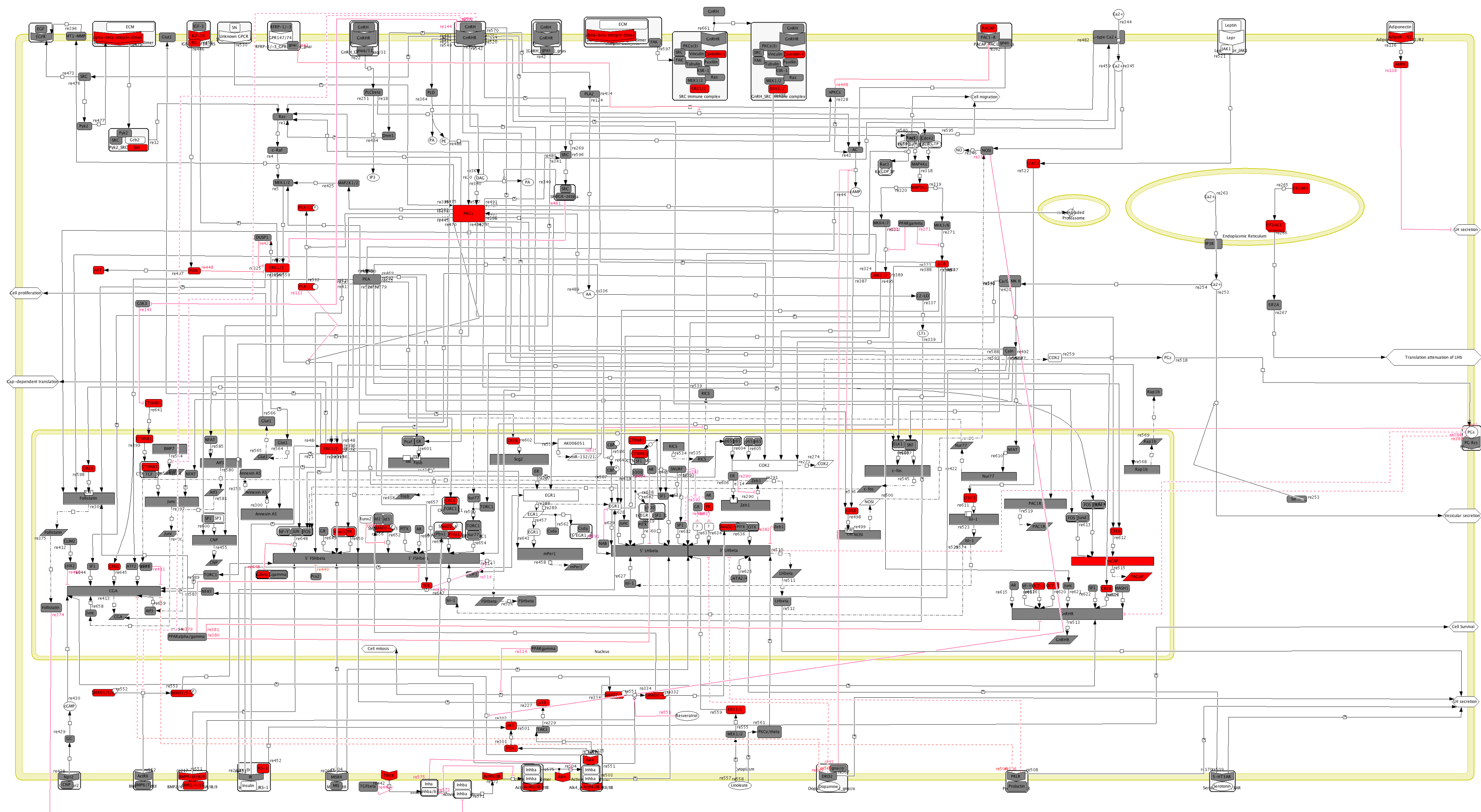


Figure 5.29 Gonadotropin releasing hormone receptor pathway

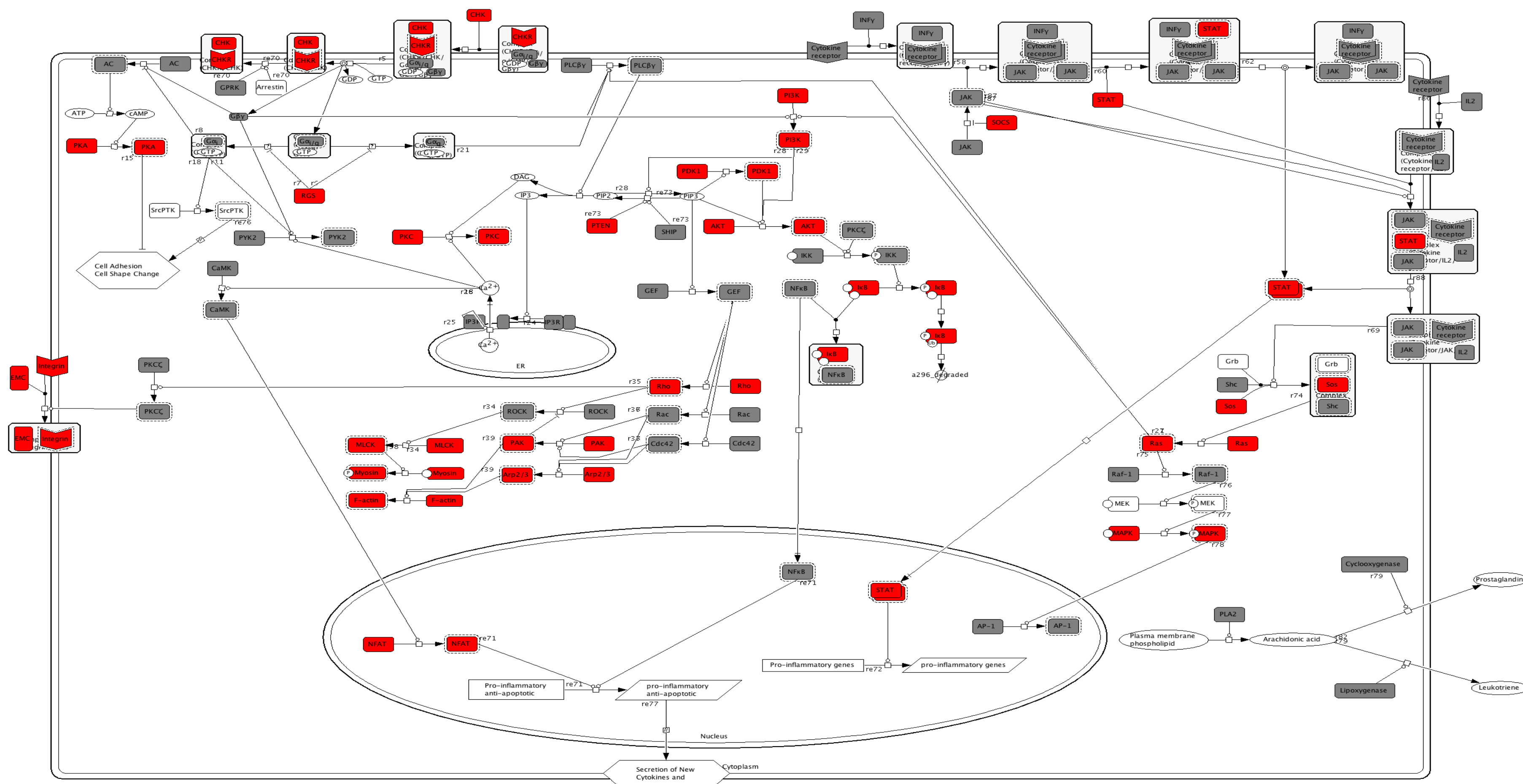


Figure 5.30 Inflammation mediated by chemokine and cytokine signalling pathway

5.4 Discussion

5.4.1 Validation study - introduction

In this study, microRNA changes identified in an initial deep sequencing study with follow-up qRT-PCR confirmation (Chapter 4) were validated using qRT-PCR. The new validation set included 37 patients who had received ECT for depression as part of the EFFECT-Dep Trial (and who were not included in the initial deep sequencing study) and 34 healthy controls. Patient and control groups did not differ significantly in terms of age or gender. The previous deep sequencing study had not found any significant differences in peripheral blood microRNA levels following ECT in the group as a whole. However, post-hoc analysis showed statistically significant changes in microRNA levels in patients in two diagnostic sub-groups, namely psychotic depression and bipolar affective disorder. Although these results differed from existing literature, where more widespread changes were seen, they were based on stringent statistical parameters, including correction for multiple testing.

5.4.2 Validation study - EFFECT-Dep patients

The results from the validation study closely mirrored the results from the preceding deep sequencing cohort. The study measured changes in the peripheral blood levels of six microRNAs before and after ECT. As in the preceding work, there were no significant changes in the group as a whole.

In contrast with the deep sequencing cohort, there were no significant changes in the levels of miR-130a-3p/5p in patients with bipolar depression. In those with psychotic depression, four candidate microRNAs were identified as being significantly changed in the deep sequencing cohort: miR-942-5p, miR-106a-5p and miR-126-3p/5p. Of these four candidate microRNAs, two were validated in this study, with miR-106a-5p and miR-126-3p both having significantly lower levels following ECT. This change was in the same direction as that observed in the deep sequencing cohort. These changes did not appear to be driven by antipsychotic medications as there were no differences in microRNA expression levels based on whether antipsychotics were prescribed or not.

5.4.3 Validation study - Patients versus healthy controls

To assess whether microRNA levels could potentially differentiate depressed patients from healthy controls, baseline levels of microRNAs were compared in these groups. MiR-130a-3p/5p were the only microRNAs that showed significant differences between patients and healthy controls in the validation cohort as a whole. MicroRNA levels and scores on the HDRS-24 at baseline were significantly correlated for miR-130a-3p (Pearson's $r=0.451$, $p<0.001$) but not for miR-130a-5p (Pearson's $r=-0.097$, $p=0.629$). No significant changes in miR-130a-3p/5p levels were observed following ECT treatment. In the psychotic subgroup of patients this comparison was repeated for the validated miR-126-3p and miR-106a-5p. In contrast to the whole group analysis, baseline levels of these microRNAs were significantly higher in the depressed psychotic patients compared to controls. Interestingly, miR-126-3p and miR-106a-5p levels were normalised at end of treatment.

5.4.4 Validation study - response status

No differences in the levels of microRNAs were observed based on response status; either at baseline or by change over time. This also held true for the psychotic subgroup of patients. There was also no significant correlation between baseline levels of microRNAs and changes in the HDRS-24.

5.4.5 Discussion of validation results

The aim of this study was to validate results from an exploratory study of microRNA changes following ECT. The results indicate that in patients with psychotic depression there is a significant downregulation of two microRNAs (miR-126-3p and miR-106a-5p) following ECT. At baseline, the levels of these microRNAs in peripheral blood were significantly higher than in healthy controls. Following ECT however, the levels of these two microRNAs had normalised to the levels seen in healthy controls. MicroRNA levels did not correlate significantly with responder status or scores on the HDRS-24. This may indicate that these changes could be related to some other aspect

of psychotic depression itself or they may be related to the mechanism of action of ECT in psychotic depression rather than a direct antidepressant effect of these microRNAs. The number of patients with psychotic depression was adequate to power the analysis of change in microRNA levels before and after ECT. However, the analysis of correlations between microRNA levels and changes on the HDRS-24 or with responder status was likely underpowered which may explain why this revealed no significant findings.

Baseline levels of miR-130a-3p/5p were also significantly higher in depressed subjects compared to controls. A post-hoc analysis of miR-130a-3p/5p expression before and after ECT in psychotic depression failed to reach statistical significance although both were decreased after ECT and miR-130a-3p was close to reaching statistical significance (miR-130a-3p $t=2.37$, $p=0.051$, miR-130a-5p $t=1.062$, $p=0.323$). Although the p-value was close to significance the deep sequencing results did not indicate a similar pattern in a separate cohort and this comparison was not part of the a priori hypotheses. The role of miR-130a-3p was therefore not further analysed in this study, although it could be a microRNA of interest in further studies with larger groups with psychotic depression.

Although the number of patients with psychotic depression was low, the findings with regard to miR-126-3p and miR-106a-5p have now held true from the initial deep sequencing exploratory study, through both confirmation and validation follow-up, using robust statistical techniques correcting for multiple testing at appropriate stages.

5.4.6 Discussion: miR-126-3p overview

Most of the literature regarding miR-126-3p comes from studies of endothelial cells where it has been identified as a key regulator of angiogenesis, vascularisation and inflammatory and immune responses (Sonntag et al., 2012, Wang et al., 2008). It has been implicated in various cancers (Vosa et al., 2013, Du et al., 2014), acute kidney injury (Aguado-Fraile et al., 2015), rheumatoid arthritis (Churov et al., 2015), and is increased by anti-Tumour Necrosis Factor-α/disease-modifying anti-rheumatic drugs in autoimmune disorder patients (Castro-Villegas et al.,

2015, Lv et al., 2015). Its implication in a number of disorders limits its utility as a biomarker on its own in depression.

The 49 gene targets of miR-126-3p that have been experimentally validated with high confidence are listed in Table 5.10, including their potential relationship with depression. Given miR-126-3p's role in angiogenesis it is no surprise that many of the genes are either directly or indirectly involved in angiogenesis, including *VEGFA*, *VCAM1*, *PI3KR2*, *PIK3CA*, *ETS1*, *AKT1*, *SIRT1*, *IGFBP2*, *IRS1*, *PTPN7*, *KRAS*, *CCNE1*, *FOXO3*, *PITPNC1*, *MERTK*, *EGFL7*, *TEK* and *BCL2*. Another set of genes are involved in cell cycle regulation, including *E2F1*, *CDKN1B*, *SPRED1*, *PLK2*, *CCNE2*, *SOX2*, *MAPK1*, *NFK1B*, *AKT1* and *TAB3*. A further set of genes are implicated in the immune response, including *VCAM1*, *IRS1*, *PIK3CG*, *CXCL12*, *CXCR4*, *TAB3*, *SSP1* and *TCF4*.

Although our samples were based on peripheral blood, miR-126-3p is expressed in the brain and its targets play an important role in neural cell functioning (Landgraf et al., 2007, Sonntag et al., 2012). Of particular interest is *EGFL7*, which is also the host gene of miR-126-3p, which is encoded in intron 7 of the *EGFL7* gene on chromosome 9 (Wang et al., 2008). This gene is a modulator of Notch signalling in neural stem cells. It has been shown to regulate neuron self-renewal and promote the differentiation of neuronal stem cells to mature neurons, thus implicating it as a key player in neurogenesis and neuroplasticity (Schmidt et al., 2009). *VEGFA* is another mRNA target of miR-126-3p that has been implicated as a key player in depression (Warner-Schmidt and Duman, 2007). Apart from *VEGFA* itself, several of the other targets are known to play a part in VEGFA signalling or interact with *VEGFA* in some way, including the PI3K pathway, IGF-1 and EGF pathways (Sonntag et al., 2012). This highlights the potential role of a microRNA to orchestrate the expression of numerous genes. This is of particular relevance in disorders such as depression, which likely involves the interplay between numerous genes affecting several biological and molecular functions.

5.4.7 Discussion: miR-106a-5p overview

MiR-106a-5p is located on the X chromosome (Xq26.2). It has not been as well studied as miR-126-3p but has been implicated in several cancers, including gastric cancer, colorectal cancer and CNS tumours such as glioma (Zhu et al., 2014, Feng et al., 2012, Chen et al., 2015b). Studies have also shown that increases in the levels of miR-106a-5p in the brains of patients with glioma were matched by increased levels of miR-106a-5p in the plasma of the same patients, supporting the utility of exploring microRNA changes in peripheral blood (Chen et al., 2015b). MiR-106a-5p is part of the miR-17 precursor family, which includes miR-17, miR-20a/b, miR-93 and others arising from a previous evolutionary gene duplication event (Tanzer and Stadler, 2004). This microRNA family has been found to be involved in monocytopoiesis (Fontana et al., 2007).

The 38 gene targets of miR-106a-5p that have been experimentally validated with good confidence are listed in Table 5.9, including their potential relationship with depression. A number of genes involve regulation of angiogenesis, many through inhibition or inducement of *VEGFA*, including *TIMP2*, *DCBLD2*, *RUNX1*, *RUNX3*, *SIRT1*, *ATM*, *MAPK14* as well as *VEGFA* itself. A number of genes were also key players in neurogenesis through cell cycle and apoptosis regulation, including *E2F1*, *CDKN1A*, *RB1*, *RBL2*, *ARID4B*, *FAS*, *TGFBR2*, *PTEN*, *CCDN1*, *BCC10*, *MAPK9* and *MAPK14*. Further genes with various functions of interest included *IL10* and *LIMK1*, both implicated in depression.

5.4.8 Discussion: Shared gene targets

Three 'high confidence' genes were shared between the two microRNAs; *VEGFA, E2F1* and *SIRT1*. As both microRNAs target these genes, the expression of these genes should be more affected than those targeted by a single microRNA. *VEGFA,* which encodes the VEGF-A protein, is a member of the platelet-derived growth factor/vascular endothelial growth factor family, and is located on chromosome 6p21 (Vincenti et al., 1996). It is a mitogen-type cytokine (a substance that encourages a cell to commence cell division) which is active in angiogenesis, vasculogenesis, increased vascular permeability and endothelial cell growth (Ferrara et al., 2003).

Furthermore, it has been implicated in neurogenesis, neuroprotection, modulation of synaptic transmission and hippocampal-dependent processes such as memory and learning (Warner-Schmidt and Duman, 2007, Jin et al., 2002).

In the brain, VEGF-A protein binds to two tyrosine kinase receptors; foetal liver kinase (Flk-1 / VEGFR-2) or fms-like tyrosine kinase 1 (Flt-1 / VEGFR-1) (Newton et al., 2013). *VEGFA* expression is tightly regulated as even small variations are associated with various diseases, from retinopathy, angiomas and cancers (Arcondeguy et al., 2013). In terms of depression, VEGF-A levels have been shown in animal models to be induced by several antidepressants, including fluoxetine, desipramine and ECS. VEGF-A-Flk-1 signalling has been implicated as a key pathway, being required and sufficient for a behavioural response in chronic (Chronic Unpredictable Stress and Novelty Suppressed Feeding) and sub-chronic (Learned Helplessness and Forced Swim Test) animal models of depression (Warner-Schmidt and Duman, 2007). VEGF-Flk-1 signalling also appears to be involved in the maturation of neuronal progenitors and plays a key role in neurogenesis induced by ECS (Segi-Nishida et al., 2008, Newton et al., 2003, Elfving and Wegener, 2012).

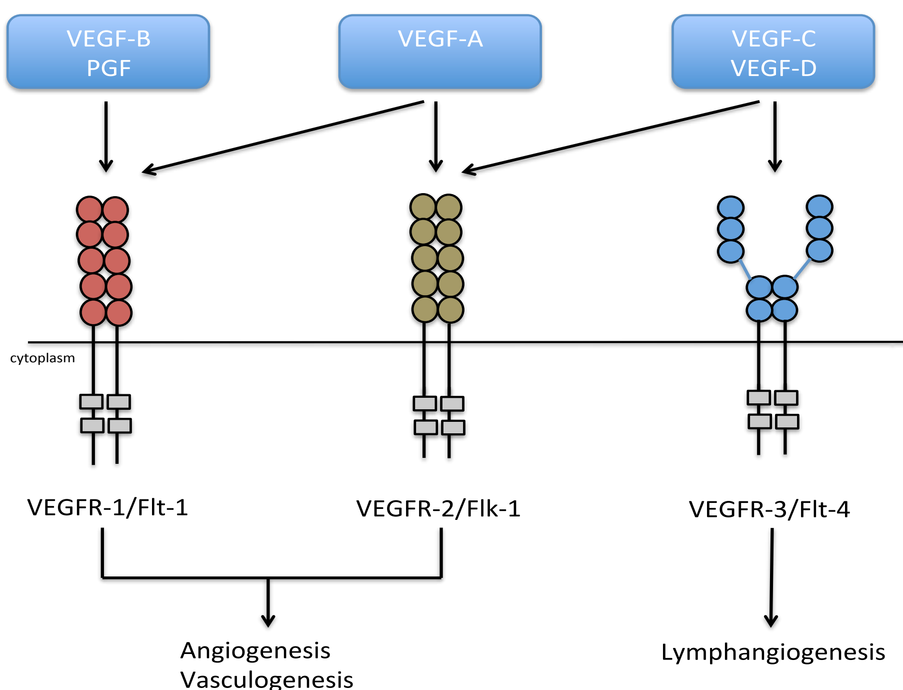


Figure 5.31 VEGF family subtypes and their receptors

In human subjects, initial studies of *VEGFA*/VEGF-A levels in patients with depression compared to controls varied in their findings, with some finding elevated *VEGFA* mRNA levels, (Iga et al., 2007, Berent et al., 2014) but others failing to replicate this when examining VEGF-A protein levels (Dome et al., 2009). Two separate recent meta-analyses have helped to clarify this issue, with an overall finding of elevated peripheral VEGF-A levels in depressed subjects versus controls (Carvalho et al., 2015, Tseng et al., 2015). Elsewhere, serum levels of VEGF-A were significantly increased one month after ECT treatment, and again, changes in VEGF-A levels corresponded significantly with changes in depression rating scores (Minelli et al., 2011). ECT in particular appears to have a powerful effect on VEGF pathways. Minelli et al (2014) showed a significant correlation between baseline *VEGFA* mRNA levels and reduction in depressive symptomatology in patients with treatment-resistant depression treated with ECT. This correlation was not seen in those treated with antidepressants alone (Minelli et al., 2014).

Other studies utilising various pharmacological antidepressants have also failed to find a relationship between treatment and VEGF-A levels, indicating that VEGF-A plays a role in the response to ECT (Clark-Raymond and Halaris, 2013). Together, these studies indicate that VEGF-A plays role in the mechanism of action of ECT, possibly through increased neurogenesis, neuroplasticity or other mechanisms.

E2F Transcription Factor 1 (E2F1*)* is a member of the E2F family of transcription factors that regulate cell proliferation and apoptosis (Morris et al., 2008). The *E2F1* gene is located on chromosome 20 (20q11.22) and consists of 7 exons (Neuman et al., 1996). It interacts closely with retinoblastoma 1 (Rb1), a tumour suppression protein, which can arrest the progression of the cell cycle from G1 to the S phase. It has been shown to play a part in hippocampal neurogenesis in adult mice, with *E2F1* knockout mice having significantly decreased production of new-born neurons in the adult brain (Cooper-Kuhn et al., 2002). It is also a mediator of retinoblastoma directed neurogenesis and neural migration (McClellan et al., 2007). In primates, treatment with the neuroprotective agent oestradiol was shown to reduce E2F1 levels in the Dorsolateral Prefrontal Cortex (DLPFC) of rhesus monkeys (Wang et al., 2004). Interestingly, it has been

shown to reduce neo-angiogenesis by inhibiting the activity of the *VEGFA* promoter (Merdzhanova et al., 2010, Qin et al., 2006). However, in depression, *E2F1* has not been implicated, or reported on, in terms its expression levels or potential role.

The third shared gene target identified was Sirtuin 1 (*SIRT1)*. SIRT1 is a member of the sirtuin family of proteins. In lower eukaryotes, this acts as a stress-response and chromatin silencing histone deacetylase with roles in nuclear transcription, DNA replication, and DNA repair (Blander and Guarente, 2004). It is associated with an increased life span in lower organisms, and thus has a potential role as an anti-ageing target in humans that has gathered much interest (Abdelmohsen et al., 2007). However, its role in humans is still being clarified. It is being linked with marked metabolic effects, with increased SIRT1 levels associated with a favourable metabolic profile through resistance to stress/damage-induced apoptosis, although increased levels may also be associated with a higher risk of cancer through the same mechanisms (Lim, 2006). Some studies have found a neuroprotective role for SIRT1 in animal models such as axonal damage and amyloid plaques (Kuningas et al., 2007, Libert et al., 2008).

Part of its neuroprotective action may be through interaction with VEGF-A (Li et al., 2015, Balaiya et al., 2012). ECS results in increased levels of SIRT1 in the hypothalamus and hippocampus (Chung et al., 2013). There is also a growing support for the potential role of *SIRT1* in depression. *SIRT1* mRNA levels in lymphocytes were lower in depressed subjects compared to controls. These levels normalised when the depressed patients achieved remission (Abe et al., 2011). These findings were supported by a recent study exploring the relationship between childhood adversity, depression and SIRT1 levels (Lo Iacono et al., 2015). Initially, a mouse model of juvenile isolation stress showed that juvenile stress, but not adult stress, resulted in decreased levels of SIRT1 and despair behaviour. This behaviour was reversed by resveratrol, a drug that increases SIRT1 levels. Moving these findings to humans, the authors found lower SIRT1 levels in patients with depression when compared to controls. This was especially evident in those who received poorer care (as defined by a retrospective self-report questionnaire) in childhood (Lo Iacono et al., 2015).

Further support for the role of *SIRT1* in depression comes from two genetic studies. In the first, a case-control study of single nucleotide polymorphisms (SNPs) in a Japanese population (cases: 450; controls: 766) found an association between rs10997875 in the *SIRT1* gene and depression. In the second study, whole-genome sequencing of over 10,000 Chinese women found two genetic loci contributing to the risk of depression in this population. One of these loci was near the *SIRT1* gene (Converge consortium, 2015).

The shared high confidence target genes of miR-126-3p and miR-106a-5p are therefore all of interest on their own in terms of depression. A STRING network analysis revealed that the protein products of these genes are also closely related and interact, for example, Forkhead Box, class O transcription factors 1 and 3 (FOXO1 and FOXO3), proteins implicated in anxiety and depression, both interact closely with both SIRT1 and VEGF-A (Polter et al., 2009). To better understand the potential sum effect of the two microRNAs, all gene targets were entered into the DAVID database for gene ontology analysis.

5.4.9 Discussion: Gene ontology

Gene ontology analysis revealed that the experimentally validated target genes were over-represented in a number of processes that may be relevant to depression or the molecular mechanism of ECT. However, a number of terms represent duplication or minor variation of the same process, for example the GO terms include such closely related terms as 'regulation of apoptosis', 'regulation of cell death' and 'apoptosis'. To remove some of this redundancy the terms were run through the REVIGO software, which selects terms on their uniqueness (Supek et al., 2011). The results of the REVIGO analysis can be seen in Figure 5.33 below. The key biological processes highlighted through this analysis were processes involved in cell cycle regulation, regulation of transferase activity and response to abiotic stimuli. Although dysregulation of cell cycle activity is most associated with cancer, they are also being linked to imbalances in neurogenesis and neuropsychiatric disorders such as depression (Patricio et al., 2013).

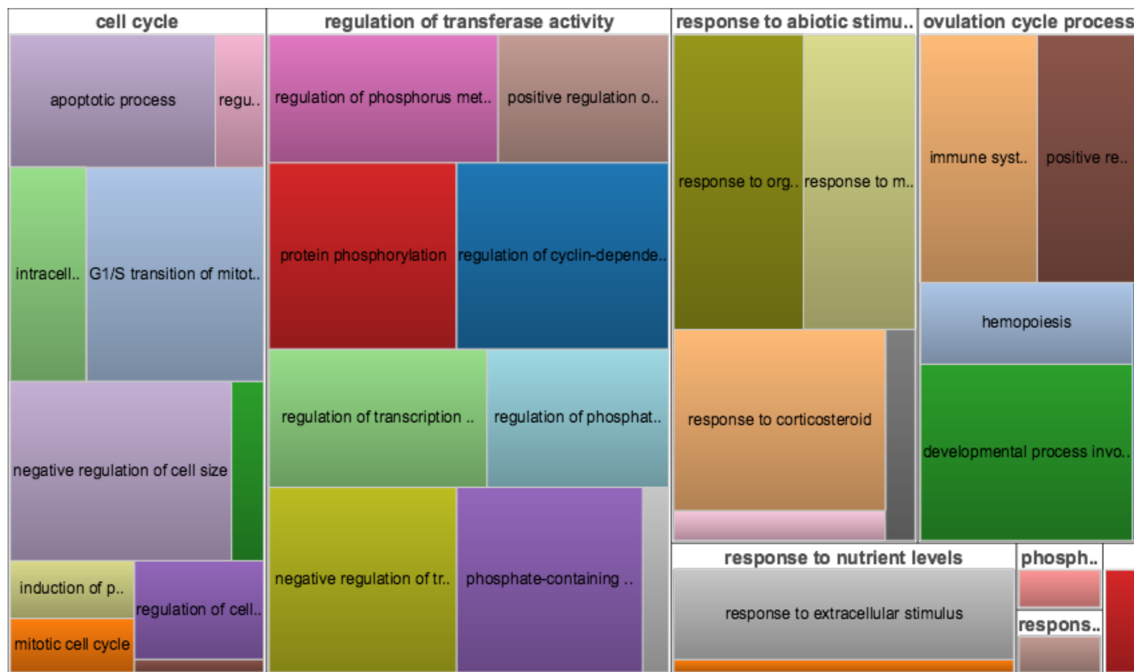


Figure 5.32 REVIGO treemap

Treemap of Gene Ontology terms for target genes. Gene ontology terms are rationalised into nested rectangles, based on their uniqueness. Within each rectangle (e.g. 'cell cycle') are a number of smaller rectangles representing the sub-branches of this group (e.g. 'apoptotic process' and 'negative regulation of cell size' are sub-branches of the 'cell cycle' branch.

Particular cell cycle regulators that have been implicated in adult neurogenesis include Cdk6-cyclin D2 and Cdk4-cyclin D1, which appear to increase the availability of the neural progenitor pool prior to neurogenesis (Artegiani et al., 2011, Beukelaers et al., 2011). As discussed earlier, E2F1, a transcription factor that is a potent regulator of the cell cycle, and one of the three shared target genes of both validated microRNAs, has been shown to play a part in hippocampal neurogenesis in adult mice. *E2F1* knockout mice show significantly decreased production of new-born neurons in the adult brain (Cooper-Kuhn et al., 2002). Several cyclin dependent kinase inhibitors have also been implicated in neurogenesis. These include *CDKN1A* (Pechnick et al., 2011) and *CDKN1B* (Andreu et al., 2015). The other major terms identified by REVIGO are too general to be useful for further comment, including 'regulation of transferase activity' and 'response to abiotic stimuli'. More specific terms of interest include 'response to

corticosteroid'. This is of interest given the role of the hypothalamic-pituitary-adrenal axis in depression, and evidence for microRNA regulation of HPA-axis dysregulation (Dwivedi et al., 2015).

As a next step a pathway analysis approach was then taken to obtain a clearer idea of the systems and processes that the target genes are involved in. Pathway analysis integrates information about the potential interaction between genes, biochemical reactions and cellular location into one schematic. The pathway analysis identified several over-represented pathways that are of interest in depression. A number of the pathways were growth factor pathways, such as VEGF, IGF, TGF-Beta, PDGF and EGF signalling pathways that have been implicated in depression, neurogenesis and neuroplasticity. In addition, the angiogenesis pathway is closely linked to these growth factor pathways, in particular the VEGF and PDGF pathways.

The second group of pathways that are closely related are cell cycle regulatory pathways, including the p53, apoptosis signalling and cell cycle pathways. Although these have come under most scrutiny in the cancer literature, as previously noted, they have also been implicated in adult neurogenesis and depression (Patricio et al., 2013).

The Ras and HIF activation pathways straddle both the growth factor and cell cycle regulation pathways, indicating a potential mechanism for 'cross-talk' between these groups of pathways, that may involve the ERK/MAP kinase signalling cascade (Ke and Costa, 2006, Di Benedetto et al., 2013, Lefloch et al., 2009). Two inflammatory pathways were identified in line with the potential role of inflammation in depression. A further pathway of interest is the CCKR signalling pathway, which has been shown to influence neurotransmission in the brain and is implicated in anxiety and depression-like behaviour in rodent models (Desai et al., 2014).

Together, the gene ontology and pathway analysis of the experimentally validated targets of significantly altered microRNAs highlight the complexity of potential networks and pathways that may be affected by changes in microRNA levels. Many of the pathways and processes that are statistically over-represented are of great interest in terms of depression, adult neurogenesis and neuroplasticity and the potential mechanism of action of ECT. In particular, the analyses point toward widespread

regulation of growth factors and cell cycle regulation processes, many of which interact.

5.4.10 Discussion: Psychotic depression

The results indicate a differential microRNA response in the peripheral blood of patients with psychotic depression compared to non-psychotic depression treated with ECT. About 20% of depressed patients will have a psychotic subtype of depression (Ohayon and Schatzberg, 2002). Current diagnostic guidelines (ICD-10 and DSM-V) list psychotic depression as a subtype of depression. However, there are several proponents arguing that it should be considered a separate diagnostic entity (Schatzberg and Rothschild, 1992, Ostergaard et al., 2012). This is based on differences in clinical presentation, prognosis, genetic and laboratory findings, as well as response to treatments. In psychotic depression a patient suffers from both the symptoms of depression (such as anhedonia, insomnia, psychomotor disturbance and poor concentration) as well as psychosis, including delusions or hallucinations. Delusional themes are often mood-congruent, such as nihilistic delusions (Rothschild, 2013). Psychotic depression is associated with higher relapse rates (Flint and Rifat, 1998), greater disability, mortality and increased use of services compared to non-psychotic depression (Rothschild, 2003).

Genetic studies have also highlighted differences between psychotic and non-psychotic depression. In a review of clinical and molecular genetic studies, Domschke (2013) noted that evidence from family, twin and adoption studies suggests there is familial aggregation of psychotic depression, especially for mood-incongruent psychotic depression (Domschke, 2013). In terms of genetic linkage studies, there is indirect support for candidate risk loci derived from studies involving schizoaffective disorder, schizophrenia, bipolar affective disorder and depression (Domschke, 2013). Association studies have identified candidate vulnerability genes for psychotic depression, but most of the sample sizes have been small. Interestingly, many of the candidate genes, such as those coding for dopamine receptors 2/4 (*DRD2 and DRD4)* and glycogen synthase kinase 3 beta (*GSK3B),* are involved in dopaminergic and

glutamatergic rather than serotonergic neurotransmission (Domschke, 2013).

Apart from genetic differences, there also appear to be real differences between psychotic and non-psychotic depression on certain laboratory tests. The strongest evidence is for greater rates of cortisol non-suppression after the dexamethasone suppression test (DST) in psychotic depression when compared to non-psychotic depression (Nelson and Davis, 1997). In addition, higher levels of blood and urinary cortisol have been observed in psychotic depression (Ostergaard et al., 2012). Dopamine beta-hydroxylase, a key catalyst for the conversion of dopamine to noradrenaline appears to have lower activity in psychotic depression, and a polymorphism (DBH*444G/A) is associated with paranoid ideation in major depression (Wood et al., 2002).

Finally, treatment response in psychotic depression differs from non-psychotic depression in several studies. ECT has been found to be particularly effective in psychotic depression, with remission rates superior to non-psychotic depression (Petrides et al., 2001). With regard to pharmacotherapy, a Cochrane Review found that although understudied, there was some evidence that the combination of an antidepressant and an antipsychotic was more effective than either treatment or placebo alone (Wijkstra et al., 2015). Psychotherapy appears to have a limited role in the acute treatment of psychotic depression (Gaudiano et al., 2007).

5.4.11 Discussion: Summary

In this validation study, the differential expression of two microRNAs (miR-126-3p and miR-106a-5p) in patients with psychotic depression remained consistent with earlier findings from an initial discovery study. Earlier findings from patients with bipolar depression were not validated in this study. Unlike the preceding work, this was a hypothesis-driven study with the addition of healthy controls and a new, separate cohort of depressed patients treated with ECT that did not overlap with the original discovery study. Although the number of patients with psychotic depression was low, an a priori power calculation indicated sufficient sample size to detect meaningful differences in microRNA expression. However, further analyses,

such as clinical and cognitive correlations were limited by the small sample size.

Both microRNAs have been linked with depression or processes linked to depression such as neuroplasticity and inflammation. Bioinformatic analysis shed further light on the numerous gene targets of the two microRNAs, including potential roles in neurogenesis and cell cycle regulation. However, it is important to stress that these findings were from peripheral blood with the limitations that this entails. MicroRNAs are actively secreted across the blood-brain barrier and are well protected from degradation, but microRNAs are also found in red and white blood cells as well as plasma (Cheng et al., 2014). It is not yet known whether peripheral changes in microRNA levels observed from antidepressant treatment reflect changes that occur in the brain (Bocchio-Chiavetto et al., 2013, Ryan et al., 2016).

5.5 Conclusion

In psychotic depression, peripheral blood miR-126-3p and miR-106a-5p are elevated at baseline compared to healthy controls. However, after ECT treatment, these differences disappear. These findings were not seen in non-psychotic or bipolar depression. The shared gene targets of the two microRNAs were *VEGFA, E2F1* and *SIRT1*. Further bioinformatic analysis highlighted the potential pathways and networks that are targeted by the two microRNAs. These pathways included several neurotrophic and neuroprotective factors as well as cell cycle regulators. Of particular interest is *VEGFA*. *VEGFA* has previously been linked to both depression and ECT, but this potential link between microRNAs, ECT and *VEGFA* is new and warrants further exploration. To this end, peripheral blood from depressed patients undergoing ECT and healthy controls was analysed for downstream mRNA changes in *VEGFA* levels. This will be presented in Chapter 6.

Chapter 6
Results

6 *VEGFA* mRNA expression in peripheral blood following ECT for severe depression

6.1 Introduction

The VEGF-A protein is a key activator of angiogenesis, vasculogenesis, increased vascular permeability and endothelial cell growth (Ferrara et al., 2003). The *VEGFA* gene is located on Chromosome 6p21 and is a member of the platelet-derived growth factor/vascular endothelial growth factor family (Vincenti et al., 1996). *VEGFA* is also implicated in neurogenesis, neuroprotection, modulation of synaptic transmission and hippocampal-dependent processes such as memory and learning (Warner-Schmidt and Duman, 2007, Jin et al., 2002). Its expression is tightly regulated, and small variations can be associated with various diseases, from retinopathy, angiomas and cancers (Arcondeguy et al., 2013). In animal models, *VEGFA* mRNA and VEGF-A protein levels have been induced by antidepressant treatments, including electroconvulsive shock (ECS), and appears to play a key role in neurogenesis induced by ECS (Segi-Nishida et al., 2008, Newton et al., 2003, Elfving and Wegener, 2012). In humans, meta-analytic evidence suggests that peripheral VEGF-A levels tend to be elevated in depressed subjects compared to controls (Carvalho et al., 2015, Tseng et al., 2015). Electroconvulsive therapy (ECT) appears to have a particularly strong effect on peripheral VEGF-A levels, also showing significant correlation with depressive symptomatology (Minelli et al., 2011). These correlational changes were not seen with other antidepressant treatments, indicating that VEGF-A may play a specific role in the response to ECT (Minelli et al., 2014, Clark-Raymond and Halaris, 2013).

In Chapter Four a discovery phase deep sequencing study with PCR confirmation identified a small number of potential microRNAs whose expression changed significantly following a course of ECT in those with psychotic depression. In Chapter Five these changes were validated in a separate cohort, including healthy controls. Following validation, two microRNAs, miR-126-3p and miR-106a-5p were identified as having elevated levels at baseline compared to healthy controls in patients with

psychotic depression. Following ECT treatment however, these differences disappeared. Bioinformatic analysis indicated that of the 1609 genes that were targeted by these two microRNAs three genes were shared targets. *VEGFA* was one of these three genes, but it also interacted with several of the pathways that were statistically over-represented by the gene targets of miR-126-3p and miR-106a-5p.

Although *VEGFA* has previously been linked to both depression and ECT, the potential link between microRNAs, ECT and *VEGFA* is new. To this end, peripheral blood samples from depressed patients undergoing ECT and controls were analysed for changes in downstream *VEGFA* mRNA levels.

6.1.1 Aims of the study

The results of the preceding microRNA studies identified *VEGFA* as an empirically derived mRNA target in patients with psychotic depression. The primary aim of this study was therefore to measure *VEGFA* mRNA levels in patients with psychotic depression treated with ECT and to compare them to non-psychotic patients treated with ECT as well as healthy controls. Secondary aims included testing for correlations between VEGFA levels and clinical (depression severity, psychosis severity) and cognitive outcomes.

6.2 Methods

6.2.1 Participants and assessment tools

This study included 97 patients with moderate to severe depression enrolled in the EFFECT-Dep Trial (see section 2.4.1). The presence or absence of a major depressive disorder, including melancholic and psychotic subtypes was confirmed by administering the mood disorder component of the research version of the Structured Clinical Interview for DSM-IV Axis 1 Disorders (First et al., 2002). Treatment resistance was measured using the Antidepressant Treatment History Form (Oquendo et al., 2003). Pre-morbid intelligence was measured by the National Adult Reading Test (Nelson HE and J, 1991).

Depression severity at baseline and end of treatment (EOT) was measured using the 24-item version of the Hamilton Depression Rating Scale (HDRS-24) (Beckham and Leber, 1985). Exclusion criteria included substance abuse in the last six months; premorbid existing cognitive impairment; other Axis I disorder; ECT in the past 6 months; inability to consent; current inflammatory, infectious, or haematological disorder. Responders were defined as those who, at end of treatment, achieved a ≥60% decrease in HDRS-24 from baseline and a final score ≤16.

Psychotic psychopathology severity was measured using items from the Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham, 1962). Five items from the BPRS - hallucinations, unusual thought content (delusions), suspiciousness, emotional withdrawal and blunted affect (BPRS-5) have been identified as having the highest clinical validity and responsiveness to change in psychotic depression (Ostergaard et al., 2014, Park et al., 2015).

In a recent meta-analysis (Semkovska and McLoughlin, 2010) our research group identified that ECT has the most pronounced sub-acute (i.e. 0-3 days post course) adverse effects upon verbal memory (delayed word list recall) and frontal executive functions. Autobiographical memory and reorientation time is also affected by ECT (Semkovska et al., 2016). Cognitive measures of executive functioning, delayed verbal memory, autobiographical memory as well as reorientation time were therefore included for correlational analysis. In addition, a measure of global cognition was included. The measures are described in detail section 2.2.3 and include: the Addenbrooke's Cognitive Examination Revised (ACE-R) (Mioshi et al., 2006) a measure of global cognition; The Columbia Autobiographical Memory-Short Form (CUAMI-SF), a widely used measure of retrograde autobiographical memory (Semkovska and McLoughlin, 2013); Part B of the Trail-Making Test (TMT-B), a measure of executive functioning (Lezak, 2012); The Free and Cued Selective Reminding Test (FCSRT), which includes a test of delayed verbal memory (Van der Linden and GREMEM, 2004) and reorientation time following ECT administration.

Fifty-three healthy controls were also recruited as part of the Mem-Dep studies (section 2.4.2) and provided baseline bloods. Exclusion criteria for healthy controls included any history of any psychiatric disorder. Depression severity was assessed with the HDRS-24.

6.2.2 Blood sampling and mRNA extraction

Blood sampling, mRNA extraction and quality analysis was carried out as described in detail in sections 2.3.1-2.3.3. Briefly, fasting whole blood samples for mRNA analysis were taken at baseline and at end of treatment (EOT) for patients treated with ECT using the PaxGene© system (Qiagen Inc., USA). For healthy controls one set of blood samples was taken. mRNA was extracted using the PaxGene© Blood RNA Kit according to the manufacturer's instructions (Qiagen Inc., USA). Quality analysis was carried out on the NanoDrop© 1000 UV-Vis Spectrophotometer (Fisher Scientific, UK) and the Bioanalyzer© 2100 (Agilent Technologies, Ireland) to establish the purity and integrity of RNA respectively.

6.2.3 Multiplex qRT-PCR

mRNA cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as described in detail in section 2.3.8. For the analysis of mRNA gene expression analysis a two-step multiplex quantitative RT-PCR (qRT-PCR) was carried out on the StepOne Plus™ Real-Time PCR system (Applied Biosystems) using TaqMan® (Applied Biosystems, UK) gene expression assays (*VEGFA*: Hs00900055_m1).

Multiplex RT-PCR has the advantage of analysing both the target of interest and an endogenous control in the same reaction. Previous work using the same blood samples had earlier established that *GAPDH* had the highest stability value of five endogenous control genes, and this was therefore chosen as the reference gene (Hs03929097_g1). The comparative C_T method was used to assess gene expression for all qRT-PCR analyses with normalisation to *GAPDH* to minimise quantification errors. The comparative C_T method compares the C_T values of one target gene to another (e.g. an internal control). Additional controls included running wells with no added template, no added reverse transcriptase as well as a random human DNA panel.

6.2.4 Ethics

Written informed consent was obtained from all participants and ethical approval was granted from the local Research Ethics Committee in St. Patrick's University Hospital.

6.2.5 Data analysis

Data analysis was carried out using SPPS version 22.0 (IBM Corporation, NY), and GraphPad Prism 6 (GraphPad Software, CA). Data are presented as means with standard deviation (SD) or number per group (% of group) where appropriate unless otherwise specified. Categorical data were tested using chi-square tests (χ^2) unless otherwise specified. All data were tested for normality and log-transformed where appropriate. Data with normal distribution were analysed using student's t-test or univariate analysis of variance (ANOVA). Where ANOVA analysis indicated significant differences existed, Fisher's least significant difference (LSD) test was applied to investigate specific differences between groups. Non-parametric data were analysed with the Wilcoxon-Signed Rank test for paired comparisons or the Mann-Whitney U test for non-paired comparisons. A p-value of less than 0.05 was considered statistically significant. Where covariates were identified that correlated with the outcome of interest, an analysis of covariance (ANCOVA) was run. Changes over time were investigated with a mixed design ANOVA / General Linear Model (GLM) (repeated-measures) with time (baseline / end of treatment) and psychosis status (yes/no) or other categorical variable as factors. For correlational analysis, data were analysed with Pearson's product-moment correlation coefficient (Pearson's r) for normally distributed variables, and Spearman's rho for non-parametric distributions testing relationships between *VEGFA* levels and clinical or cognitive outcomes. Baseline correlations were explored using multiple regression where appropriate, and end of treatment correlations were explored using ANCOVA to correct for baseline scores. As multiple correlations were tested, significance was set at $p<0.01$.

With regard to power, it is estimated that there is over 80% power to detect a statistically significant difference in mRNA levels between patients and controls with around 26-42 subjects in each group, whereas for

repeated measures tests, 16 subjects are required to have 80% power. These calculations are based on results from two previous peripheral blood mRNA studies involving *VEGFA*, and alpha error of 5% (Berent et al., 2014, Iga et al., 2007).

With regard to correlational analysis, our group have previously reported medium-large mean effect sizes (e.g. -1.1) for sub-acute impairments in episodic memory and executive functioning soon after completing ECT (Semkovska and McLoughlin, 2010). ECT has a similar therapeutic effect size (0.91) (UK ECT Review Group, 2003). Sample sizes of at least 60 per group are sufficient to achieve a power of 80% with up to six predictors to detect a medium to large correlation (Harris, 2001).

6.3 Results

6.3.1 Participant details

Demographic and baseline clinical characteristics are listed in Table 6.1 below. EFFECT-Dep Trial patients were matched to healthy controls on the basis of gender (χ^2=0.957, p=0.620) and socio-economic group (χ^2=14.233, p=0.076). The EFFECT-Dep Trial patients were older (t=2.670, p=0.008), more depressed (t=29.438, p<0.001), consumed less alcohol (U=5.761, p=<0.001) but were more likely to be smokers (χ^2=12.426, p<0.001) than their healthy control counterparts. Alcohol consumption may have been lower in the EFFECT-Dep Trial patients as they were all hospital in-patients at the time of recruitment.

Of note, higher age has been associated with lower *VEGFA* mRNA levels in skeletal muscle and peripheral nerves (Wagatsuma, 2006, Pola et al., 2004). However, the relationship is not clear, as higher age has elsewhere been associated with elevated *VEGFA* levels in rat hepatocytes (Kang et al., 2005). With regard to alcohol consumption, this has been shown to increase *VEGFA* mRNA and VEGF-A protein expression in melanomas in mice (Tan et al., 2007). With regard to smoking, two separate studies in humans (one in villous tissue, one in serum) have shown that smoking does not appear to affect *VEGFA* levels (Kawashima et al., 2014, Berent et al., 2014).

Variable	EFFECT-Dep Group (n=97)	Healthy Controls (n=53)	Formal test
Age, mean (SD), range	56.2 (14.3)	49.7 (14.4)	t=2.670 p=0.008
Gender, female n (%)	60 (61.9)	37 (69.8)	χ^2=0.957 p=0.620
Socio-economic Group, n (%)			
1	21 (21.6)	15 (28.8)	
2	11 (11.3)	13 (25.0)	χ^2=14.233
3	25 (25.8)	11 (19.2)	p=0.076
4	23 (23.7)	5 (9.6)	
5	17 (17.5)	8 (15.4)	
Alcohol consumption, median units per week, (range)	0 (0-40)	8 (0-35)	U=5.761 p=<0.001
Smokers, No. (%)	41 (39.4)	7 (13.5)	χ^2=12.426 p<0.001
HDRS Baseline	30.0 (6.4)	2.9 (2.2)	t=29.438 p<0.001
Episode duration, median weeks, (range)	20.0 (4-104)	n/a	n/a
Treatment-resistant, n (%)	69 (71.1)	n/a	n/a
Previous ECT, n (%)	27(27.8)	n/a	n/a
Psychotic subtype, n (%)	21 (21.6)	n/a	n/a
Bipolar depression, n (%)	22 (22.7)	n/a	n/a
Psychotropic medications, n			
SSRI	26 (26.8)	n/a	n/a
SNRI	39 (40.2)		

TCA	29 (29.9)	
Mirtazapine	38 (39.2)	
Lithium	41 (42.3)	
Antipsychotics	65 (67.0)	
BZD	59 (60.8)	
Pregabalin	10 (10.3)	
Hypnotic^	51 (52.6)	
Valproate	8 (8.2)	
Trazodone	6 (6.2)	
Buproprion	4 (4.1)	
Lamotrigine	9 (9.3)	
MAOI	1 (1.0)	

Table 6.1 Demographic and clinical details of patients and controls
BZD: Benzodiazepines; HDRS: Hamilton Depression Rating Scale; MAOI: Monoamine oxidase inhibitor; n/a: not applicable; SNRI: Serotonin and noradrenaline reuptake inhibitor; SSRI: Selective serotonin reuptake inhibitor; TCA: Tricyclic antidepressants. Episode duration capped at 104 weeks. Treatment resistance measured using Antidepressant Treatment History Form (ATHF); ^ Zopiclone, Zolpidem, Lormetazepam or Triazolam.

The primary aim of this study was to explore differences in peripheral blood *VEGFA* mRNA levels in those with and without psychotic depression treated with ECT. A comparison between these two groups can be found in Table 6.2 below. The patients with psychotic depression did not differ significantly from non-psychotic patients in terms of age, gender, socio-economic group, alcohol consumption, smoking status, episode duration, polarity of depression or ECT electrode placement. Psychotic patients did however have a higher baseline depression severity level, higher pre-morbid intelligence and lower levels of treatment-resistance.

Variable	Psychotic depression (n=21)	Non-psychotic depression (n=76)	Formal test
Age, mean (SD), range	61.4 (12.1)	54.8 (14.6)	t=-1.924 p=0.057
Gender, female n (%)	12 (57.1)	48 (63.2)	χ^2=0.252 p=0.621
Socio-economic Group, n (%)			
1	4 (19.0)	17 (22.4)	
2	4 (19.0)	7 (9.2)	χ^2=2.609
3	6 (28.6)	19 (25.0)	p=0.625
4	3 (14.3)	20 (26.3)	
5	4 (19.0)	13 (17.1)	
Pre-Morbid IQ, mean (SD)	113.4 (5.9)	108.0 (6.8)	t=-0.273 p=0.008
Alcohol, median units per week (range)	0 (0-40)	0 (0-20)	U=-0.082 p=0.935
Smokers, No. (%)	6 (28.6)	35 (46.1)	χ^2=2.061 p=0.213
HDRS Baseline	34.9 (8.7)	28.6 (4.9)	t=-4.279 p=0.004
Episode duration, median weeks, (range)	14 (7-104)	20 (4-104)	U=-0.443 p=0.658
Electrode placement, unilateral, n (%)	12 (57.1)	31 (40.8)	χ^2=1.783 p=0.219
Treatment resistant depression*, n (%)	5 (23.8)	64 (84.2)	χ^2=29.234 p<0.001
Bipolar depression, n (%)	5 (23.8)	17 (22.4)	χ^2=0.019 p=0.549

Psychotropic medications, n			
SSRI	7 (33.3)	19 (25.0)	$
SNRI	8 (38.1)	31 (40.8)	
TCA	4 (19.0)	25 (32.9)	
Mirtazapine	7 (33.3)	31 (40.8)	
Lithium	9 (42.9)	32 (42.1)	
Antipsychotics	17 (81.0)	49 (64.5)	
BZD	14 (66.7)	41 (53.9)	
Pregabalin	0 (0.0)	10 (13.2)	
Hypnotic	11 (57.1)	40 (52.6)	
Valproate	1 (4.8)	7 (9.2)	
Trazodone	1 (4.8)	5 (6.6)	
Buproprion	1 (4.8)	3 (3.9)	
Lamotrigine	3 (14.3)	6 (7.9)	

Table 6.2 Comparison of psychotic and non-psychotic patients
Demographic and clinical comparison of psychotic and non-psychotic patients receiving ECT. BZD: Benzodiazepines; HDRS: Hamilton Depression Rating Scale; IQ: Estimated by National Adult Reading Test; n/a: not applicable; SNRI: Serotonin and noradrenaline reuptake inhibitor; SSRI: Selective serotonin reuptake inhibitor; TCA: Tricyclic antidepressants; * Treatment resistance as measured by Antidepressant Treatment History Form; $: For descriptive purposes, no statistical testing performed. Pre-Morbid IQ assessed using National Adult Reading Test (NART).

6.3.2 mRNA quality assessment

The purity of RNA in the samples was assessed by measuring the absorbance ratio at 260/280 (A260/280) nm on the NanoDrop© 1000 UV-Vis Spectrophotometer. A minimally accepted threshold of 1.8 was set for A260/280 values (Farrell, 2010). With regard to RNA integrity, all samples had a RIN value above 7.0, indicating excellent quality.

6.3.3 *VEGFA* mRNA expression at baseline

As age and alcohol have been associated with differential *VEGFA* expression and differed between groups the effect of these variables on baseline mRNA expression were explored. Age was weakly, but significantly, correlated with baseline *VEGFA* levels (Pearson's r =0.142, p=0.047). The number of alcohol units consumed per week was negatively correlated with baseline *VEGFA* levels (Spearman's rho = -0.224, p=0.002). Age and alcohol units per week were therefore retained as covariates. An ANCOVA of baseline *VEGFA* values, with age and alcohol units per week as covariates and patient group (patients/healthy controls) as fixed factors revealed a significant main effect of patient group, F(1,144) = 27.688, p=<0.001, partial eta squared 0.161.

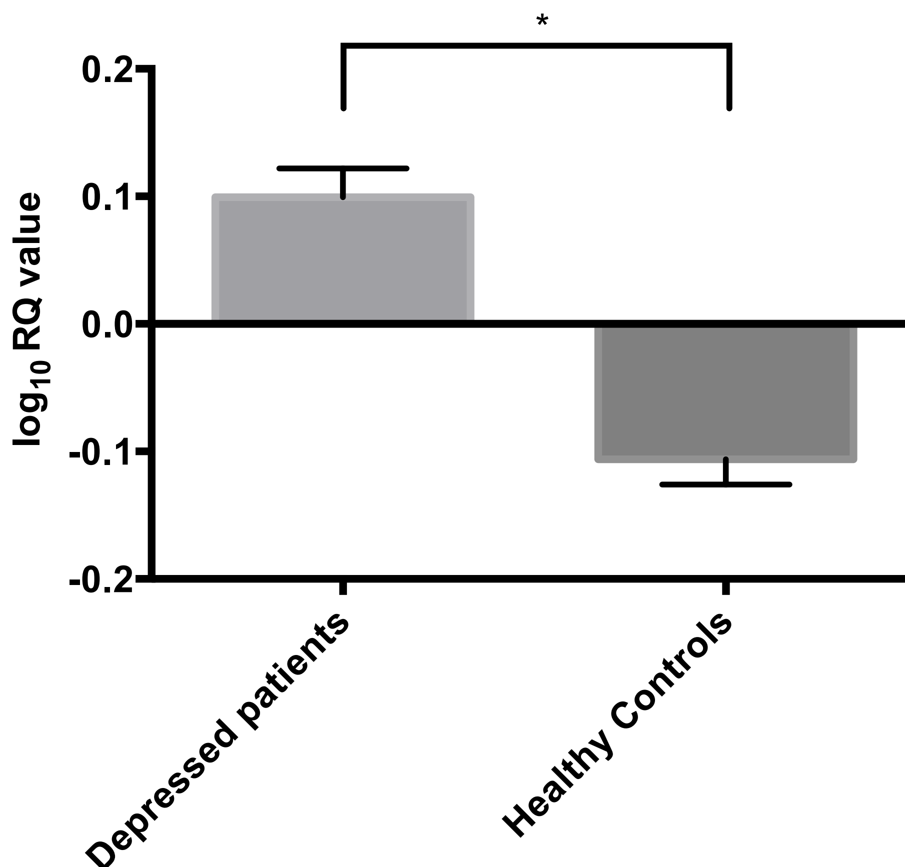


Figure 6.1 Baseline *VEGFA* mRNA expression

Relative quantification (RQ) values for depressed patients (n=97), and healthy controls (n=53). *p=<0.001. Data are presented as means and SEM.

There was no main effect of age ($F(1,143)=0.057$, $p=0.812$) or alcohol consumption ($F(1,143)=0.023$, $p=0.880$). There were no significant interaction terms between patient group type and the covariates.

When the subgroup of psychotic patients treated with ECT was analysed separately from the total ECT group, the *VEGFA* levels were found to be even higher (post-hoc tests revealed the psychotic group had significantly higher levels than both the non-psychotic group ($p=0.021$) as well as the healthy groups ($p<0.001$). Non-psychotic patients also had significantly higher *VEGFA* levels than healthy controls ($P<0.001$).

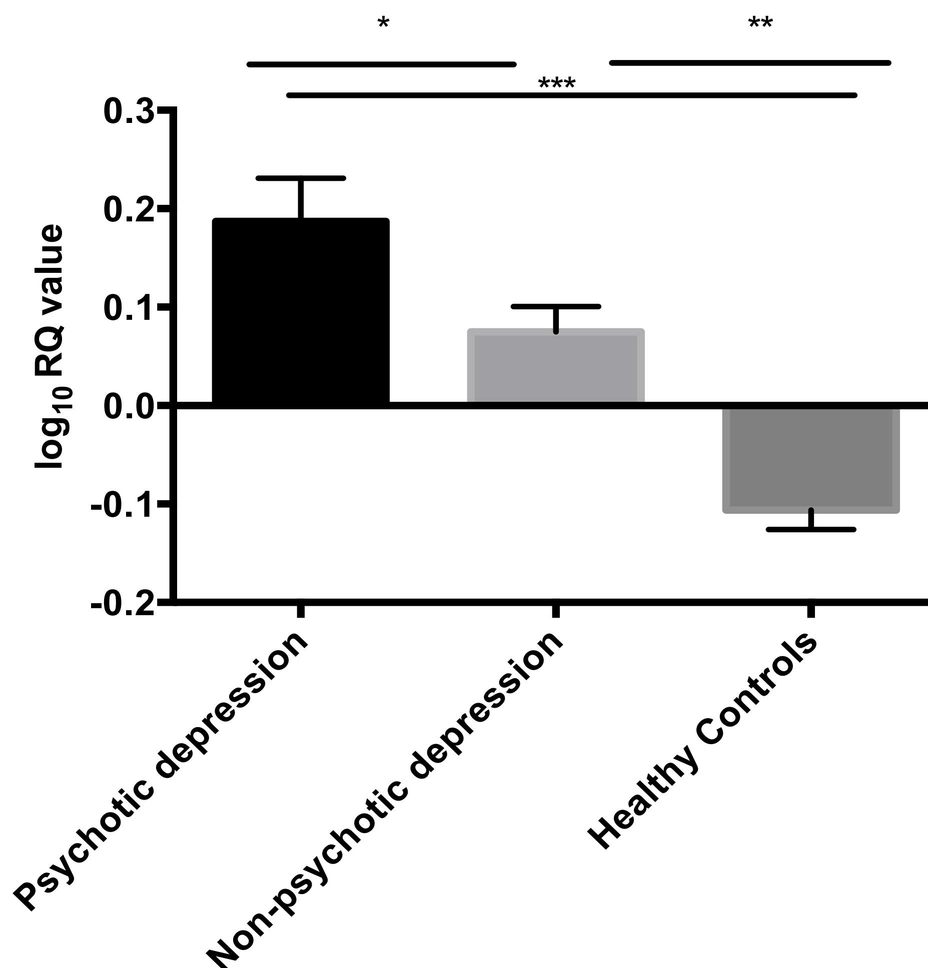


Figure 6.2 Baseline *VEGFA* mRNA expression by psychosis status
RQ values for psychotic depression treated with ECT (n=21), non-psychotic depression treated with ECT (n=76) and healthy controls (n=53) are shown. * $p=0.021$ vs. non-psychotic group, ** $p<0.001$ vs. control group. ***$p<0.001$ vs. control group. Data are presented as means and SEM.

The two ECT (psychotic and non-psychotic depression) groups differed significantly with regard to baseline depression severity (HDRS-24), pre-morbid IQ (NART) and antidepressant treatment resistance (ATHF) NART scores did not correlate with baseline *VEGFA* levels (Pearson's r=-0.004, p=0.972). Treatment resistance was not associated with any significant difference in *VEGFA* levels (t=1.274, p=0.206). Depression severity alone correlated significantly with baseline *VEGFA* levels (Pearson's r=0.302, p=0.003) and therefore this was entered as a covariate in an ANCOVA model with psychosis as a fixed factor. This ANCOVA indicated a significant main effect of baseline depression severity, F(1,94) = 5.950, p=0.017, but no significant effect of psychosis status, F(1,94) = 0.948, p=0.333. *VEGFA* levels did not differ on the basis of the polarity of depression (t=-1.752, p=0.083).

6.3.4 *VEGFA* mRNA expression after ECT

Changes over time were investigated with a mixed design ANOVA / General Linear Model (GLM) (repeated-measures) with time (baseline / end of treatment) and psychosis status (yes/no) as factors. This revealed a significant effect on time (F(1,76)=7.105, p=0.009) and also a significant time x psychosis interaction (F(1,76)=7.340, p=0.008), see Figure 6.3. Post-hoc analysis indicated that there was no significant change in *VEGFA* levels in the non-psychotic group (t=-0.046, p=0.963), but a significant decrease in *VEGFA* levels was observed in the psychotic group after ECT (t=2.915, p=0.010), see Figure 6.4 below. Polarity of depression had no effect on *VEGFA* expression from baseline to end of treatment (F(1,76) =2.066, p=0.155).

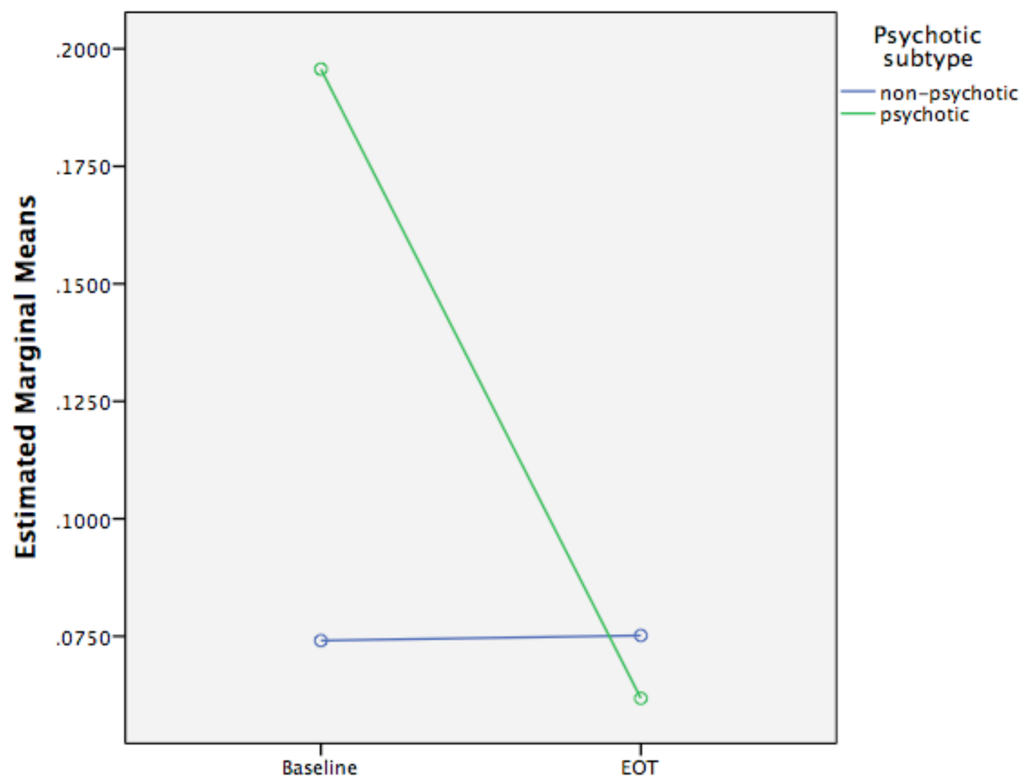


Figure 6.3 Interaction plot of *VEGF-A* levels

Interaction plot of *VEGF-A* levels before and after a course of ECT in psychotic and non-psychotic patients. The plot shows that *VEGFA* levels do not change significantly for non-psychotic patients (blue line), whereas for psychotic patients (green line) there is a significant decrease.

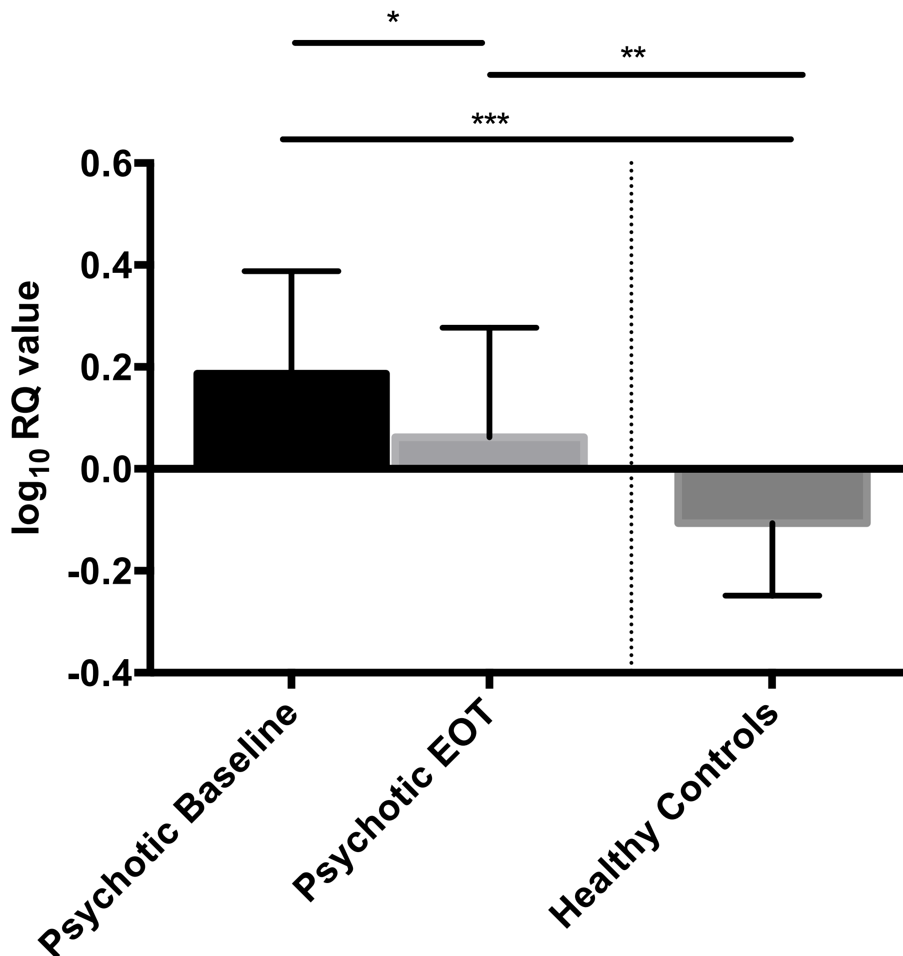


Figure 6.4 *VEGF-A* mRNA levels over time

Relative quantification (RQ) values for psychotic depression before and after ECT (n=21) and healthy controls (n=53). * Psychotic baseline vs. psychotic EOT p=0.001, ** Psychotic EOT vs. healthy controls p=<0.001, *** Psychotic baseline vs. healthy controls p=<0.001. Data are presented as means and SEM.

6.3.5 *VEGFA* responders versus non-responders

Responders were defined as those who at end of treatment achieved a ≥60% decrease in HDRS-24 from baseline and a final score ≤16. The number of responders in the EFFECT-Dep Trial group was 53 (54.6%). Baseline levels of *VEGFA* did not differ between responders and non-responders (t=0.365, p=0.716). There was no difference in *VEGFA* levels from baseline to end of treatment based on responder status; GLM repeated measures between subjects F(1,76)=0.147, p=0.703. The number of

responders in the psychotic group was 15 (71.4%) and 50.0% in those with non-psychotic depression (χ^2=3.048, p=0.081). There was no difference in baseline *VEGFA* levels between responders and non-responders in the psychotic subgroup (t=0.025, p=0.980). Equally, there was no significant difference in *VEGFA* levels from baseline to end of treatment based on responder status in the psychosis group, GLM repeated measures between subjects F(1,16)=0.018, p=0.896.

6.3.6 Role of antipsychotic medication in *VEGFA* expression

Given the observed differences in *VEGFA* levels due to the presence of psychosis, the potential impact of antipsychotic medications was explored. There was no statistically significant difference in the proportion of patients on antipsychotics (psychotic group n=17 (81.0%), non-psychotic group n=49 (64.5%), χ^2= 2.055, p=0.192). There was no significant difference in *VEGFA* levels at baseline (t=-1.683, p=0.096) or in change in *VEGFA* levels from baseline to EOT (t=0.947, p=0.347) based on antipsychotics being prescribed.

6.3.7 Correlational analyses

6.3.7.1 Depression severity and *VEGFA* levels

At baseline, depression severity correlated weakly, but significantly with *VEGFA* levels, (Pearson's r=0.302, p=0.003). In the psychotic subgroup no significant correlation between baseline depression severity and *VEGFA* levels was observed (Pearson's r=0.175, p=0.448). To assess whether baseline levels of *VEGFA* could predict changes in depression they were correlated with change in depression scores as measured by the HDRS-24. However, this correlation failed to reach statistical significance (Pearson's r=-0.195, p=0.055). There was also no significant correlation between baseline *VEGFA* levels and change in depression scores in the psychotic subgroup (Pearson's r=0.061, p=0.792).

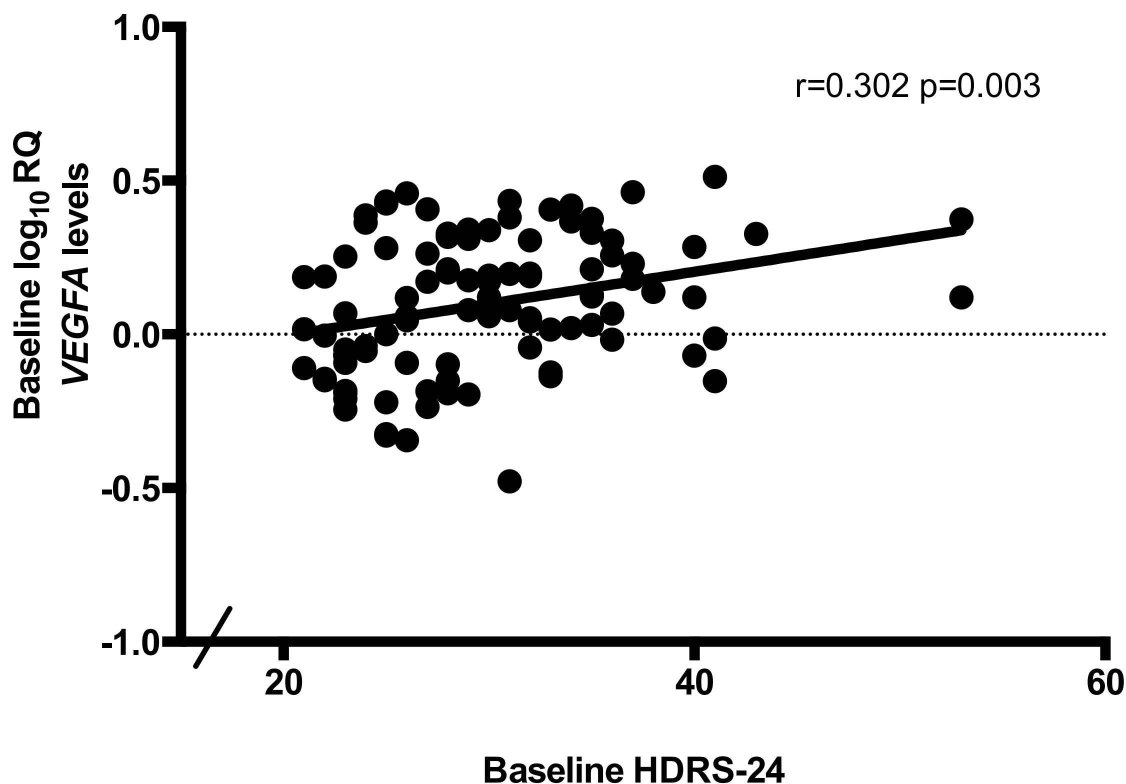


Figure 6.5 Correlations: baseline depression severity
Correlation between baseline depression severity and *VEGFA* levels in depressed patients (n=97).

In depressed patients, there was also no significant correlation between the change in depression scores and the change in *VEGFA* mRNA in either the main group (Pearson's r = -0.096, p=0.402), or in the psychotic subgroup (Pearson's r=-0.096, p=-0.402).

6.3.7.2 Psychotic symptomatology and *VEGFA* levels

Psychotic psychopathology severity was measured using five items (hallucinations, unusual thought content (delusions), suspiciousness, emotional withdrawal and blunted affect (BPRS-5)) from the Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham, 1962). Mean baseline BPRS-5 scores in the psychotic group were 11.86 (SD 3.23), which had lowered to 6.24 (SD 2.14) at end of treatment.

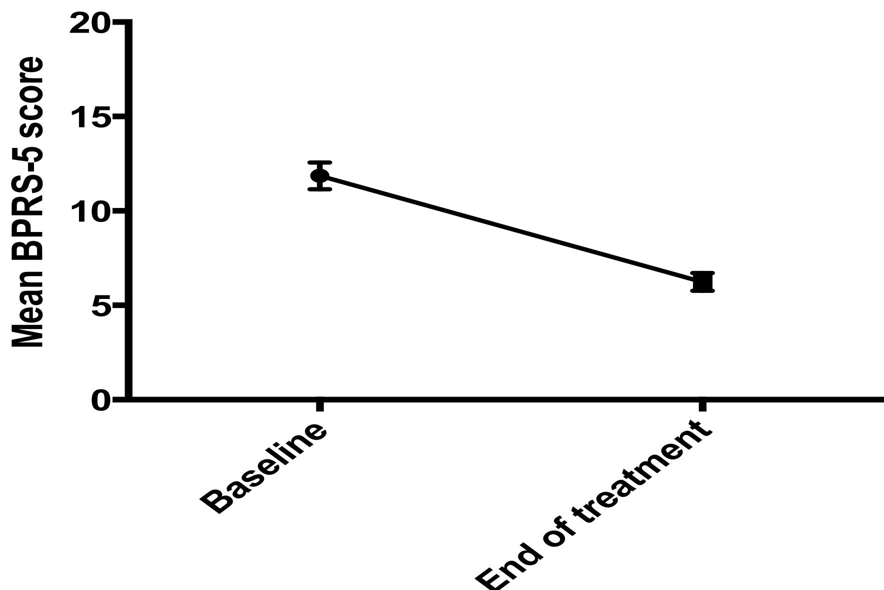


Figure 6.6 BPRS-5 Scores following ECT

Change in BPRS-5 scores after ECT in patients with psychotic depression (n=21). Data are means with SEM.

At baseline, BPRS-5 scores moderately correlated with *VEGFA* levels (Pearson's r=0.505, p=0.020).

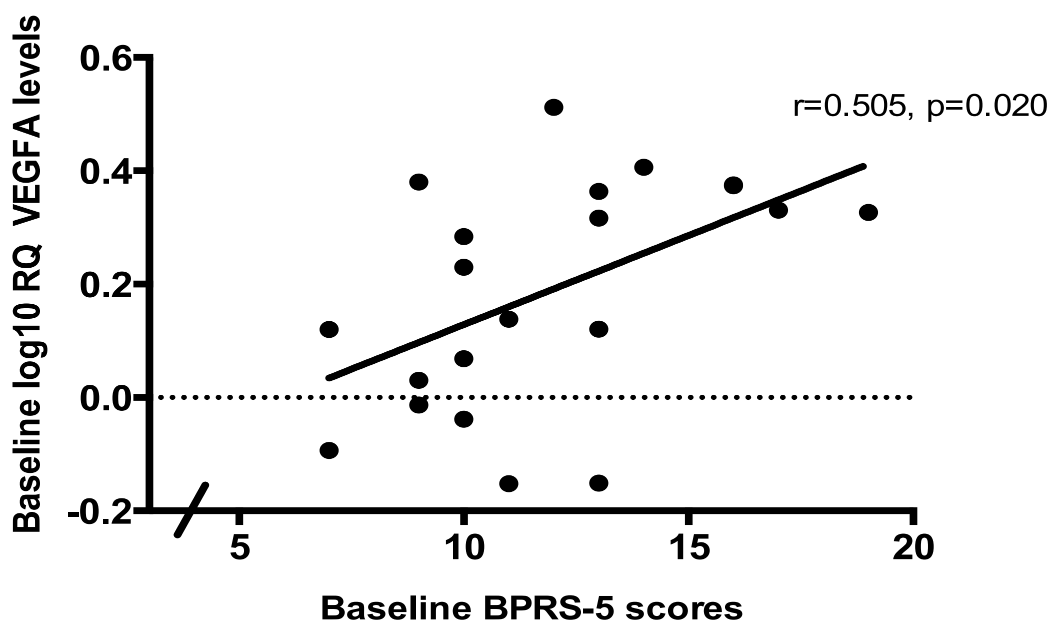


Figure 6.7 Correlations: *VEGFA* and BPRS-5

Correlations between baseline *VEGFA* levels and BPRS-5 scores in patients with psychotic depression (n=21)

To assess whether baseline levels of *VEGFA* could predict changes in psychotic symptomatology they were correlated with change in BPRS-5 scores. The two measures were significantly correlated (Pearson's r=-0.473, p=0.031).

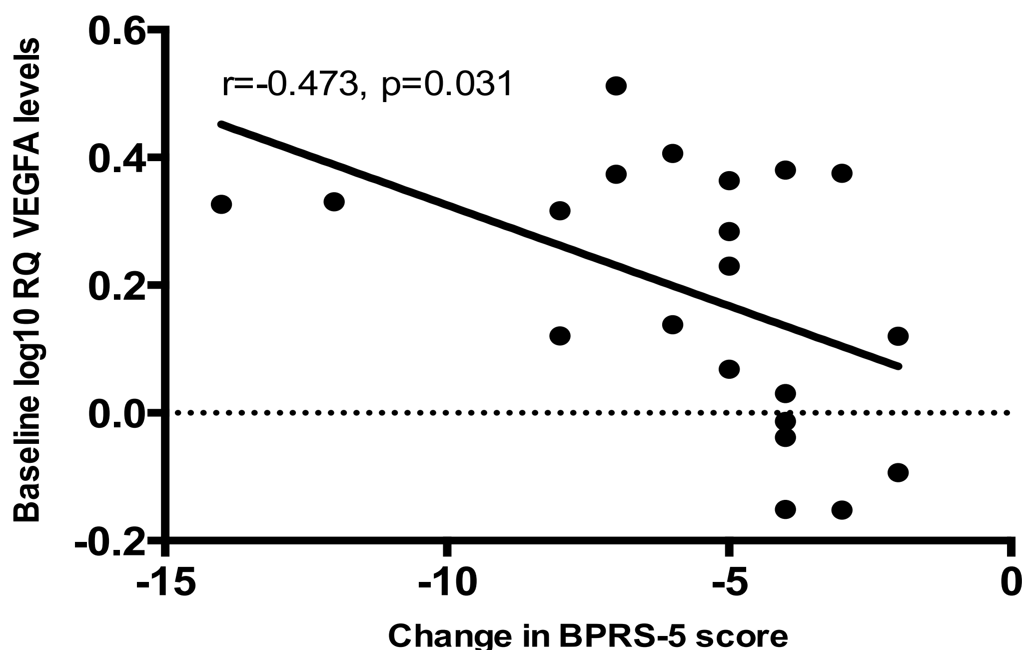


Figure 6.8 Correlations: Baseline *VEGFA* and change in BPRS-5
Correlations between baseline *VEGFA* levels and change scores on BPRS-5 in psychotic patients (n=21).

Both baseline depression severity (as measured by HDRS-24) and psychotic symptomatology severity (as measured by the BPRS-5) were significantly correlated with *VEGFA* baseline levels. Baseline HDRS-24 and BPRS-5 scores, however, were themselves not significantly correlated (Pearson's r=0.095, p=0.682). The HDRS-24 (see section 2.2.2.2) covers a wide range of depressive symptoms, only some of which tap into psychotic symptomatology, as reflected by the lack of correlation on the two measures in this psychotic subgroup of patients. To explore the association between baseline *VEGFA* levels and baseline scores on the HDRS-24 and BPRS-5, I performed a linear regression analysis with baseline *VEGFA* as the dependent variable and baseline HDRS-24, BPRS-5 and age as independent variables. This model significantly predicted baseline *VEGFA* levels

(F=3.631, p=0.034, R^2=0.391). The individual coefficients for the independent variables was significant for BPRS-5 (Beta:0.027, p=0.039), but not for HDRS-24 (Beta:0.001, p=0.845) or age (Beta:0.006, p=0.086). Change in BPRS-5 scores did not correlate significantly with change in *VEGFA* levels (Pearson's r=0.346, p=0.160).

6.3.7.3 Cognitive variables and *VEGFA* levels

6.3.7.3.1 General cognition and *VEGFA* levels

General cognition was measured using the total score on the Addenbrooke's Cognitive Examination-R, which provides a global measure of cognition. At baseline, ACE-R scores differed significantly between EFFECT-Dep Trial patients (n=80) and healthy controls (n=46) (t=-6.963, p<0.001).

In EFFECT-Dep Trial patients, a multiple regression was run to predict baseline ACE-R scores from pre-morbid intelligence (NART), depression severity, electrode placement, presence of psychosis and baseline *VEGFA* levels. Pre-morbid intelligence has previously been shown to be correlated with performance on tests of general cognition (Alves et al., 2013). Although the model significantly predicted ACE-R scores (F=2.659, p=0.030) it did not explain a large amount of the variance in ACE-R scores (R^2=0.166). Neither the presence of psychosis (p=0.750) or baseline *VEGFA* levels (p=0.550) contributed significantly to the model indicating a lack of correlation of these variables with ACE-R levels. Only pre-morbid intelligence contributed significantly to the model (p=0.002). Controlling for pre-morbid intelligence there was no significant correlation between baseline ACE-R scores and *VEGFA* levels (see Figure 6.9).

With regard to changes over time, an ANCOVA of end of treatment ACE-R values, with baseline ACE-R scores and EOT *VEGFA* levels as covariates and electrode placement and presence of psychosis as fixed factors revealed a significant main effect of baseline ACE-R levels, F(1,57) = 14.526, p=<0.001. There was no main effect of psychosis, F(1,57) = 0.256, p=0.615, electrode placement, F(1,57) = .345, p=0.560, or *VEGFA* levels, F(1,57) = 0.547, p=0.463. There were no significant interaction effects.

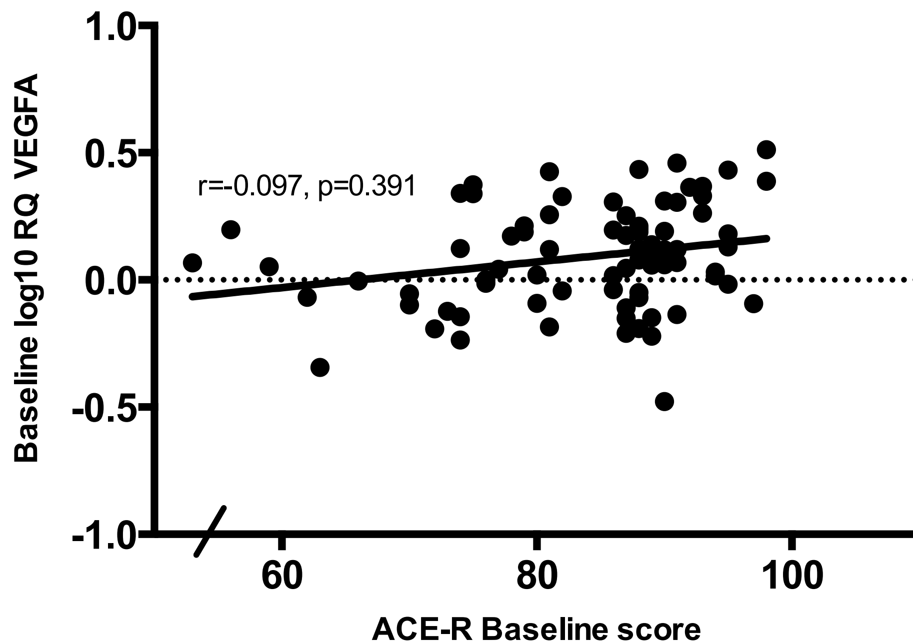


Figure 6.9 Correlations: ACE-R and *VEGFA*

Correlations between ACE-R scores and *VEGFA* levels at baseline (n=80).

6.3.7.3.2 Autobiographical memory and *VEGFA* levels

At baseline, scores on the Columbia Autobiographical Memory-Short Form (CUAMI-SF) differed significantly between patients (n=91) and healthy controls (n=18), (t=-3.621, p<0.001). Baseline CUAMI-SF scores were comparable between psychotic (n=18) and non-psychotic (n=78) patients (t=1.015, p=0.321). Initial multiple regression modelling failed to identify a significantly predictive model and there was no significant correlation between baseline CUAMI-SF and *VEGFA* levels (Pearson's r=-0.169, p=0.080). At end of treatment, the CUAMI-SF measures recall consistency as a percentage of baseline performance rather than being directly compared to baseline scores. There was no difference in end of treatment scores on the CUAMI-SF between psychotic and non-psychotic groups (t=-0.681, p=0.498). There was no significant correlation between *VEGFA* levels and scores on the CUAMI-SF (corrected for depression severity) at end of treatment (Pearson's r=0.027, p=0.824) in the group as a whole or for the psychotic subgroup (Pearson's r=-0.024, p=0.935).

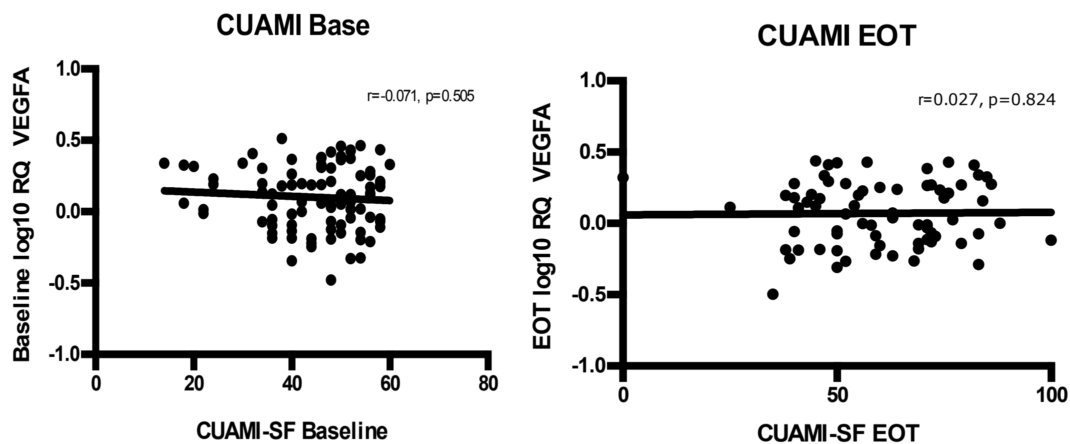


Figure 6.10 Correlations: CUAMI-SF and *VEGFA*

Correlations between CUAMI-SF scores and *VEGFA* levels at baseline (n=91) and EOT (n=90).

6.3.7.3.3 Trail-making test-B and *VEGFA* levels

At baseline, TMT-B scores differed significantly between patients (n=65) and healthy controls (n=40), (t=5.075, p<0.001). Initial multiple regression modelling failed to identify a predictive model beyond baseline depression scores. Due to missing values for this outcome, the number of subjects with psychotic depression (n=11) was too low to make any meaningful correlations. A partial correlation analysis was therefore carried out of baseline TMT-B scores and *VEGFA* levels corrected for baseline depression severity. This indicated there was no significant correlation between *VEGFA* levels and TMT-B scores (corrected Pearson's r=-0.021, p=0.837).

With regard to changes over time, an ANCOVA of end of treatment TMT-B values, with baseline TMT-B scores, EOT *VEGFA* levels and EOT depression severity as covariates, and electrode placement and presence of psychosis as fixed factors, revealed a significant main effect of baseline TMT-B levels, F(1,43)=8.299, p=0.007. There was no main effect of psychosis, F(1,43)=0.019, p=0.892, electrode placement, F(1,43)=3.554, p=0.067, or *VEGFA* levels, F(1,43)=2.302, p=0.138. There were no significant interaction effects.

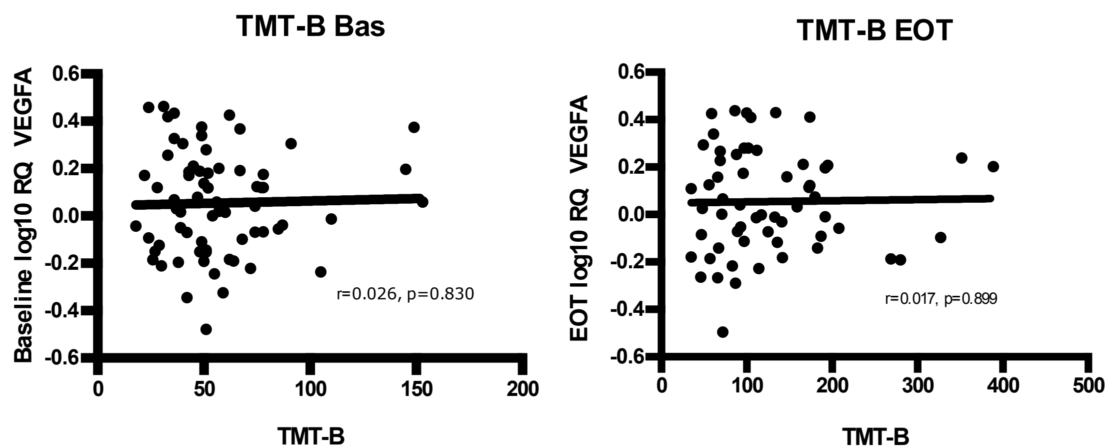


Figure 6.11 Correlations: TMT-B and *VEGFA*

Correlations between TMT-B scores and *VEGFA* levels at baseline (n=65) and EOT (n=74).

6.3.7.3.4 FCSRT and *VEGFA* levels

Baseline scores on the Free and Cued Selective Reminding Test (FCSRT) were significantly poorer in EFFECT-Dep Trial patients (n=66) compared to healthy controls (n=11), t=-3.688, p<0.001. An initial multiple regression failed to identify any significant explanatory variables. Neither the presence of psychosis (p=0.664) nor baseline *VEGFA* levels (p=0.518) contributed significantly to the model, indicating a lack of correlation of these variables with FCSRT levels.

With regard to changes over time, an ANCOVA of end of treatment FCSRT values, with baseline FCSRT scores, age and EOT *VEGFA* levels as covariates and electrode placement and presence of psychosis as fixed factors revealed a significant main effect of baseline FCSRT levels, F(1,57)=17.063, p=<0.001. There was no main effect of psychosis (F(1,57)=0.288, p=0.594), electrode placement (F(1,57)=1.321, p=0.684), age (F(1,57)=0.192, p=0.663) or *VEGFA* levels (F(1,57)=1.564, p=0.217). There were no significant interaction effects. There were no significant correlations between baseline (r=-0.110, p=0.271) or EOT (r=0.063, p=0.650) *VEGFA* levels and their respective FCSRT scores.

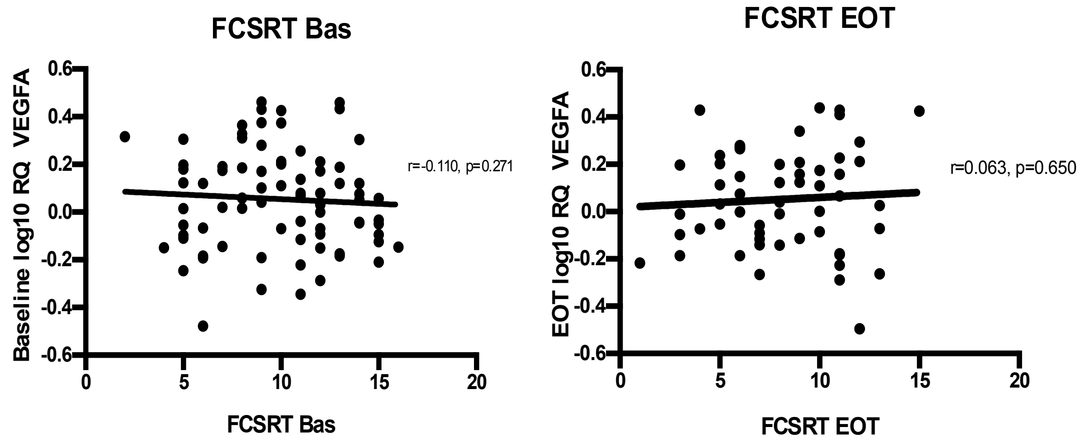


Figure 6.12 Correlations: FCSRT and *VEGFA*

Correlations between FCSRT (delayed) scores and *VEGFA* levels at baseline (n=66) and EOT (n=73).

6.3.7.3.5 Reorientation time

Median time to reorientation after ECT in the EFFECT-Dep Trial group was 22.5 minutes (range 10-60, capped at 60 minutes). There was no significant difference in reorientation time by psychosis (Mann Whitney Z = -0.289, p=0.773), but a quicker recovery time was observed with unilateral electrode placement (median recovery time 18.85 versus 25.50 minutes, Mann Whitney Z = 2.596, p=0.009). There was no significant correlation between reorientation times and baseline *VEGFA* levels (Spearman's rho = -0.069, p=0.505) or end of treatment *VEGFA* levels (Spearman's rho = -0.192, p=0.095).

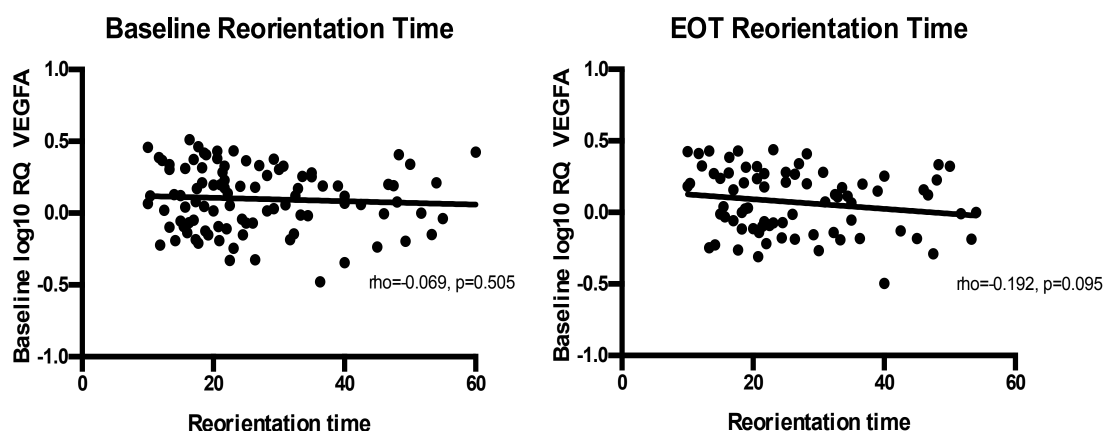


Figure 6.13 Correlations: Reorientation time and *VEGFA*

Reorientation time measured in minutes, n=96.

6.4 Discussion

6.4.1 *VEGFA* analysis - introduction

This study of *VEGFA* mRNA expression in peripheral blood included 97 hospital in-patients treated with ECT and 43 healthy controls. The groups were matched in terms of gender and socio-economic group, but differed in that the ECT group tended to be older, consumed less alcohol and were more likely to be smokers than healthy controls.

As the microRNA changes observed in Chapter 4 and Chapter 5 were limited to patients with psychotic depression, this group of patients was also analysed separately. When the psychotic sub-group was compared to the non-psychotic subgroup, psychotic patients had higher baseline depression severity and NART scores (pre-morbid intelligence). However, they were less likely to meet criteria for treatment resistant depression, which may be due to ECT being used as an earlier treatment option in psychotic depression as recommended by various international guidelines (Leadholm et al., 2013). Of the ECT sample, 21.6% had psychotic depression, in line with epidemiological literature which estimates that about 20% of depressed patients will have a psychotic subtype of depression (Ohayon and Schatzberg, 2002). Variables that differed between groups were used as potential covariates in the expression analyses.

6.4.2 Baseline *VEGFA* expression

At baseline, *VEGFA* levels were significantly higher in the EFFECT-Dep Trial patients compared to healthy controls. For the psychotic subgroup, *VEGFA* levels were significantly higher than the non-psychotic ECT group. As the psychotic sub-group had higher depression severity, this was used to adjust *VEGFA* levels in an ANCOVA, which removed the significant difference between psychotic and non-psychotic groups referred for ECT. This suggested that baseline depression scores were correlated with *VEGFA* levels, and this was supported by correlational analysis, indicating a weak correlation between baseline depression severity and baseline *VEGFA* levels,

(Pearson's r=0.302, p=0.003). This correlation was not seen in the psychotic sub-group, but was likely to have been underpowered. Significant correlations were seen between baseline psychotic symptomatology scores (BPRS-5) and baseline *VEGFA* levels. Change scores on the BPRS-5 also correlated with baseline *VEGFA* levels, even after correcting for depression severity and age.

In Chapter 4 and Chapter 5 miR-126-3p and miR-106a-5p levels were found to be significantly higher in a small subgroup of patients with psychotic depression compared to healthy controls. Based on typical microRNA-mRNA interactions, where microRNAs negatively regulate their target mRNA one might have expected that *VEGFA* mRNA levels would be lower in patients with psychotic depression compared to controls. However, in this study, the reverse was found. Although miR-126-3p has a binding site in the *VEGFA* mRNA sequence, indicating it can negatively regulate *VEGFA* expression, it has also been shown that through miR-126-3p's interactions with other genes, *VEGFA* expression can actually be increased (Hong et al., 2014, Fish et al., 2008). The interactions between miR-126-3p and *VEGFA* is visualised in Figure 6.14 below using the mirTarBase Tool (Chou et al., 2016). Similarly, miR-106a-5p has an extensive network of target genes that can target *VEGFA* beyond its direct relationship, see Figure 6.15 below. Of note, in breast cancer, miR-106a and *VEGFA* are positively correlated, indicating there are more complex relationships between miR-106a and *VEGFA* than simple direct binding and ensuing negative regulation (KuanHui and Walker, 2011).

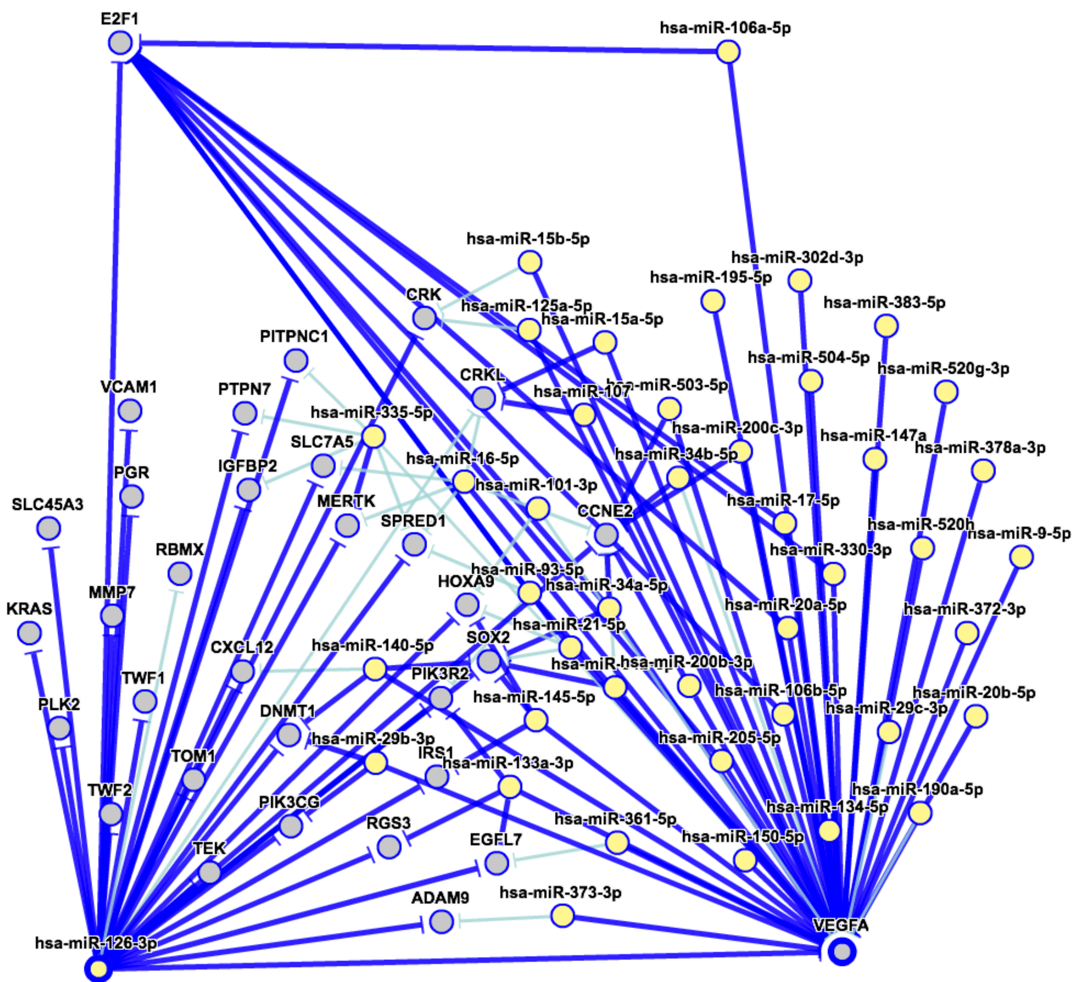


Figure 6.14 MiR-126-3p and *VEGF-A* interactions

MiR-126-3p and *VEGFA* have been placed in the bottom corners for identification. MiR-106a-5p (the other significantly regulated microRNA from earlier work) and *E2F1* (shared gene target of both microRNAs along with *VEGFA)* have been placed in the top corners. Although miR-126-3p directly targets *VEGFA*, it also targets a number of other targets that in turn have the potential to negatively regulate *VEGFA.* One possibility is therefore that the net effect is a positive correlation between miR-126-3p and *VEGFA.* Visualised using mirTarBAse. Blue lines: strong evidence, cyan: weaker evidence.

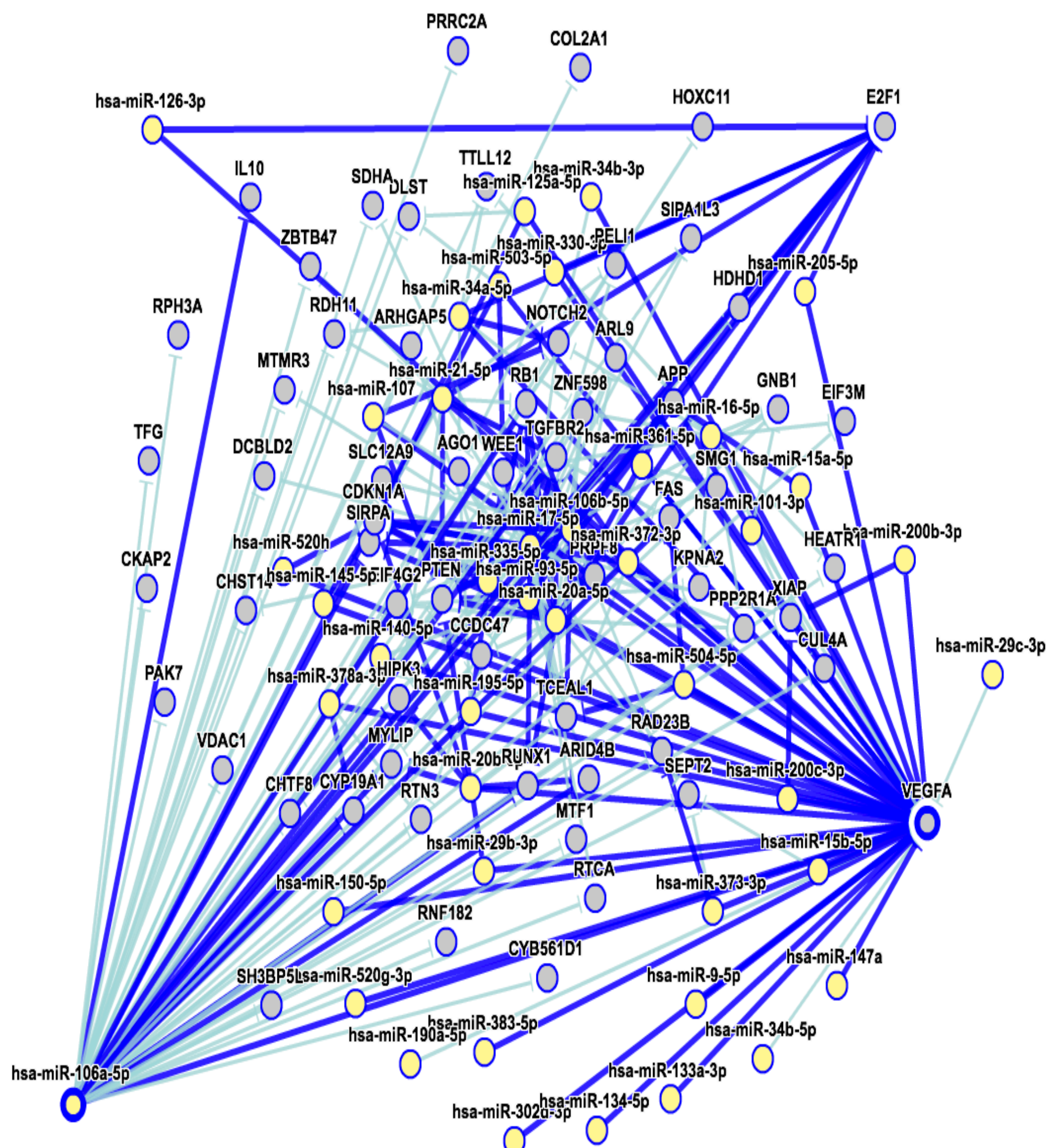


Figure 6.15 MiR-106a-5p and *VEGFA* interactions

MiR-106a-5p and *VEGFA* have been placed in the bottom corners for identification. MiR-126-3p and *E2F1* (shared gene target of both microRNAs along with *VEGFA)* have been placed in the top corners. Although miR-106a-5p directly targets *VEGFA*, it also targets a number of other targets that in turn have the potential to negatively regulate *VEGFA.* The net effect can therefore be a positive correlation between miR-106a-5p and *VEGFA.* Visualised using mirTarBase. Blue lines: strong evidence, cyan: weaker evidence.

6.4.3 *VEGFA* and depression

The indirect relationships between microRNAs and *VEGFA* levels are not just theoretical. Work in animal models has shown that miR-126-3p, which is the most studied of the two microRNAs discussed in this chapter, works in part by inhibiting negative regulators of the *VEGFA* pathway (Fish et al., 2008). In particular, miR-126-3p downregulates *SPRED1* and *PIK3R2*, both negative regulators of the VEGFA signalling pathway (Suarez and Sessa, 2009, Fish et al., 2008, Kuhnert et al., 2008, Wang et al., 2008). In humans, there has been no study to date investigating the relationship between miR-126-3p levels and *VEGFA* levels in depression. However, one study of 115 patients with pre-eclampsia compared to 115 healthy controls found lower levels of both miR-126-3p and *VEGFA* in those with pre-eclampsia (r=0.79) (Hong et al., 2014), indicating a positive correlation. Together, these results indicate that the elevated levels of both miR-126-3p and miR-106a-5p and *VEGFA* is in line with theoretical and experimental findings elsewhere.

With regard to the depression literature, animal models have shown that chronic antidepressant treatment leads to elevated *VEGFA* levels in the hippocampus (Warner-Schmidt and Duman, 2007, Newton et al., 2003). It is therefore possible that the elevated *VEGFA* levels seen in peripheral blood may be due to the effect of antidepressant treatment because nearly all patients (one patient was referred for ECT due to a lack of tolerance of pharmacotherapy) were on antidepressants as well as other psychotropics (see Table 6.1). The differences between psychotic and non-psychotic groups were not likely due to the presence of antipsychotics, as no differences were seen when the groups were analysed on the basis of the presence of antipsychotics. Most groups investigating the role of peripheral *VEGFA* / VEGF-A levels in depression have focused on serum protein levels (VEGF-A). Only two groups have measured peripheral *VEGFA* mRNA levels in depressed subjects compared to healthy controls. The first such study, with 32 depressed subjects and 32 healthy controls found higher levels of *VEGFA* mRNA in the peripheral leucocytes of depressed subjects (Iga et al., 2007). The depressed patients had not received antidepressant therapy prior to the commencement of the study. The second study also found

elevated levels of *VEGFA* mRNA in 38 depressed subjects who were antidepressant-naive compared to 38 healthy controls (Berent et al., 2014).

Studies measuring serum VEGF-A protein levels have shown more variation, but a recent meta-analysis including 872 patients and 882 controls concluded that peripheral blood VEGF-A levels were significantly higher in depressed subjects compared to controls (Hedges's g=0.435, p=<0.001) (Tseng et al., 2015). Of note, a sensitivity analysis of the effect of the presence of antidepressants at baseline (were patients drug-naive or not) did not alter the overall findings. Age was negatively correlated with VEGFA levels and there was evidence of publication bias against small, negative studies. A separate meta-analysis by a different research group with similar numbers (n=1633) reported broadly similar findings (Hedges's g=0.343, p<0.01) (Carvalho et al., 2015). These findings are in contrast to results of other neurotrophic factors, such as BDNF, where deficits are associated with depression (Brunoni et al., 2014, Castren et al., 2007).

With regard to psychosis, no published reports to date were identified that provided data on *VEGFA*/VEGF-A levels in psychotic depression. In a study of first episode psychosis (not depression), serum VEGF-A levels did not differ between cases (n=15) and healthy controls (n=15) (Murphy et al., 2014). At baseline, there was no correlation between VEGF-A levels and BPRS scores. However, following 12 weeks of quetiapine treatment, although there was no significant change in VEGF-A levels from baseline, there was a positive correlation between change in VEGF-A levels and BPRS scores (r=0.382, p=0.024). Another study of first episode psychosis also failed to identify any difference in baseline VEGF-A levels in cases (n=24) and controls (n=24) (Di Nicola et al., 2013). In schizophrenia/ schizoaffective disorder, serum VEGF-A levels were elevated in cases (n=96) compared to healthy controls (n=83) (Pillai et al., 2015). These levels were inversely correlated with prefrontal cortex brain volume and positively correlated with Interleukin-8 levels, suggesting a link between serum VEGF-A and inflammation. In contrast to this study, another recent study of 50 schizophrenia subjects and 50 healthy controls found VEGF-A levels to be significantly lower at baseline, and levels increased following six weeks of antipsychotic treatment (Lee et al., 2015). No genetic

polymorphisms of the *VEGFA* gene have been implicated in schizophrenia to date (Gao et al., 2015).

Together, the findings from the literature are therefore in line with the results of this study comparing patients with psychotic depression to healthy controls.

6.4.4 ROC analysis

To assess whether baseline *VEGFA* levels could differentiate between depressed patients (n=97) and healthy controls (n=53) a Receiver Operating Characteristic (ROC) curve was drawn up. The accuracy of a diagnostic marker is measured by the area under the ROC curve (AUC). An area of 1 would indicate a perfect test, whereas an area of 0.5 represents mere chance. For *VEGFA* levels, the AUC was 0.770, (standard error 0.037), with an estimated sensitivity of 65% and a specificity of 77%, see Figure 6.16 below. As a single biomarker, this would not be of any great clinical utility, but it could be a candidate as one of a panel of biomarkers.

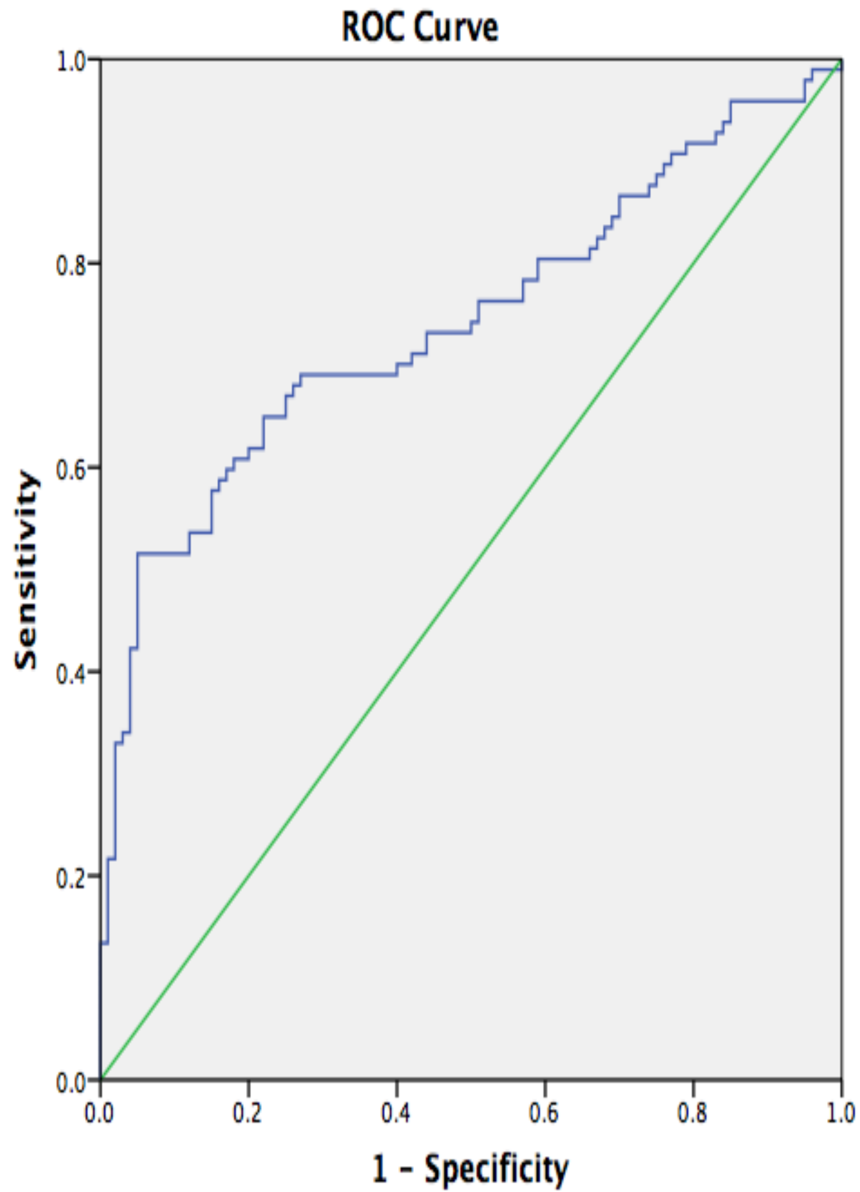


Figure 6.16 ROC Analysis - whole group analysis

ROC of *VEGFA* levels and depressed/control classification. Depressed patients n=97, healthy controls n=53.

When only those with psychotic depression were compared to healthy controls the AUC was slightly improved at 0.868, with a standard error 0.051 ($p<0.001$), and an estimated sensitivity of 81% and a specificity of 73%, see Figure 6.17 below.

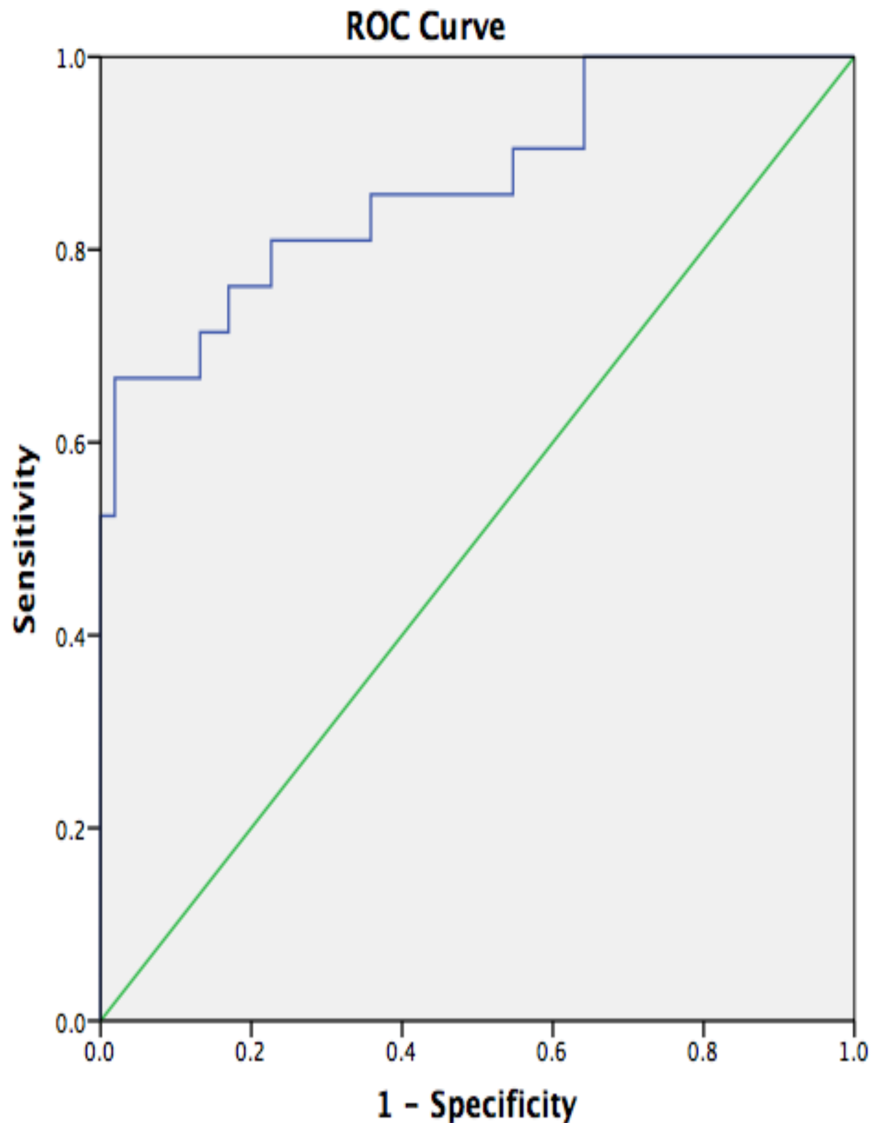


Figure 6.17 ROC Analysis - Psychotic group

ROC of *VEGFA* levels and depressed/control classification in psychotic patients. Psychotic depression n=21, healthy controls n=53

With regard to presence or absence of psychosis on the basis of *VEGFA* levels, a ROC analysis indicated an AUC of 0.641 with a standard error of 0.067 (p=0.049), with low sensitivity (52%) and specificity (58%), see Figure 6.18 below.

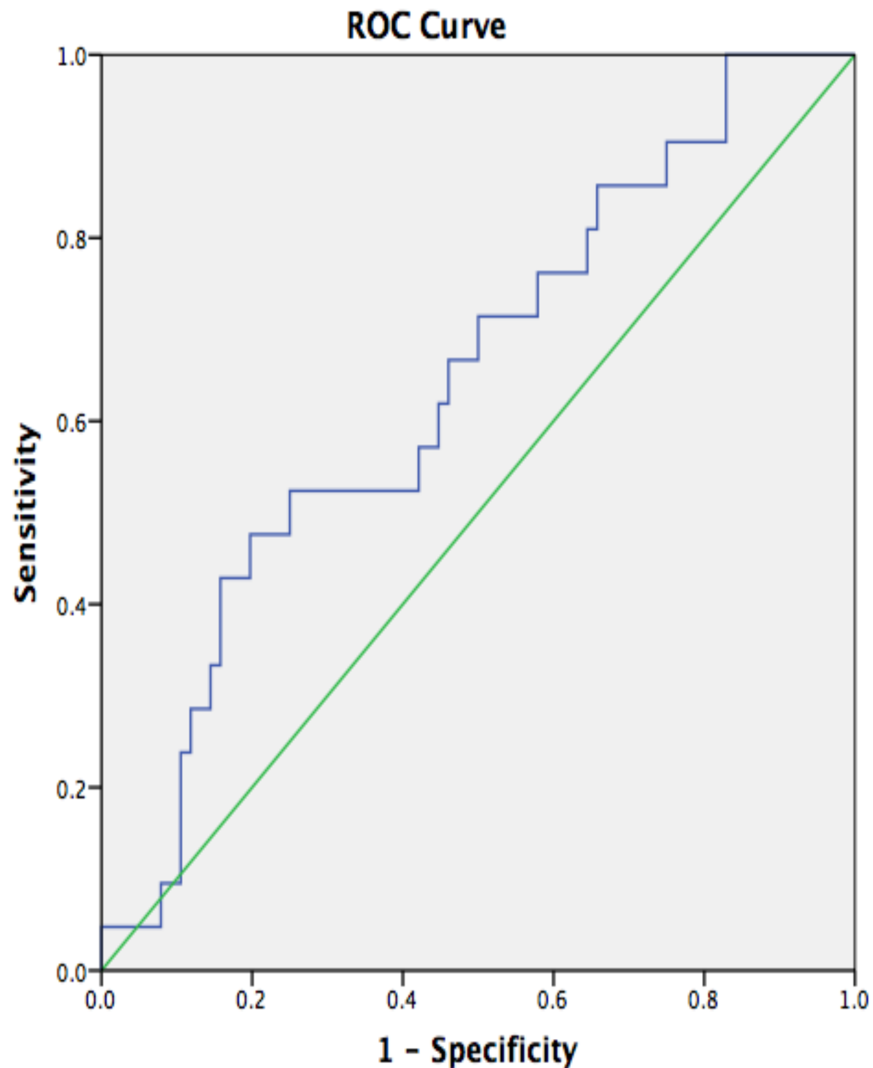


Figure 6.18 ROC Analysis - Psychosis status

ROC of *VEGFA* levels and psychotic/non-psychotic classification. Psychotic patients n=21, non-psychotic patients n=76.

6.4.5 Change in *VEGFA* expression following ECT

Changes in *VEGFA* levels following ECT were significant in the psychotic subgroup of patients only, with a medium to large effect size (Cohen's *d*) of 0.649. At end of treatment, *VEGFA* mRNA levels were closer to, but still significantly higher than healthy controls. These changes were therefore in line with the observed changes in peripheral microRNA levels in patients with psychotic depression treated with ECT. The observed reduction in peripheral *VEGFA* levels differs from animal studies that have suggested an increase in brain levels of *VEGFA* following various antidepressant treatments, including ECS (Warner-Schmidt and Duman, 2007, Newton et al., 2003). Studies of changes in peripheral *VEGFA* mRNA levels are limited, as most studies have focused on VEGF-A protein levels. One study found no change in *VEGFA* levels in peripheral blood following eight weeks of Paroxetine (Iga et al., 2007).

With regard to VEGF-A protein levels, a systematic review (but not meta-analysed) of studies investigating VEGF-A levels in depression indicated a lack of consistency of findings in terms of the effect of antidepressant treatments (including ECT) on VEGF-A levels (Clark-Raymond and Halaris, 2013). In terms of studies investigating the relationship between ECT and VEGF-A, one study found no significant difference in VEGF-A levels between baseline and at end of treatment, but did find a significant difference between VEGF-A levels at baseline and one month after treatment (Minelli et al., 2011). It is therefore possible that if blood samples had been collected at a further time-point beyond end of treatment different results may have been observed in this study.

The results from the present study indicate that peripheral *VEGFA* levels are only altered in a sub-group of patients with psychotic depression, in line with findings from earlier microRNA work. Polarity of depression did not have a significant impact on *VEGFA* changes. No significant changes were seen in non-psychotic depression, in line with other studies of peripheral *VEGFA* mRNA or VEGF-A protein levels. Changes in *VEGFA* levels correlated significantly with changes on a scale of psychotic symptomatology (BPRS-5) but not with a broader depression rating scale (HDRS-24) supporting the psychosis-*VEGFA* interaction.

6.4.6 Correlations with clinical and cognitive outcomes

Baseline depressive and psychotic symptom severity correlated with baseline *VEGFA* levels. Changes in psychotic symptom severity correlated with baseline VEGFA levels only. Otherwise there were no significant relationships between *VEGFA* levels and other clinical or cognitive outcomes. Of note, significant changes in *VEGFA* levels were seen in the psychotic subgroup of patients only. As there were a limited number of psychotic patients (n=21), the correlational analyses involving this group with significant *VEGFA* changes was therefore likely to have been underpowered. Therefore, the results do not eliminate the possibility of a relationship between *VEGFA* levels and other clinical or cognitive outcomes.

6.4.7 Discussion: Summary

In this study, peripheral *VEGFA* levels in patients treated with ECT were found to be higher than in healthy controls, and this difference was even more pronounced for those with psychotic depression. These findings are line with existing evidence from the literature and the results from the previous chapters. Following ECT treatment, there was a significant reduction in *VEGFA* levels in patients with psychotic depression only. This was also in line with results from previous chapters involving microRNAs, but in contrast to some of the limited literature in this area, particularly in contrast to CNS findings in animal models. Of note, animal models of depression do not include psychotic depression, which is difficult to represent in a behavioural model (Koch, 2006).

A limitation of this study is that *VEGFA* levels were from a peripheral source whereas depression is a neuropsychiatric illness, and it is still unclear to what extent peripheral changes reflect central nervous system changes. The only study of cerebrospinal fluid (CSF) and *VEGFA* or VEGF-A product involved 43 medication-free suicide completers and 20 healthy controls (Isung et al., 2012a). CSF levels of VEGF-A were lower in cases compared to controls in contrast to the evidence from peripheral sources. Serum VEGF-A levels did not correlate with CSF VEGF-A levels. Lower levels

were also associated with a higher risk of completing suicide in the future (Isung et al., 2012b). However, from a practical point of view, a biomarker based on cerebrospinal fluid (CSF) is unlikely to be acceptable to patients and a peripheral biomarker would be of great benefit to clinicians and patients alike.

It is also not clear what the relationship between elevated levels of *VEGFA* and depression is. One hypothesis is that in the depressed state the blood-brain barrier becomes hyper-permeable, allowing VEGF-A to pass through to the brain where it may have neuroprotective effects (Tseng et al., 2015, Clark-Raymond and Halaris, 2013). Also, mRNA expression in blood is similar to brain, and particularly so for genes relevant to depression such as neurotransmitter receptors, neurotrophins and cytokines (Liew et al., 2006, Sullivan et al., 2006, Hepgul et al., 2013). There is also evidence that there is 'cross-talk' between the CNS and periphery in processes such as inflammation and the hypothalamic-pituitary-adrenal axis, both thought to be involved in the pathophysiology of depression (Zunszain et al., 2011).

Although this study was adequately powered to explore differences in *VEGFA* levels between depressed patients and healthy controls, including the psychotic subgroup, it was likely underpowered for the sub-group correlational analysis involving psychotic patients.

6.4.8 Conclusions

In depressed patients undergoing ECT, baseline *VEGFA* levels are significantly higher than healthy controls, in line with current literature. In patients with psychotic depression, baseline levels were higher again compared to non-psychotic patients. Following ECT, there was a significant decrease in *VEGFA* levels in psychotic depression only. These findings suggest that peripheral blood *VEGFA* levels are a state marker in depression and may be involved in the response to treatment in psychotic depression.

To date, no study reporting on *VEGFA* / VEGF-A in psychotic depression could be identified in the literature. This is therefore the first study to show i) raised peripheral levels of *VEGFA* in psychotic depression, ii) a significant correlation between baseline *VEGFA* levels and psychotic

symptomatology and iii) A significant decrease in *VEGFA* levels following ECT.

Chapter 7
Conclusions and future directions

7 Conclusions and future directions

7.1 Introduction

Depression is one of the most common psychiatric disorders and a leading contributor to the global burden of disease (Vos et al., 2012). Electroconvulsive therapy (ECT) is the most powerful treatment available for depression but its use is limited by both a limited understanding of its molecular mechanism of action as well as concerns about its cognitive side-effects (UK ECT Review Group, 2003). In this thesis, a comparison of the clinical and cognitive side-effects of two electrode placements (bitemporal and high-dose right unilateral ECT) was investigated through a systematic review and meta-analysis. In addition, the potential role played by microRNAs (small, non-coding RNA molecules) in ECT was investigated by measuring its expression in peripheral blood. The expression of a downstream mRNA target of differentially expressed microRNAs was subsequently investigated.

Although microRNAs have been extensively studied in the cancer field, the psychiatric microRNA literature is nascent and, to date, there have been no reports on microRNA expression changes in depressed patients receiving ECT (Ryan et al., 2016). A series of studies was therefore carried out to answer the following questions:

- What is the peripheral blood microRNA profile of depressed patients before and after ECT and how does this compare to healthy controls?
- What are the gene targets of differentially expressed microRNAs and do these also change with ECT treatment?
- Do microRNA/mRNA levels correlate with clinical and cognitive outcomes and could they serve as potential biomarkers?

7.2 A comparison of the effectiveness and cognitive side-effects of bitemporal and high-dose right unilateral ECT for depression

The patients receiving ECT studied in this thesis were participants in a randomised controlled trial of bitemporal and high-dose right unilateral ECT for depression (Semkovska et al., 2016). To further compare these two forms of ECT, a systematic review and meta-analysis was carried out that included seven randomised controlled trials. High-dose brief-pulse right unilateral ECT was as effective as brief-pulse bitemporal ECT for the treatment of depression, and has some cognitive advantages. The immediate clinical implication of this is that high-dose right unilateral ECT should be considered as a first-line option when prescribing ECT.

7.3 Whole blood microRNA expression following ECT for severe depression

Contrary to expectation, there were no widespread changes in microRNA levels after a course of ECT. The only changes that remained significant through the initial discovery, confirmation and validation studies were in patients with psychotic depression. The discovery phase and confirmation studies are summarised in section 4.4, and the validation study is discussed in more detail in section 5.4.5.

The changes observed were fewer than expected at the outset. This expectation was based on previous studies of microRNA expression in depression. It should be noted that the majority of these studies had not carried out correction for multiple testing (despite testing hundreds to thousands of microRNAs) or carried out any validation steps of their findings. In the present study, a conservative approach with regard to multiple testing and validation was used. This was necessitated by the exploratory nature of the initial hypotheses but lends weight to the eventual findings.

Two microRNAs, miR-126-3p and miR-106a-5p were significantly elevated in patients with psychotic depression at baseline when compared to healthy controls. After ECT treatment, the levels of these microRNAs

normalised. These molecular changes were specific to psychotic depression, lending support to previous work indicating that psychotic depression differs from non-psychotic depression on clinical, genetic and biochemical grounds (Ostergaard et al., 2012).

7.4 Gene targets of differentially expressed microRNAs

Two microRNAs (miR-126-3p and miR-106a-5p) were differentially expressed in psychotic depression compared to healthy controls but normalised following ECT. A single microRNA can target hundreds of mRNAs and bioinformatic analysis revealed a high number of genes, molecular and biological processes as well as signalling pathways targeted by miR-126-3p and miR-106a-5p (see sections 5.4.6. - 5.4.9.). In terms of specific genes, *VEGFA, SIRT1* and *E2F1* were shared gene targets of both microRNAs. *VEGFA* has been studied the most in depression, but this has been mostly limited to studies of serum expression of VEGF-A protein (Clark-Raymond and Halaris, 2013). *SIRT1,* a histone deacetylase involved in epigenetic regulation, has recently been implicated in depression in both genome-wide association studies (GWAS) and clinical studies (Converge consortium, 2015, Li et al., 2015, Lo Iacono et al., 2015). The third shared gene target *E2F1* has not previously been implicated in depression.

Moving from specific genes to a broader overview of the molecular and biological processes involving genes targeted by miR-126-3p and miR-106a-5p revealed that cell cycle regulation, regulation of transferase activity and response to abiotic stimuli were particularly over-represented. Although these terms are broad, it is interesting to note the high number of cell cycle regulation processes involved, given their role in adult neurogenesis, one of the proposed mechanisms of action of ECT in depression (Duman et al., 1997). Pathway analysis, which integrates gene interactions, biochemical reactions and cellular location, revealed a number of over-represented pathways relevant to depression and the potential mechanism of ECT. These included several growth factor pathways (VEGF, IGF, TGF-Beta, PDGF, EGF) and cell cycle regulatory pathways implicated in

depression and neuroplasticity (Krishnan and Nestler, 2008, Patricio et al., 2013).

Together these findings fit with existing theories of the possible molecular basis of ECT, but more importantly identify potential specific gene targets (*VEGFA, SIRT1, E2F1)* that warrant further study. Based on previous literature findings, *VEGFA* was selected for downstream analysis as a candidate gene of high interest. In this study, *VEGFA* mRNA levels were raised in psychotic depression as well as non-psychotic depression compared to healthy controls (see section 6.4.2.) Following ECT, there was a significant decrease in *VEGFA* levels in the psychotic depression group only (see section 6.4.5.). Although classical microRNA-mRNA interactions would have predicted lower *VEGFA* levels, a review of the literature combined with bioinformatic analysis indicated that these results were in line with current literature and may reflect the inhibition of negative regulators of the *VEGFA* pathway by miR-126-3p and miR-106a-5p (Fish et al., 2008). The results lend further support to the existence of molecular differences between psychotic and non-psychotic depression and its response to ECT.

7.5 Clinical and neurocognitive correlations

With regard to microRNAs, significant differences between patients and healthy controls were only evident in patients with psychotic depression. The number of patients with psychotic depression with samples available for microRNA analysis was low. This limited the extent of clinical and neurocognitive correlational analysis due to insufficient power, which had been based on the total group rather than just those with psychotic depression. Correlational analysis was therefore limited to depression scores (24-item Hamilton Depression Rating Scale - HDRS) / response status. No significant correlations between miR-126-3p or miR-106a-5p levels and HDRS scores / response to treatment were observed. Whether this was due to a lack of power or whether the changes in microRNA levels are related to some other aspect of psychotic depression or the mechanism of action of ECT remains unclear.

VEGFA mRNA levels correlated weakly, but significantly with depression scores (HDRS-24) and moderately with psychotic symptomatology (psychotic items from Brief Psychiatric Rating Scale - BPRS-5) at baseline. Baseline *VEGFA* levels also correlated significantly with change scores of psychotic symptomatology, but not depression scores. No correlations between *VEGFA* levels and cognitive outcomes were seen. In terms of biomarker potential, baseline *VEGFA* levels showed some promise as a potential biomarker, particularly in differentiating psychotic depression from controls, but this finding should be tempered by the low numbers involved and would need to be replicated and validated in separate cohorts (Papakostas et al., 2013).

Electrode placement had little impact on *VEGFA* expression, suggesting that the cognitive differences observed with these two forms of ECT in the meta-analysis study are not associated with peripheral *VEGFA* levels.

7.6 Future directions

The work described in this study opens several avenues for further work. Although brief-pulse high-dose right unilateral ECT has advantages over standard bitemporal ECT further refinements are possible. Ultra-brief pulse ECT (pulse-width 0.3ms) appears to offer an improved cognitive side-effect profile at the cost of clinical efficacy, but most studies comparing this to brief-pulse right unilateral ECT have employed a pulse-width of 1.0ms for the brief-pulse stimulus. It would be interesting to instead examine the effectiveness and cognitive side-effects of ECT delivered at a pulse-width of 0.5ms (Sienaert, 2015). Other potential modifications of ECT include Focal Electrically Administered Seizure Therapy (FEAST) which is currently undergoing open-label investigation (ClinicalTrials.gov:NCT02462551) (Spellman et al., 2009), and magnetic seizure therapy (Cretaz et al., 2015).

The differential expression of miR-126-3p and miR-106a-5p in patients with psychotic depression treated with ECT is a novel finding. These microRNAs have not been reported in other studies of microRNA changes in depression, but whether this is due to the changes being specific to psychotic depression or ECT remains uncertain. Future work including

patients with psychotic depression treated with other modalities would be helpful in this regard. It would also be interesting to investigate the abundance of these microRNAs in cerebrospinal fluid (CSF) or in post-mortem brain tissue.

VEGFA mRNA was selected for downstream analysis as one of three shared gene targets of miR-126-3p and miR-106a-5p. Significant changes in *VEGFA* expression was seen in psychotic depression treated with ECT, a finding that has not been reported in the literature to date. These preliminary findings should ideally be validated in a separate cohort of patients treated with ECT. Also, the study of *VEGFA* expression in psychotic depression not treated with ECT would be helpful to disentangle whether it is psychotic depression, ECT, or the combination of the two that are driving the changes observed here. As *VEGFA* mRNA levels were raised, it suggests that other components regulating *VEGFA* expression are involved. Two gene targets of miR-126-3p that have been shown to negatively regulate *VEGFA* expression are *SPRED1* and *PIK3R2*. It would be interesting to measure the levels of these mRNAs in the same samples used in this study. This could test the hypothesis that a reduction in *SPRED1* and *PIK3R2* may be behind the elevated *VEGFA* levels observed here.

The other two shared gene targets of miR-126-3p and miR-106a-5p were *SIRT1* and *E2F1*. The study of these mRNAs may also yield interesting results, particularly *SIRT1*, which has already been identified in two GWAS studies in depression (Converge consortium, 2015, Kishi et al., 2010). Downstream analysis of the protein abundance in peripheral blood of all three shared gene targets would also be of interest, and serum samples of patients and controls involved in this study are available. Although *VEGFA* alone is unlikely to represent a biomarker of depression with sufficient utility, it could potentially be a candidate as part of a panel of biomarkers in psychotic depression along with *SIRT1* and *E2F1*.

The findings in this project, together with future work, could therefore be instructive in further improving the clinical and molecular understanding of both ECT and the neurobiology of psychotic depression.

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Appendix

List of papers published during this work

KOLSHUS, E., DOUGLAS, L. & DUNNE, R. 2011. Antidepressant augmentation and combination in unipolar depression: strong guidance, weak foundations. *Irish Journal of Psychological Medicine,* 28**,** i-ix.

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Antidepressant augmentation and combination in unipolar depression: strong guidance, weak foundations

Erik Kolshus, Leonard Douglas, Ross Dunne

Depression will be the second leading contributor to the global burden of disease by 2020.[1] In Ireland, in 2009, 6061 people were hospitalised with depressive disorders.[2] This represents a significant economic and social burden. There is growing awareness of the difficulty in treating depression with medications alone. The likelihood that a patient will achieve remission with the first antidepressant tried is around 30%, and the rates are similar for the second antidepressant tried. This falls to around 15% after three trials.[3] Many patients are exposed to pharmacotherapy for extended periods of time with little beneficial effect, but often with side-effects. Patients are therefore in great need of clear information with regard to their chance of success. Clinicians are in need of clear guidance on prescribing strategies which have proven efficacy. However, this guidance often discusses treatment strategies based on varying levels of evidence. Guiding bodies may approach the problem from varying perspectives. The UK National Institute for Health and Clinical Excellence (NICE) has a clear government mandate with regard to provision of not only effective but cost-effective treatments. The British Association of Psychopharmacology (BAP) is an independent body of interested researchers and therefore may discuss prescribing options from the point of view of tertiary care institutions, and university centres. The South London and Maudsley NHS Foundation Trust publish the popular Maudsley guidelines. These are perhaps more pragmatic in nature, but include very low levels of evidence, including case series.

The American Psychiatric Association (APA) is an independent member association which also publishes guidelines. These are published in the *American Journal of Psychiatry* and the latest guidelines were published in October 2010.

All these bodies attempt to weigh their advice according to the level of evidence available and aim to provide clinical guidance in difficult situations. The burden on guiding organisations is to provide some direction and clarity in areas that are often unclear or controversial. Clinical guidelines are one method of providing support and guidance to busy clinicians. However, this clinician-centered approach has limitations. The onus is on the authors of the guidance to provide ever-more treatment options. This may mean that conclusions about the efficacy of medications is overstated or the limitations of the literature not fully explored in explanatory notes.

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Method

The aim of this paper is to examine the basis of recommendations for pharmacological combination and augmentation therapies in treatment resistant depression (TRD) and the levels of evidence supporting their use.

We performed a search of PubMed and the Cochrane Library using key terms "antidepressant augmentation", "antidepressant combination" and "treatment resistant depression" from inception to May 2011. Inclusion criteria were human trials investigating psychopharmacological approaches to treatment resistant depression in adults and other papers reviewing this issue in English. Exclusion criteria included other physical therapies or psychotherapies, studies in children or adolescents and studies specifically looking at other axis I disorders. We found 808 papers, which was reduced to 238 papers following removal of duplicates and papers not meeting criteria.

Although there are no specific guidelines in Ireland, we commonly refer to UK or US guidelines and we therefore reviewed NICE, BAP, Maudsley and APA prescribing guidelines. The NICE, BAP and APA guidelines include a detailed methodology of how their reviews and recommendations were derived. The Maudsley guidelines do not provide these details.

Our review of the literature assessed studies using the principles laid out by the Oxford Centre for Evidence-based Medicine (CEBM)[4] *(see Table 1)*. The studies with the highest level of evidence were given the most credence.

Background

Depression rarely responds to a 'one size fits all' approach. Combination and augmentation treatment for depression is common in clinical practice.

A survey from the Royal Australian and New Zealand College of Psychiatrists found that 79% of Australian psychiatrists prescribe combination antidepressants and 89% felt that general practitioners should be given more advice on this.[5] A study by Valenstein et al found that 22% of patients in a large sample (n=220,502) of veterans in the United States received either

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Table 1: CEBM Levels of evidence

Level of evidence	Description
1a	Systematic review of RCT's with homogeneity
1b	Individual RCT with narrow Confidence Interval
1c	All or none study
2a	Systematic review of cohort studies with homogeneity
2b	Individual cohort study and low quality RCT's.
2c	"Outcomes" Research; Ecological studies
3a	Systematic review of case-control studies with homogeneity
3b	Individual case control study
4	Case series (and poor quality cohort or case-control studies)
5	Expert opinion without explicit critical appraisal OR based on physiology, bench research or "first principles".

combination or augmentation antidepressant therapy.[6] Despite its common occurrence, there is a lack of consensus in clinical guidelines on how to proceed if initial antidepressant treatment proves unsuccessful.

Pharmacotherapy remains a cornerstone in the treatment of moderate to severe depression. Other evidence-based treatments that have an important role include psychological interventions and physical treatments such as electroconvulsive therapy (ECT).[7] Although low-intensity psychosocial interventions may be sufficient in mild to moderate depression, higher intensity interventions are required to treat moderate to severe depression. The NICE and APA guidelines provide a thorough review of the evidence for the various psychotherapies, with strong support for Cognitive Behavioural Therapy[8] and Interpersonal Psychotherapy[9] in the treatment of depression, both as a stand-alone option or in combination with pharmacotherapy.

If pharmacotherapy is the preferred option, clinical guidelines, such as those provided by NICE[10] suggest initial treatment should be a single Selective Serotonin Reuptake Inhibitor (SSRI) for at least six to eight weeks at an adequate dose. This is based on the NICE Guideline Development Group (GDG) examination of clinical trials, which found that if there was inadequate response to an antidepressant at two weeks, there was still a 40% chance of the patient achieving a response after eight weeks. If there was no response at four weeks, the chance of a response after eight weeks was still 20%. After six to eight weeks of non-response however, only a minority went on to a response over the following weeks.

Although about two-thirds of patients respond to this approach, this leaves about one-third where there is either an incomplete or no response. Treatment resistance refers to an inadequate response to at least one antidepressant given for an adequate duration with adequate compliance. Some insist on two failed trials.[11] Treatment resistance does not refer to medication trials terminated due to side-effects. Unfortunately, there is not a generally agreed exact definition of treatment resistance, which adds to the lack of clarity in this area. The reported prevalence of treatment resistance varies from 20-50% [12,13] and the recent large publicly funded STAR-D trial found decreasing response rates in people who had already required multiple trials of antidepressants.[14]

In drug trials an adequate response is typically defined as a 50% reduction in symptoms on a symptom rating scale such as the Hamilton Depression Rating Scale (HAM-D). A partial response to treatment is usually defined as a 25-50% reduction. There are however some cases where even a 'full' response is inadequate. If an initial HAM-D score is 30, a 50% reduction would still leave a HAM-D score of 15, indicating a significant level of depression.

Combination antidepressant therapy strictly means the use of two separate antidepressants in combination, the rationale being that the agents have differing modes of action. Augmentation strategies on the other hand, involve adding another compound to an antidepressant to boost the antidepressant effect of the initial drug. This compound may not be a first-line antidepressant or suitable in isolation, but have shown some efficacy in combination.

There are several issues to consider before considering combination or augmentation of antidepressant therapy in treatment resistant depression.[15]

First one should ask whether the diagnosis is correct. Some people with personality disorders are likely to score highly on rating scales for depression but the associated distress is unlikely to respond to medication[16]. It is also important to consider undiagnosed bipolar affective disorder. One should also assess for co-morbid conditions that may influence treatment, such as substance abuse and anxiety disorders, as well as underlying medical conditions *(see Table 2)*.

Substance misuse disorders are more common in the psychiatric than the general population. Substance misuse can lead to a worsening of depressive symptoms and treatment resistance, but depression can also lead to a worsening of substance misuse.[17] It is important to clarify in these instances where there is co-morbidity to assess whether one or both of the disorders are currently active.

This underlines the necessity of a thorough re-assessment, including chart review and extensive collateral history in patients who have treatment resistant episodes as some causes of treatment resistance could be eliminated.[18]

An adequate period of time and an optimal dose should be used. If patients cannot tolerate the highest dose of a medication due to side effects this does not have the same prognostic implications as the failure to respond.

A population survey by Broly[19] revealed that up to 10% of the Caucasian population may be either extensive or poor CYP2D6 metabolisers which means there can be wide variations in blood medication levels and dosage requirements. Other pharmacokinetic issues which may hinder drug efficacy include p-glycoprotein. P-glycoprotein is a basement membrane transport pump active in the blood brain barrier which inhibits drug entry. Recent studies have shown this may account for a significant variation in response to drugs.[20]

The metabolising status of the individual patient becomes irrelevant if they are not taking the medication so adherence should be assessed, and social factors that may be maintaining the depression should be addressed, again highlighting the necessity of thorough re-evaluation.

Table 2: When one antidepressant is not enough

Checklist	√
Confirm Diagnosis Collateral History Old Notes / Chart review Mood diaries	
2. Rule out Co-morbid conditions Substance misuse disorders Anxiety disorders Medication related Medical illness Thyroid (subclinical) Steroids Inflammatory disorders	
3. Adequate dose	
4. Adequate trial period	
5. Adherence	
6. Address social factors maintaining depression	

There may also be gender-specific issues such as mood disorders in the menopausal period[21] that should be taken into account. If these factors do not shed any light on the inadequate response, options include switching to another antidepressant, adding another antidepressant, adding an augmenting agent, physical treatments such as ECT or a psychological therapy such as CBT or IPT if not used already.[22] In treatment resistant depression, the psychotherapy with the strongest evidence is CBT,[23] with the STAR*D trial also finding it to be generally as effective as second-step pharmacological therapy.[24]

There are several arguments in favour of combination or augmentation strategies:

- It is postulated that the added compound can act via a different neurotransmitter, through a synergistic effect or through modulation of second messenger systems.[25]
- With substitution, the patient may lose all the gains they may have made when the first antidepressant is stopped. This is especially the case in partial responders, where the loss of even limited gains may be a serious and dangerous setback.
- It can be demoralising for patients after several weeks on one agent to have to start all over again with a new agent.
- When one switches an antidepressant it may involve several weeks of a washout period or time spent titrating dosages up and down.

Theoretically, combination strategies can therefore offer a quicker response than monotherapy[26] due to time saved in washout and titration. However, only limited evidence exists for faster response with certain combinations and these have not necessarily, included treatment resistant patients.[27] Potential drawbacks of a combination or augmentation approach include:

- Increased number of potential drug interactions
- Increased risk of side-effects with the possibility of reduced compliance.
- Increased economic cost
- Lack of clarity as to which pharmacological agent is causing a clinical improvement.[28]

Combining two agents may increase toxicity so the risk of adverse effects would be expected to be higher,[29] but the evidence base suggests that this approach is generally well tolerated, and that the second agent does not substantially alter the side effect profile of the initial antidepressant.[30,31] In some cases the second agent may even reduce side-effects induced by the first drug, such as the use of buspirone to ameliorate sexual side-effects of SSRIs.[32]

There are important differences within the SSRI group in terms of pharmacokinetics and drug interactions despite their similar clinical efficacy, therefore a knowledge of the individual pharmacokinetic properties of the agents used in combination is essential. Fluoxetine, for example, has a much longer half-life than the other SSRIs.

Review of the evidence

The STAR*D trial

The American STAR*D trial (Sequenced Treatment Alternatives to Relieve Depression) was a large, publicly funded, open-label effectiveness trial divided into several levels. The aim was to test the effectiveness of various approaches to the treatment of depression in a clinical setting. The mean HRDS17 score at baseline was 19 indicating a moderate level of depression. Patients not responding to the first level of treatment (citalopram only) would go on to a series of options in the second level. If this was not successful they would go on to the third level and so on. It is an important study, but there are some limitations to bear in mind when reviewing the results. No placebo was used in the trial, and randomisation was often limited by patient choice. Caution should be used in comparing treatments offered at different levels. Patients who previously have not responded to the treatments offered in STAR-D were excluded from the trial which may have inflated the remission rates.[33] The STAR-D trial found that co-morbid axis I and III conditions as well as anxious features were associated with higher rates of treatment resistance.[34] These patients may also have a slower response to treatment and can be more susceptible to side-effects.[35]

Augmentation strategies

1. Lithium

Lithium is one of the most widely used and best-studied augmentation strategies, and is one of the few combinations supported by NICE as a next-step treatment in those who do not respond to first-line treatment. It is also supported by the BAP and the Maudsley Guidelines as a first choice treatment for refractory depression. [36] It is recommended with moderate confidence by the APA.

The exact mechanism of action is unknown, however it is thought to enhance serotonin release, possibly by reducing the negative feedback on serotonergic neurons[37], and also up-regulated dopamine second messenger signaling[38]. It has been used with various monoamine reuptake inhibitors, at doses between 600 to 1200 mg. A meta-analysis by Crossley and Bauer[39] in 2007 found that there was a relationship between dose and efficacy, suggesting doses above 800mg may be required to be of benefit. This relationship is important as lithium is often used as an augmenting agent in lower doses than in lithium monotherapy. If low doses are used, the evidence suggests that there are still drawbacks in terms of side-effects and monitoring, but

Table 3: Features of serotonin syndrome

Features of serotonin syndrome
1. Agitation or restlessness
2. Confusion or delirium
3. Autonomic instability (changes in blood pressure and heart rate)
4. Hyperthermia
5. Nausea
6. Diarrhoea
7. Myoclonus

little clinical benefit.

The Crossley and Bauer meta-analysis showed convincing evidence for lithium as an effective augmentation strategy, but not for its use to accelerate antidepressant response. The study found lithium was three times more effective than placebo as an augmenting agent, with a Number Needed to Treat (NNT) of five to achieve remission.[40] It is worth noting however, that most of the studies augmented lithium with tricyclic antidepressants (TCAs) rather than SSRI's, and the numbers were small (a total of 269 patients from a total of 10 RCTs). Some of the trials also included both bipolar and unipolar depressed patients and there were large differences in dosages.

Regarding the combination of lithium and a SSRI, there are case reports of serotonin syndrome, as well as instances of hypomania and mania, absence seizures and delirium.[41] This risk appears to be higher with fluoxetine, whereas citalopram appears safe.[42]

One arm of the STAR-D trial compared lithium augmentation with tri-iodothyronine (T3) augmentation of citalopram. Patients at this level had already failed two treatments for depression. 15.9% of patients on lithium achieved remission and there were frequent reports of side-effects.[43] Although this study reported a favourable response for T3 over lithium, this was not statistically significant. Also, the mean dose of lithium given was 859mg. Given the findings from the Crossley meta-analysis, this may reflect insufficient dosing for many patients in the STAR-D trial.

2. Tri-iodothyronine (T3)

T3 has been used to augment monoamine oxidase inhibitors (MAOIs), TCAs and SSRIs in non-hypothyroid patients.

The use of T3 is not recommended by the NICE guidelines as only one paper met their stringent criteria (it appeared to show only a subgroup of responders), but it is supported by the BAP, the APA, and the Maudsley guidelines. Small doses (25-50 mcg/day) are used, but due to possible interference with thyroid function, should be discontinued if there is no response within three weeks. The mechanism of its effect is complex but there is evidence that thyroid hormone has substantial effects on brain neurochemistry, neuronal plasticity and gene expression.[44]

The STAR-D trial, discussed above, found remission rates of 24.7% when T3 was added as a third-step option. This was higher than for lithium augmentation (but not statistically significantly)[45] and had fewer side-effects.

A meta-analysis by Aronson et al[46] including 292 patients, showed moderately large improvements with T3 augmentation, mostly of TCAs.[47] There was a large degree of statistical heterogeneity between the studies, and only four of the eight included studies in this meta-analysis were randomised controlled trials (RCTs). When the non-RCT's were excluded, a sub-analysis failed to find any significant advantage for T3.

A more recent meta-analysis in 2009 by Papakostas et al examined T3 augmentation of SSRIs only.[48] Four trials, with 444 patients were included. Again, there was a large degree of heterogeneity between studies. The pooled results failed to find a significant difference between SSRI alone and SSRI with T3 augmentation.

Studies examining doses above 50mcg of T3 are rare. The evidence is limited to an open trial using dose of 100 mcg[49] and a case series with doses up to 150mcg[50], both with 17 patients. Although they showed some promise, there are marked methodological issues due to the nature of the study design.

Together, these results indicate the need for further evidence to clarify the role of T3 as an augmenting agent.

3. Atypical antipsychotics

Antipsychotics are effective in psychotic depression but there is also gathering evidence for the use of atypical antipsychotics in non-psychotic depression. In the 2009 NICE guidelines, aripiprazole, olanzapine, quetiapine and risperidone were recommended as augmenting agents for the first time. Along with the NICE guidelines, the BAP, APA and Maudsley guidelines also support the use of these antipsychotics, with the BAP guidelines reporting stronger evidence for olanzapine and quetiapine. The Maudsley guidelines limit their recommendation of olanzapine for augmenting fluoxetine only.

Aripiprazole was the first of these agents to receive a US Food and Drug Administration (FDA) approval as an augmenting agent in treatment-resistant depression in 2007, followed by olanzapine and quetiapine. Their approval was based on large, drug-company funded trials that have yet to be replicated. The combination of olanzapine and fluoxetine is for sale as Symbyax in the US. In 2010, quetiapine was given a licence in the UK as an augmenting agent. This was followed at the start of 2011 in Ireland. To date, quetiapine remains the only antipsychotic licenced as an augmentation agent in depression in Ireland.

The exact mechanism by which they exert an antidepressant effect is unclear, but it is thought that blockade of 5HT2A receptors plays a key part,[51] and they also have 5HT2C and dopaminergic activity. As yet there is no proven independent antidepressant effect.

A meta-analysis by Nelson et al in 2009 [52] found that olanzapine, risperidone, quetiapine and aripiprazole were an effective adjunct in treatment resistant depression, with an overall number needed to treat of nine to achieve remission acording to indivdual trial criteria. The largest effect-size was for Risperidone (OR =2.63) followed by Aripirazole, with Quetiapine and Olanzapine equally effective. This meta-analysis included a large number of patients (3480) and there was a low level of heterogeneity between the studies.

The meta-analysis points out that the rates of discontinuation specifically attributable to side effects were significant for

olanzapine, quetiapine, and aripiprazole. The use of antipsychotics are associated with serious adverse events, such as tardive dyskinesia as well as the more common side-effects such as sedation and weight gain. The odds of discontinuation due to side-effects for any reason were non-significant for risperidone, olanzapine and aripirazole, but raised for quetiapine.

A pooled analysis[53] of the two main RCTs supporting the use of quetiapine in depression found that 35% and 44% (300 mg XR and 150mg XR respectively) of the improvement in the HAM-D scores was due to improvement in sleep.

There was also a suggestion of publication bias, with a surplus of small trials showing a large effect, however, when these were removed the outcomes were unchanged. Keitner commented that there are still a number of unanswered questions regarding the use of atypical antipsychotics, such as optimum dosage, necessary duration of treatment and importantly, the long-term effects of antipsychotic use.[54]

It therefore appears atypical antipsychotic augmentation may be helpful in the short-term, but the risk-reward balance for longer use remains unknown.

4. Lamotrigine

Lamotrigine is an anti-epileptic that works by inhibiting glutamate and is used in bipolar affective disorder. The NICE guidelines report that there is insufficient evidence to recommend its use as an augmenting agent in unipolar depression. The BAP and APA guidelines give it some limited support. The Maudsley guidelines recommend it as a second-line augmenting agent. It was not included in the STAR-D trials.

The evidence for its use in unipolar depression is based on retrospective chart reviews and small open trials, only some of which were randomised[55]. Actual randomised controlled trials, have been completely underpowered to detect an effect of lamotrigine, with numbers ranging from 15-40, the larger trials showing some effect on secondary outcomes.[5657] There is very limited evidence for the use of lamotrigine in this group of patients. It requires slow titration and carries a risk of Stevens-Johnson syndrome.

5. Pindolol

Pindolol is a beta-adrenergic antagonist which selectively blocks pre-synaptic 5HT1A receptors. It is not supported by NICE, APA or BAP guidelines and is only recommended as a second-line augmenting agent in the Maudsley guidelines.

Its mechanism of action is thought to be through inhibition of the negative feedback from increased serotonin levels that occurs with the use of SSRI's. [58] It has been thought to accelerate the antidepressant response to SSRI's. [59]

Its role as an augmenting agent remains unclear with a large degree of heterogeneity in studies of its potential benefit. One recent systematic review of pindolol augmentation with SSRI's only, concluded it had an overall beneficial clinical effect, most notably in the first four weeks of treatment. [60] This study included 889 subjects, but was limited by a high degree of heterogeneity. Both unipolar and bipolar, treatment resistant and treatment naive in-patients and outpatients were included. Although there appeared to be a benefit over placebo at 4 weeks, by 6 weeks there was no statistically significant benefit to pindolol augmentation suggesting that if it is effective it is in speeding response, rather than enhancing overall response rates..

Combination strategies

1. Mirtazapine and SSRI/SNRI

One of the more common true combination strategies is the use of Mirtazapine and a SSRI or SNRI. Mirtazapine, a tetracyclic antidepressant is a noradrenergic and specific serotonergic antidepressant (NaSSA) and is recommended as a first choice combination agent by the NICE and Maudsley Guidelines. It is also supported by the BAP and APA guidelines. In the STAR-D trials the combination of venlafaxine and mirtazapine only achieved a 13.7% remission rate, but was used as a fourth-line treatment and so only in the most resistant of cases.[61] It compared favourably with the use of tranylcypromine (a MAOI), especially in terms of side-effect burden and ease of use.

Mirtazapine is thought to increase noradrenaline (NA) transmission through antagonism of $\alpha 2$ adrenoreceptors as well as action on serotonin reuptake. The combination of mirtazapine and high-dose venlafaxine was dubbed "California Rocket Fuel" by Stahl in Essential Psychopharmacology[62], due to its theoretical synergy. Mirtazapine has also been used with high dose venlafaxine to block 5HT2 and 5HT3 receptors with the aim of reducing sexual and anxiety side-effects[63]. The use of duloxetine, another SNRI, with Mirtazapine was reported by Meagher et al who dubbed this combination "Limerick Rocket Fuel"[64]. As with the use of any two serotonergic agents there is a risk of serotonin syndrome.

The evidence-base is chiefly from randomised controlled trials. The NICE guidelines identified one RCT comparing mirtazapine augmentation with placebo by Carpenter et al from 2002[65], which found mirtazapine augmentation resulted in a statistically significant improvement in mean end-point in depression scores and response, but not for remission. This study had small numbers (26 patients), the duration of the study was short, (four weeks) and there was heterogeneity in terms of diagnosis, antidepressants used and dosages used.

Since the NICE guidelines were published a new RCT examining the role of mirtazapine as an augmenting agent has been published by Blier et al.[66] They concluded that combining mirtazapine with either fluoxetine, venlafaxine or bupropion was more clinically effective than fluoxetine alone, with the strongest results for a mirtazapine and venlafaxine combination. There was a statistically significant difference in favour of combination treatment for mean HAM-D scores and remission rates, with a NNT for remission of between 3-5 versus fluoxetine alone. This study had a relatively large sample of 105 patients split into four groups. The randomisation process is somewhat unclear, but the groups were largely similar in terms of demographics and drop-out rates. The dose of fluoxetine may have been sub-optimal favouring the combination treatment arms, and there was a lack of estimate of the precision of the findings.

In both the Carpenter and Blier trial weight gain was associated with mirtazapine vs placebo. This was statistically significant in the larger Blier trial, despite their choice of "weight-neutral" agents to combine with mirtazapine. There was a mean increase of 2.7 kg over a six-week period in those groups a mirtazapine combination.

2. Bupropion and SSRI

Bupropion is a dopamine and noradrenaline reuptake inhibitor licenced for smoking cessation in Ireland and the UK. It is licenced as an antidepressant in the United States and the

Table 4: Overview of augmentation strategies

Augmentation agent	Proposed mode of action	Used with	Dose	Potential issues
Lithium	Enhances serotonin release. Action on GSK3Beta/Akt signalling complex,	TCA, SSRI, MAOI	0.6-1.2g*	1. Careful monitoring required. Special attention to TFT/U&E 2. Serotonin Syndrome 3. Tolerance to side-effects can be an issue
T3	Exact mode of action unclear.	TCA, SSRI, MAOI	25-50* mcg	1. Monitor thyroid function.
Olanzapine	5HTa2 receptor activity	SSRI (fluoxetine)	12.5mg*	1. Metabolic syndrome 2. Sedation
Quetiapine	5HTa2 receptor activity	SSRI, SNRI	300-600mg*	1. Weight gain 2. Sedation 3. Hypotension
Risperidone	5HTa2 receptor activity	SSRI, SNRI TCA	0.5-2mg*	1. Hyperprolactinaemia 2. Weight gain 3. Hypotension
Aripiprazole	5HTa2 receptor activity. Partial agonist 5HT1a	SSRI, SNRI	5-20mg*	1. Akathisia, restlessness
Lamotrigine	Glutamate inhibition ?5HT1a activity	SSRI	200mg*	Steven-Johnson syndrome
Pindolol	Blocks pre-synaptic 5HT1a receptor	SSRI, SNRI	7.5-15mg*	May only affect speed of response
Omega-3 triglycerides	Anti-inflammatory effect	SSRI, SNRI TCA, MAOI	1-2g*	Appears safe
Folate	Increased levels of 5HT, DA, NA	SSRI, TCA	2-5mg*	Dosage trials underway

**Indicative doses only. Not to be used as a prescribing guideline. Please review relevant literature.*

combination with an SSRI is supported by the APA. The Maudsley guidelines recommend it as a first-line augmenting agent due to the findings from the STAR-D trial, which found a remission rate of 29.7% when bupropion was combined with citalopram. The limitations of the open-label STAR-D trial have been discussed previously, and it should be noted that bupropion was offered as an early step in the STAR-D algorithm. The remaining evidence base for bupropion and SSRI combination is from small open trials or case reports and as such is not supported by the NICE or BAP guidelines.

It is also thought to be helpful in ameliorating sexual side-effects from SSRIs. [67]

3. Buspirone and SSRI

Buspirone, a pre- and post-synaptic 5HT agonist is sometimes combined with antidepressants, but the evidence is mixed. It is not recommended by the BAP or NICE guidelines. The NICE guidelines specifically state that there is insufficient evidence for its use.[68] The Maudsley guidelines recommend buspirone augmenation as a second-line augmenting agent on the basis

Table 5: Overview of combination strategies

Combination agents	Proposed mode of action	Used text	Dose*	Potential issues
Mirtazapine	α2 adrenoreceptor antagonism 5HT2, 5HT3 antagonism	SSRI, SNRI	30-45mg*	1. Serotonin syndrome 2. Sedation
Bupropion	NA and DA reuptake inhibition	SSRI	up to 400 mg*	Not licenced for depression in Ireland
Buspirone	5HT1 agonist	SSRI	up to 60mg*	May not be as well tolerated as bupropion. (based on STAR*D)
TCA	5HT and NA increase CYP2D6 inhibtion leading to elevated TCA levels	SSRI	Lower dose than if used alone*	1. Serotonin syndrome 2. Monitor plasma concentrations 3. ECG monitoring recommended
MAOI	Synergy of NA and 5HT increase	TCA	Lower dose than if used alone*	1.Serotonin syndrome 2. Avoid highly serotonergic TCA's like clomipramine, imipramine 3. High-risk combination

Indicative doses only. Not to be used as a prescribing guideline. Please review relevant literature.

of the STAR-D trials. The STAR-D trials showed similar remission rates to buproprion (30.1%) but with a higher burden of side-effects. The APA supports its use where anxiety is a prominent feature.[69]

There are some positive case reports and open label trials[70], but two randomised controlled trials failed to find a significant advantage over placebo[71,72]. The first of these trials had an unusually large placebo response of 46.7% and outcomes were only measured on the Clinical Global Impressions-Improvement (CGI-I) scale. The trial also became open-label after only four weeks.

4. TCA and MAOI

The combination of two of the 'older' classes of antidepressant has been used since the 1960's. Its use was limited as the agents either had very similar mechanism of action or the combination was potentially dangerous. One of the first combinations was the use of a MAOI and TCA. This had limited efficacy and frequent occurrence of serotonin syndrome,[73] and is generally not recommended, though the Maudsley guidelines give it some cautious support. Gillmann argues that it is only the TCAs with potent serotonergic action (imipramine and clomipramine) that carry this risk.[74]

5. TCA and SSRI

The combination of a TCA and a SSRI is also generally considered potentially hazardous due to the risk of serotonin syndrome but is used sporadically. The Maudsley and APA guidelines give it some support. Open trials have been encouraging, but the only double-blind trial found that high dose SSRI monotherapy was as good as a SSRI and TCA or SSRI and lithium combination.[75] Mean serum lithium and desipramine levels were low however, indicating these groups may have been under-treated. There was also no placebo group in this study.

As SSRIs can inhibit the cytochrome P450 system (CYP) it is argued that the benefits from this combination is chiefly derived from raised TCA levels due to inhibition of its metabolising enzyme, however, this is just as likely to contribute to toxicity as response.

The SSRIs differ greatly in terms of their inhibitory action on the cytochrome P450 system. Fluvoxamine has a strong inhibitory action on a range of CYP enzymes. Fluoxetine and paroxetine have strong inhibitory action on CYP2D6. Reports of toxicity due to combinations using fluvoxamine, fluoxetine and paroxetine and TCAs are therefore predictable. Sertraline, citalopram and escitalopram have fewer drug interactions and would therefore be preferable agents in a combination strategy.[76]

The combination of an SSRI with another SSRI cannot be recommended due to the risk of serotonin syndrome (*see Table 3*).

Other strategies

There are a number of other augmenting agents that have been suggested to have a potential benefit in treatment resistant depression. These are not supported by the NICE guidelines, which are more conservative than the other guidelines. The NICE guidelines, after its review of the evidence, specifically recommend against the routine use of anticonvulsants (including carbamazepine and valproate) and benzodiazepines beyond two weeks.

The BAP guidelines include more candidates in their recommendations with the caveat that they only be "considered in specialist centres with careful monitoring".[77] Their list of candidates include tryptophan, modafinil and other stimulants, oestrogen in perimenopausal women and antiglucocorticoids (metyrapone).

BAP also give some support to the use of omega-3 fatty acids and there is also some evidence that folate may be beneficial. A meta-analysis of folate augmentation by Taylor et al[78] included two RCTs. The trials were relatively homogenous, however, there were only 124 patients included in the meta-analysis and confidence intervals were wide. Due to the small number of trials, the use of funnel plots to establish a publication bias is of limited value. A new randomised controlled trial (FolATED)[79] is under way and may shed more light on the role of folate augmentation in depression.

The APA give some limited support to omega-3 fatty acids, folate, stimulants and anticonvulsants in "individual circumstances".[80] They also give some support to the use of anxiolytics and sedatives in the short-term where anxiety is a prominent co-morbid complication.

The Maudsley guidelines have an even more extensive list of agents that are listed as third-line agents, but stress that the evidence-base is limited and "prescribers must familiarise themselves with the primary literature before using these strategies".[81] The evidence base for these include small randomised trials, open trials, case series, case reports and animal studies. These may be useful in specific circumstances.

The Maudsley list include amantadine, cabergoline (dopamine agonist), clonazepam, mecamylamine (nicotinic antagonist), metyrapone, tryptophan, yohimbine (pre-synaptic alpha 2-adrenergic antagonist), zinc, ziprasidone (atypical antipsychotic) and modafinil.

Conclusions

Treatment resistance is a common phenomenon and psychiatrists should familiarise themselves with the available options. The strategies discussed in this paper should not be viewed as the only option when antidepressants fail, but rather as alternatives in the therapeutic arsenal. There are a number of guidelines in use, and there are differences between them. The current evidence-base treatment strategies varies from solid to weak. The best evidence for augmenting or combining treatments remains for the use of lithium with TCAs.

There will be circumstances when one might choose agents outside the general recommendations, or using a medication off-licence, but this should ideally be done by specialists who are familiar with their use and have adequate clinical facilities to monitor their effects. One should also bear in mind that the treatment that has been proven to be the most acutely effective treatment for severe treatment-resistant depression remains ECT.

It is important that the patient is informed of the rationale and evidence for the proposed treatment, especially if one is using a strategy that has a limited or no evidence-base. Nonetheless, given the burden of depression on individuals and society, every effort should be taken to treat it.

Clinicians must remember that treatment guidance is often created from less than systematic reviews of the literature and may use a very subjective evaluation of varying levels of evidence. This must be further criticised and interpreted by the treating clinician. Often, the best available evidence is less than gold standard. In fact, it may be unpersuasive. However, its very discussion in the guidance of supervising and leading bodies encourages the use of treatments with meagre evidence. We must recognise the difficulty in generating good quality research on resistant depression, and should not deprive our patients of treatment options because the research evidence does not meet criteria of certainty. Patients so treated should be informed that their regimen is based on more tentative evidence, as otherwise failure to respond is likely to jeopardise their relationship with their treating clinician and their expectations for the future. It also keeps us honest, because while as clinicians we feel obliged to offer more and more treatment alternatives, we must appreciate that the best evidence does not always support doing "something different".

Declaration of interest: None

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Multiple Choice Questions: Module 11

1. Regarding treatment resistance:

In drug trials, an adequate response to a medication is typically defined as a 50% reduction in symptom score	T	F
The likelihood that a patient will achieve remission with the first antidepressant tried is around 50%	T	F
Initial treatment with an SSRI should be for at least 6-8 weeks at an adequate dose	T	F
Two trials of medications terminated due to side-effects indicates treatment resistance	T	F
Reported prevalence of treatment resistance is around 10%	T	F

2. Regarding lithium augmentation:

Lithium is an effective augmentation agent at lower doses than those used in lithium monotherapy	T	F
Lithium is contraindicated with SSRIs	T	F
Lithium is thought to inhibit serotonin release	T	F
Lithium augmentation has a NNT of 5 to achieve remission	T	F
The strongest evidence is for lithium augmentation with TCAs	T	F

3. Other augmentation strategies:

T3 augmentation is reserved for hypothyroid patients	T	F
The safety of long-term augmentation with atypical antipsychotics is yet to be established	T	F
Stevens-Johnson syndrome is associated with lamotrigine	T	F
Pindolol augmentation has a benefit over placebo, but only after six weeks of treatment	T	F
The STAR*D trial found Lithium to be superior to T3 augmentation	T	F

4. Combination strategies:

Mirtazapine is a dopamine and noradrenaline reuptake inhibitor	T	F
Bupropion may ameliorate sexual side-effects of SSRIs	T	F
TCA and MAOI combinations have a high risk of serotonin syndrome	T	F
Citalopram, escitalopram and sertraline are drugs of choice in a combination strategy	T	F
SSRIs can be safely combined with other SSRIs	T	F

5. General recommendations:

Guidelines always reflect gold standards of treatment	T	F
Patients should not be advised that their treatment is based on tentative evidence as this may jeopardise the therapeutic relationship with their clinician	T	F
The randomised controlled trial is the highest level of evidence when appraising a treatment	T	F
ECT is the most acutely effective treatment for severe treatment resistant depression	T	F
CBT has proven efficacy in treatment resistant depression	T	F

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Relapse Following Successful Electroconvulsive Therapy for Major Depression: A Meta-Analysis

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High rates of early relapse following electroconvulsive therapy (ECT) are typically reported in the literature. Current treatment guidelines offer little information to clinicians on the optimal nature of maintenance therapy following ECT. The aim of this study was to provide a systematic overview of the existing evidence regarding post-ECT relapse. A keyword search of electronic databases was performed for studies appearing in the peer-reviewed literature before January 2013 reporting on relapse rates in responders to an acute course of ECT administered for a major depressive episode. Meta-analyses were performed where appropriate. Thirty-two studies with up to 2 years' duration of follow-up were included. In modern era studies of continuation pharmacotherapy, 51.1% (95% CI = 44.7–57.4%) of patients relapsed by 12 months following successful initial treatment with ECT, with the majority (37.7%, 95% CI = 30.7–45.2%) relapsing within the first 6 months. The 6-month relapse rate was similar in patients treated with continuation ECT (37.2%, 95% CI = 23.4–53.5%). In randomized controlled trials, antidepressant medication halved the risk of relapse compared with placebo in the first 6 months (risk ratio = 0.49, 95% CI = 0.39–0.62, $p < 0.0001$, number needed to treat = 3.3). Despite continuation therapy, the risk of relapse within the first year following ECT is substantial, with the period of greatest risk being the first 6 months. The largest evidence base for efficacy in post-ECT relapse prevention exists for tricyclic antidepressants. Published evidence is limited or non-existent for commonly used newer antidepressants or popular augmentation strategies. Maintenance of well-being following successful ECT needs to be improved.
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Keywords: depression; relapse; electroconvulsive therapy; meta-analysis

INTRODUCTION

Electroconvulsive therapy (ECT) is a highly effective acute treatment for major depression (Eranti *et al*, 2007; Kellner *et al*, 2010; The UK ECT Review Group, 2003). Although remission rates exceed those seen with other somatic treatments, high rates of relapse, especially early relapse, are observed and acknowledged as a major clinical problem (Kellner *et al*, 2006; Sackeim *et al*, 2001). Consolidating and prolonging remission is a key clinical challenge surrounding ECT use (Kellner 2013).

Following introduction of the first effective antidepressants, continuation antidepressant monotherapy following ECT appeared to minimize the likelihood of relapse. Early research from the United Kingdom demonstrated the efficacy of antidepressants over placebo with 6-month relapse rates in tricyclic antidepressant (TCA)- or monoamine oxidase inhibitor (MAOI)-treated patients of about 20% compared with 40–70% in untreated or benzodiazepine-only-treated patients (Imlah *et al*, 1965; Kay *et al*, 1970; Seager and Bird, 1962). However, more recent studies are less favorable, with relapse rates typically about 40–50% at 6 months despite vigorous continuation therapy, such as antidepressant–lithium combination or continuation ECT (C-ECT; Kellner *et al*, 2006; Prudic *et al*, 2013; Sackeim *et al*, 2001). Of note, in a more recent trial where patients were randomized to TCA monotherapy, TCA–lithium combination, or placebo, TCA monotherapy was not significantly more effective than placebo in preventing relapse (Sackeim *et al*, 2001).

Higher rates of relapse in recent decades may be due to historical changes in ECT patient populations (Sackeim, 1994). ECT is a unique treatment in psychiatry that predates modern psychopharmacology. Once used as first-line treatment for severe depression in often medication-naive patients, its use nowadays is reserved for a minority of patients with severe, chronic, difficult-to-treat depression where several treatment steps have usually been unsuccessful. Such treatment-resistant patients are generally less likely to achieve full remission and, when they do, are prone to relapse (Fekadu *et al*, 2009).

The negative impact of medication resistance on ECT outcomes had been noted decades ago (Bruce *et al*, 1960; Hamilton, 1974) and was subsequently demonstrated by studies showing that patients with established medication resistance have worse acute (Prudic *et al*, 1996; Prudic *et al*, 1990) and longer-term (Sackeim *et al*, 1990) outcomes.

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A recent meta-analysis confirmed that acute remission rates with ECT are lower in treatment-resistant patients (48%) compared with those in whom medication resistance had not been established (65%) (Heijnen *et al*, 2010).

Currently, there is no agreement on what constitutes optimal post-ECT relapse prevention treatment. The American Psychiatric Association guidelines on ECT, now over a decade old, recommend continuation therapy with either pharmacotherapy or C-ECT for virtually all patients (American Psychiatric Association, 2001). However, no specific guidelines on choice of agent or duration of treatment exist. Most experimental work over the past 3 decades has focused primarily on optimizing ECT treatment parameters (eg, electrode placement, stimulus dose, and pulse width) to produce the best possible balance between clinical and neuropsychological outcomes. These studies unequivocally show that ECT is a powerful treatment option capable of producing full remission where other treatments have failed (Dunne and McLoughlin, 2012; Eranti *et al*, 2007; Kellner *et al*, 2010; Loo *et al*, 2012; Sackeim *et al*, 2009). However, given that relapse following ECT is a key clinical problem, we carried out a systematic review of all existing evidence, randomized and observational, to provide an overview of current knowledge on this important question.

MATERIALS AND METHODS

Search Strategy

An electronic literature search of PubMed, Embase, CINAHL, PsycINFO, and Cochrane Library databases was performed up to January 2013 with no time, language or other restrictions. Keywords used were (ECT OR electroconvulsive therapy OR convulsive therapy) AND (depression OR depressive OR mood disorder OR bipolar disorder OR affective disorder OR melancholi*) AND (long term OR follow up OR relapse OR prognosis OR mortality OR maintenance OR continuation). Hand-searches of reference sections of previous reviews and included studies were carried out.

Following exclusion of database duplicates and clearly ineligible reports, judging by title and abstract screening, two reviewers (AJ, EK) independently evaluated for eligibility all studies retained for full-text screening. Where studies met inclusion criteria (described below), the reviewers independently extracted data from reports. Information regarding study design, ECT treatment parameters, sample characteristics, type of continuation therapy, type of outcome measure, definition of relapse, valid sample size at each follow-up, cumulative number of relapses at each time point, and cumulative number of dropouts at each time point was extracted. Discrepancies were resolved by joint re-evaluation of reports.

When extracting relapse proportions from reports, preference was given to information in the body of texts and tables. Where the study explicitly reported relapse rates only for the study endpoint but where patients were assessed at multiple intermediate time points, survival curves were examined; where it was deemed that the number of relapses could be extracted from graphs, this was done jointly by the reviewers. Where studies met inclusion criteria but data were reported in a non-extractable format, we contacted the authors. Given the literature age span, this was not always possible as authors were sometimes untraceable or deceased.

Study Eligibility Criteria

The following inclusion criteria were applied:

(1) prospective study reported in a peer-reviewed publication;
(2) participant age $\geqslant 18$ years;
(3) an acute course of ECT was administered for treating a major depressive episode (unipolar or bipolar) diagnosed by clinical judgement or formal diagnostic criteria (eg, DSM-IV);
(4) those deemed to be ECT responders or remitters were prospectively followed-up and monitored for relapse;
(5) relapse was operationally defined by the original investigators and reported in a categorical fashion (ie, as the percentage of the initial responder or remitter sample who relapsed);
(6) relapse was ascertained on the basis of clinical judgement or by using formal diagnostic criteria and/or pre-specified cutoff scores on clinician-rated depression severity rating scales (eg, Hamilton Depression Rating Scale); and
(7) clinical outcome assessment was carried out $\geqslant 3$ months following the last ECT session.

Exclusion criteria:

(1) case studies or series with $N < 10$;
(2) retrospective studies;
(3) prospective studies where relapse was not established directly via patient interview but instead on the basis of proxy measures (eg, rehospitalization rates), mailed self-report questionnaires, or information obtained from third-parties (eg, patients' relatives or treating physicians);
(4) presence of non-affective psychosis, dementia, neurological disease, or unstable medical conditions in the sample; and
(5) unmodified ECT.

Outcomes

Relapse rate was defined as the proportion of the original ECT responder or remitter sample that subsequently experienced a return of depressive symptoms deemed to be significant enough to merit the designation of relapse by the original investigators. Specific criteria for relapse varied between the studies; original investigators' definitions were retained. Studies using inadequate measures of relapse likely to underestimate its true prevalence (eg, rehospitalization rates only) were excluded.

The primary outcome was cumulative relapse proportion at the 6-month follow-up after last ECT for which we expected most data would be available. In all primary analyses, only samples treated with antidepressant pharmacotherapy were included, because virtually all ECT patients today receive long-term prophylactic therapy most commonly administered in the form of medication. We also carried out secondary analyses of relapse rates on C-ECT,

which is used less frequently than medication. C-ECT is a form of relapse prevention where the patient continues to receive ECT after the acute course at a reduced schedule. It is indicated in patients with a past history of good ECT response where antidepressant continuation therapy was either ineffective or could not be tolerated at therapeutic doses (American Psychiatric Association, 2001). Other secondary analyses investigated relapse rates on placebo or no maintenance treatment.

Additional secondary outcomes were relapse rates at 3, 12, and 24 months after last ECT, again in patients receiving antidepressant medication. Finally, to investigate the relative efficacy of different relapse prevention strategies, we aimed to calculate relative risks (RRs) of relapse in randomized controlled trials (RCTs) of different continuation therapies at 3, 6, and 12 months where at least two studies comparing the same strategy were available.

Statistical Analyses

All analyses were based on study completers. Attrition rates for each study were recorded. Mean relapse proportions with 95% confidence intervals (CIs) were calculated by pooling samples using a random-effects model (DerSimonian and Laird, 1986), as we expected substantial differences in study designs and patient populations. Heterogeneity was assessed using the I^2 statistic (Higgins *et al*, 2003). Where substantial heterogeneity was observed and where sufficient data were available, random-effects meta-regression analyses with unrestricted maximum likelihood estimation were carried out to explore possible sources of heterogeneity. Pre-specified covariates investigated were mean age, proportion of psychotic patients, and proportion of medication-resistant patients. Planned subgroup analyses compared study designs (trial *vs* observational), relapse criteria (standardized symptom rating scale *vs* clinical judgement), and whether concomitant pharmacotherapy was allowed during the index ECT course. To investigate the possibility of changes in relapse rates over time, a cumulative meta-analysis was carried out for the primary endpoint (6 months).

For head-to-head comparisons of different continuation therapies, RRs with 95% CIs and numbers needed to treat (NNT) were calculated.

Publication bias was assessed by visual inspection of funnel plots where >10 studies were available. All statistical analyses were carried out using Comprehensive Meta Analysis Version 2.2 software (Borenstein *et al*, 2011).

Results

Search Results

The computerized search retrieved 4198 results (Figure 1). Hand-searches identified four additional eligible studies. Following exclusion of database duplicates and initial exclusion of ineligible studies, 194 titles were retained for full-text screening. Of these, 32 studies met inclusion criteria and provided extractable data either from published reports or contact with original authors (Supplementary Table 1).

Relapse Rate at 6 Months

By 6 months following ECT, 34.0% (95% CI = 27.2–41.5%, $I^2 = 76\%$) of patients ($N = 844$) treated with continuation pharmacotherapy had relapsed. Because long-term outcomes are believed to have worsened over the many decades of ECT use, we performed a cumulative meta-analysis with each study added to the previous ones in chronological order (Figure 2a). Beginning with the first controlled studies of continuation pharmacotherapy in the 1960s, relapse rates held at around 20%. As modern studies of more treatment-resistant patients and clearer reporting of methodology began to be conducted, relapse rates rose towards present-day levels. It should be noted that following the publication of three important early trials (Imlah *et al*, 1965; Kay *et al*, 1970; Seager and Bird, 1962), with the exception of one small trial in 1984 (Krog-Meyer *et al*, 1984), no other prospective long-term follow-up studies of continuation pharmacotherapy meeting inclusion criteria were found between 1970 and the early 1990s, perhaps coinciding with diminishing use of ECT. Given this gap in evidence, it is unclear when precisely the shift in relapse rates might have occurred.

Due to the historical trend observed in the data, we carried out a sensitivity analysis where only modern post-DSM-III studies of pharmacologically treated patients ($N = 710$) were included in the meta-analysis. Relapse rate across these studies was 37.7% (95% CI = 30.7–45.2%, $I^2 = 70\%$) (Figure 2b). Visual inspection of the funnel plot showed no evidence of publication bias (data not shown).

Due to remaining high heterogeneity, we performed random-effects meta-regressions to investigate the possible contribution of study characteristics on outcome. As only a small number of studies reported relevant moderators, multivariate analyses could not be conducted; hence, each moderator was modelled separately. In modern studies, there was no effect of baseline medication resistance on likelihood of relapse ($p = 0.429$). However, there was a suggestion of lower relapse rates in samples with a greater percentage of psychotic patients ($p = 0.004$) and a higher mean age ($p = 0.038$).

Methodological factors appeared to influence outcome. In subgroup analyses, studies using clinical judgement to determine relapse reported lower rates (28.3%, 95% CI = 17.1–43.1%) than studies using cutoff scores on depression rating scales (41.7%, 95% CI = 34.8–48.9%). Studies where concomitant pharmacotherapy was permitted during the ECT course had lower relapse rates (29.2%, 95% CI = 18.0–43.6%) than those where maintenance pharmacotherapy was begun after the course (41.6%, 95% CI = 35.0–48.6%). Naturalistic studies (39.1%, 95% CI = 29.2–50.0%) and controlled trials (36.1%, 95% CI = 26.9–46.4%) of continuation pharmacotherapy did not differ in relapse rates.

Relapse Rates at 3, 12 and 24 Months

By 3 months following ECT, 27.1% of patients ($N = 350$) on continuation pharmacotherapy had relapsed (95% CI = 20.5–34.8%, $I^2 = 48\%$) (Figure 3a), and by 1 year ($N = 348$) 51.1% (95% CI = 44.7–57.4%, $I^2 = 27\%$) had relapsed (Figure 3b). Only three prospective studies with a

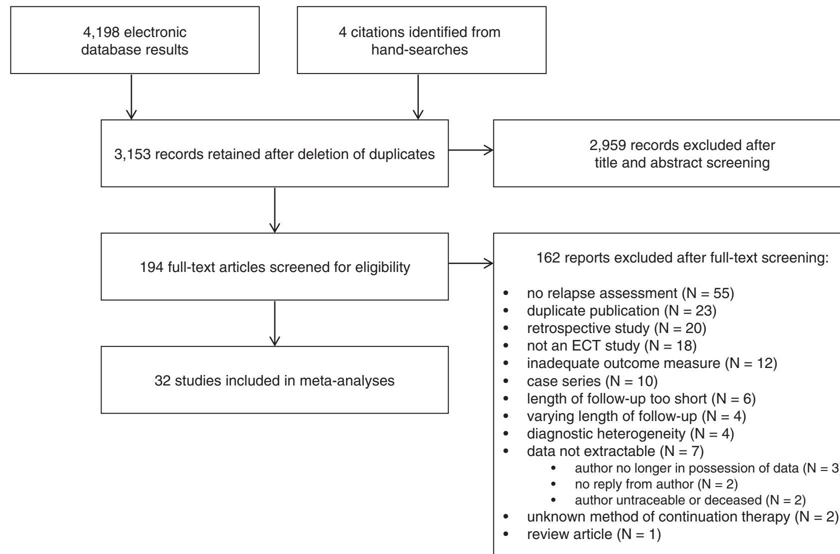


Figure 1 Study flow diagram.

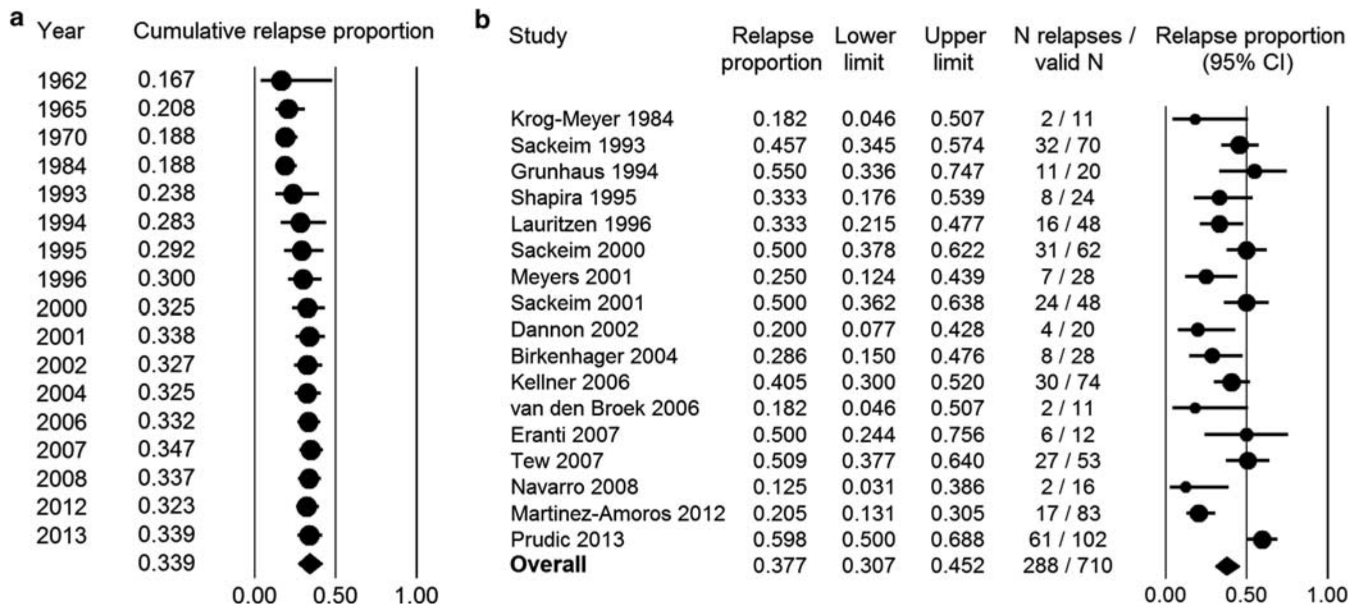


Figure 2 Outcomes at 6 months following ECT. Panel (a) shows a cumulative meta-analysis of relapse rates at 6 months following ECT across all eligible studies from 1962 onwards. Panel (b) shows relapse rate at 6 months following ECT in modern-era studies.

2-year follow-up were found: two investigating outcomes in psychotic elderly patients ($N=28$) treated with nortriptyline monotherapy (Flint and Rifat, 1998; Navarro *et al*, 2008) and one in a general adult sample ($N=83$) maintained on treatment-as-usual pharmacotherapy (Martinez-Amoros *et al*, 2012). Relapse rate at 2 years was 50.4% (95% CI = 41.2–59.6%, $I^2=0$) (Figure 3c).

Relapse Rates With C-ECT

At 6-month follow-up, relapse rate across the four eligible C-ECT samples ($N=146$) was 37.2% (95% CI = 23.4–53.5%, $I^2=57\%$), a virtually identical relapse rate to the figure for modern-era pharmacologically treated patients presented above (37.7%). Given the similarity in 6-month relapse rates in medication and C-ECT samples, we also carried out a meta-analysis of all eligible modern-era studies where patients were treated with any form of recognized continuation therapy, pharmacological or C-ECT. Across 19 eligible studies ($N=1001$), 39.5% of patients had relapsed (95% CI = 31.9–47.7%, $I^2=81\%$).

When the two studies (Kellner *et al*, 2006; Wijkstra *et al*, 2000) where patients ($N=86$) were treated with C-ECT only and where no concomitant medication was permitted were analyzed separately, relapse rate at 6 months rose to 45.4% and heterogeneity was eliminated (95% CI = 35.2–55.9%, $I^2=0$). For 1 and 2-year follow-ups, only two studies at each time point met inclusion criteria. Patients in these studies were treated with C-ECT and pharmacotherapy combination therapy. Relapse rate at 12 months ($N=33$) was 20.5%

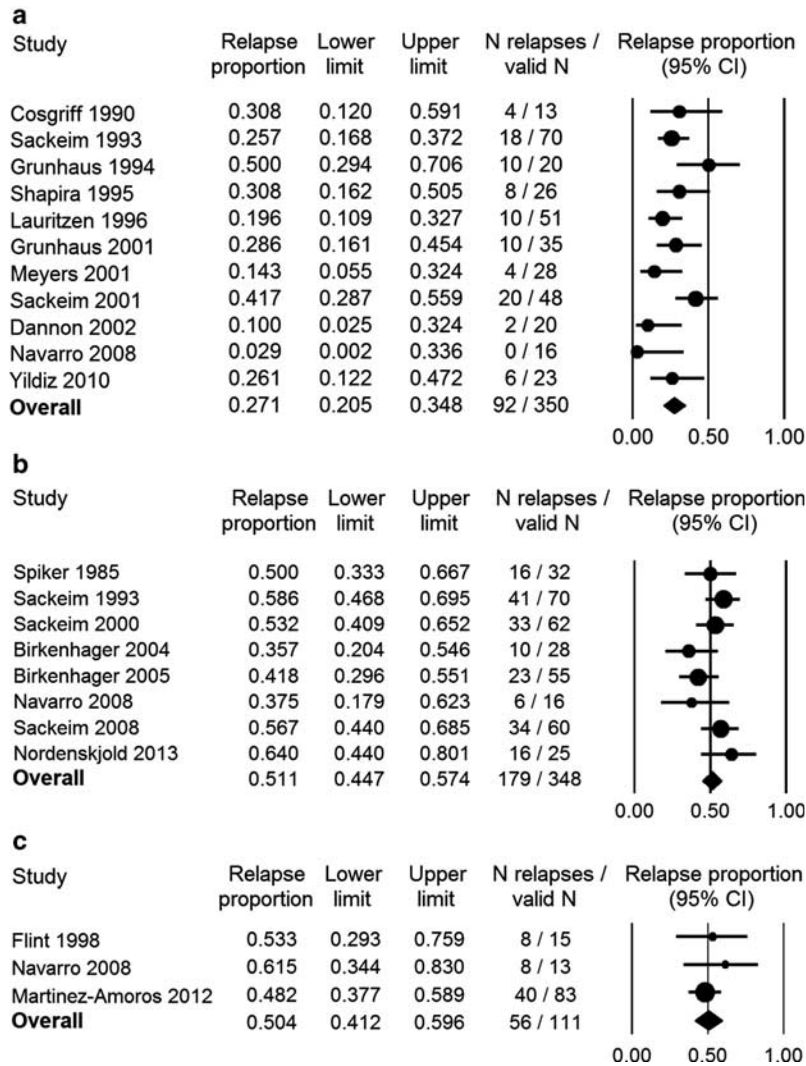


Figure 3 Outcomes at 3, 12, and 24 months following ECT. Panels a, b, and c show relapse rates at 3, 12, and 24 months following ECT, respectively.

(95% CI = 3.0–68.1%, $I^2 = 73\%$), and at 24 months ($N = 56$) it was 30.3% (95% CI = 2.9–86.4%, $I^2 = 85\%$). High levels of heterogeneity were present in the analyses.

Relapse Rates in Untreated Samples

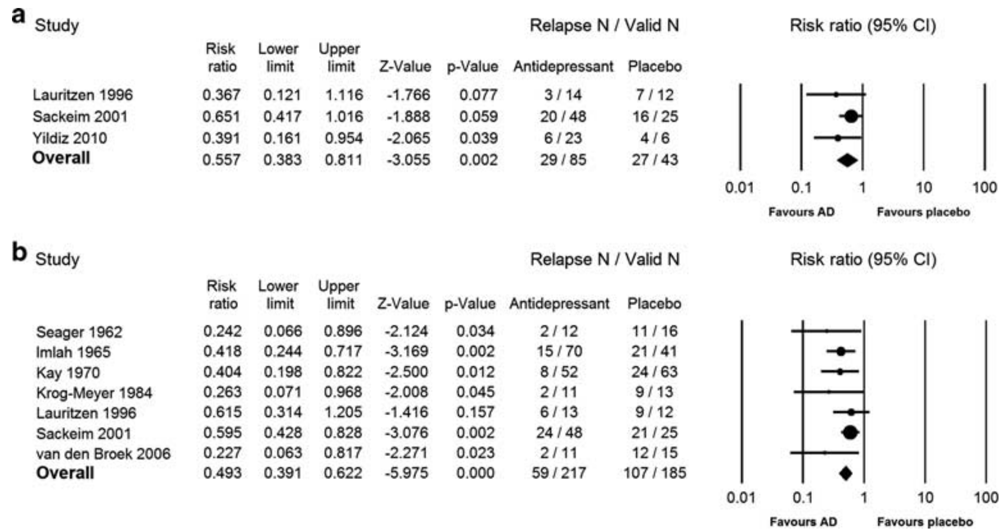
To examine the long-term efficacy of a course of ECT in the absence of continuation treatment, studies reporting outcomes in unmedicated patients were meta-analyzed. Two studies published in 1973, both with a 3-month follow-up, reported relapse in ECT responders not permitted to take antidepressant medication during follow-up (Arfwidsson *et al*, 1973; Barton *et al*, 1973). By 3 months after ECT, 47.9% had relapsed (95% CI = 38.1–57.9%, $I^2 = 0$). No modern studies featuring entirely untreated (including no placebo) samples were found.

Next we analyzed relapse rates in placebo-treated samples where some non-specific benefit can be expected. Three RCTs (Lauritzen *et al*, 1996; Sackeim *et al*, 2001; Yildiz *et al*, 2010) provided extractable data at 3 months and seven (Imlah *et al*, 1965; Kay *et al*, 1970; Krog-Meyer *et al*, 1984; Lauritzen *et al*, 1996; Sackeim *et al*, 2001; Seager and Bird, 1962; van den Broek *et al*, 2006) at 6 months. Relapse rates were 62.7% (95% CI = 47.6–75.8%, $I^2 = 0$) at 3 months and 65.5% (95% CI = 49.7–78.5%, $I^2 = 72\%$) at 6 months. As with active continuation therapy, relapse rates were substantially lower in earlier placebo samples. When only modern day RCTs (Krog-Meyer *et al*, 1984; Lauritzen *et al*, 1996; Sackeim *et al*, 2001; van den Broek *et al*, 2006) are considered ($N = 65$), relapse rate on placebo reached 78.0% (95% CI = 66.1–86.5%, $I^2 = 0$) at 6 months.

RR of Relapse on Continuation Antidepressant Pharmacotherapy *vs* Placebo

RRs of relapse in RCTs of active relapse prevention strategies *vs* placebo were investigated at 3 and 6 months after ECT (Figure 4a and b).

For the 3-month follow-up, three placebo-controlled RCTs ($N = 128$) provided extractable data: two (Lauritzen *et al*, 1996; Yildiz *et al*, 2010) evaluating selective serotonin reuptake inhibitor (SSRI) monotherapy *vs* placebo and the other (Sackeim *et al*, 2001) comparing TCA monotherapy and TCA–lithium combination to placebo. The first



a

Study	Risk ratio	Lower limit	Upper limit	Z-Value	p-Value	Relapse N / Valid N Antidepressant	Placebo
Lauritzen 1996	0.367	0.121	1.116	-1.766	0.077	3 / 14	7 / 12
Sackeim 2001	0.651	0.417	1.016	-1.888	0.059	20 / 48	16 / 25
Yildiz 2010	0.391	0.161	0.954	-2.065	0.039	6 / 23	4 / 6
Overall	0.557	0.383	0.811	-3.055	0.002	29 / 85	27 / 43

b

Study	Risk ratio	Lower limit	Upper limit	Z-Value	p-Value	Relapse N / Valid N Antidepressant	Placebo
Seager 1962	0.242	0.066	0.896	-2.124	0.034	2 / 12	11 / 16
Imlah 1965	0.418	0.244	0.717	-3.169	0.002	15 / 70	21 / 41
Kay 1970	0.404	0.198	0.822	-2.500	0.012	8 / 52	24 / 63
Krog-Meyer 1984	0.263	0.071	0.968	-2.008	0.045	2 / 11	9 / 13
Lauritzen 1996	0.615	0.314	1.205	-1.416	0.157	6 / 13	9 / 12
Sackeim 2001	0.595	0.428	0.828	-3.076	0.002	24 / 48	21 / 25
van den Broek 2006	0.227	0.063	0.817	-2.271	0.023	2 / 11	12 / 15
Overall	0.493	0.391	0.622	-5.975	0.000	59 / 217	107 / 185

Figure 4 Relative risk (RR) of relapse in patients treated with pharmacotherapy *vs* placebo at 3 and 6 months following ECT. Panels a and b, respectively, show the RR of relapse in patients maintained on active antidepressant pharmacotherapy *vs* placebo at 3 and 6 months following ECT.

meta-analysis measured RR of relapse in patients treated with any antidepressant pharmacotherapy *vs* placebo. RR of relapse on medication was 0.56 (95% CI = 0.38–0.81, $p = 0.002$, NNT = 3.5, $I^2 = 0$). Next, the two studies ($N = 55$) comparing SSRI monotherapy *vs* placebo were separately analyzed. Pooled analysis showed SSRI monotherapy to be significantly more effective than placebo in preventing relapse at 3 months (RR = 0.38, 95% CI = 0.19–0.77, $p = 0.007$, NNT = 2.7, $I^2 = 0$).

At 6 months, two meta-analyses could be carried out: one featuring any antidepressant pharmacotherapy *vs* placebo; another featuring TCA monotherapy *vs* placebo. No meta-analyses of other medication classes or combination strategies *vs* placebo could be carried out for the 6-month time point as only one study evaluated efficacy of an MAOI *vs* placebo (Imlah *et al*, 1965), one study compared an SSRI with placebo (Lauritzen *et al*, 1996), while one study featured a TCA–lithium combination treatment group *vs* placebo (Sackeim *et al*, 2001). Across the seven included studies (Imlah *et al*, 1965; Kay *et al*, 1970; Krog-Meyer *et al*, 1984; Lauritzen *et al*, 1996; Sackeim *et al*, 2001; Seager and Bird, 1962; van den Broek *et al*, 2006; $N = 402$), continuation pharmacotherapy halved the risk of relapse compared with placebo at 6 months (RR = 0.49, 95% CI = 0.39–0.62, $p < 0.0001$, NNT = 3.3, $I^2 = 0$). Patients in these studies were predominantly treated with TCAs. When TCA monotherapy samples are considered separately, this strategy was found to reduce the RR of relapse slightly further (RR = 0.44, 95% CI = 0.29–0.66, $p < 0.0001$, NNT = 3.2, $I^2 = 36\%$). In all included studies where TCAs were used, with the exception of one trial that compared nortriptyline with placebo (Sackeim *et al*, 2001), TCA monotherapy was significantly more effective than placebo. Other included studies used either imipramine (Imlah *et al*, 1965; Seager and Bird, 1962; van den Broek *et al*, 2006) or amitriptyline (Kay *et al*, 1970; Krog-Meyer *et al*, 1984) monotherapy.

No placebo-controlled RCTs of continuation pharmacotherapy with a 1-year (or longer) follow-up were identified. No meta-analyses of head-to-head comparisons of different active relapse prevention strategies could be carried out as only one study contained the same comparison.

DISCUSSION

Relapse rates following ECT are disappointingly high and appear to have increased over time. In patients treated with continuation pharmacotherapy, the main focus of our investigation, relapse was highest in the first 6 months, plateauing afterwards. In present day clinical practice, nearly 40% of ECT responders can be expected to relapse in the first 6 months and roughly 50% by the end of first year.

A course of ECT, in the absence of active continuation therapy, does not appear to have much lasting effect. In early trials where no continuation therapy was permitted, half of all patients who responded to ECT relapsed within 3 months (Arfwidsson *et al*, 1973; Barton *et al*, 1973). This suggests that the natural course of depressive illness severe enough to warrant ECT is a prompt return to depression in the absence of long-term treatment. When modern placebo samples were analyzed, relapse rates were even higher, approaching 80% at 6 months. In the current ECT practice, therefore, we recommend that initial gains are consolidated with vigorous maintenance therapy.

Nonetheless, these findings need to be interpreted in the context of superior acute remission rates with ECT compared with other existing treatments for treatment-resistant depression. A meta-analysis investigating acute outcomes found ECT to be more effective than pharmacotherapy (The UK ECT Review Group, 2003). Although our systematic review did not identify any long-term studies directly comparing outcomes in ECT *vs* medication-treated patients, when our results are compared with the existing literature on short- and longer-term antidepressant effectiveness in refractory MDD, similar outcomes are observed. In the STAR*D study (Rush *et al*, 2006), relapse rates were predictably higher in patients entering follow-up after more previous failed treatment steps. During the 1-year follow-up, remitters from the third and fourth successive treatment steps relapsed at rates of 43 and 50%, respectively. These

long-term outcomes in medication-treated patients with similar degree of treatment resistance to modern ECT samples are very similar to our findings of a 51% relapse rate 1 year following ECT. Acute remission rates for every treatment step in STAR*D, however, were much lower compared with those typically observed in ECT trials, hence more patients overall can be expected to benefit from ECT.

Our systematic review cannot offer clear guidance on what type of continuation therapy works best and for which patients. Many ECT patients routinely receive continuation therapy with the same medication(s) that failed to elicit a clinical response before ECT, a counterintuitive strategy (Sackeim, 1994). To our knowledge, no evidence is available to suggest this practice might be effective, although no particular evidence to the contrary exists either. Our meta-analysis suggests that continuation pharmacotherapy is significantly more effective than placebo at both 3- and 6-month follow-ups. Most available evidence consists of trials of older antidepressants, such as imipramine and amitriptyline. Our search of the published literature could not identify any placebo-controlled trials of some of the most commonly used newer-generation antidepressants, such as serotonin-norepinephrine reuptake inhibitors, mirtazepine, or popular augmentation strategies with mood stabilizers (other than lithium) or atypical antipsychotics. Even for SSRIs, published evidence is relatively sparse. ECT research has favored the use of TCAs; however, as TCAs produce many undesirable side-effects, carry an overdose risk, and cannot be tolerated at adequate doses by many patients, efficacy of newer antidepressants with more favorable side-effect profiles merits further investigation. Also requiring future study is the optimization of treatment schedules for C-ECT, which has thus far tended to be used with fixed dosing schedules in prospective studies. This may have underestimated its true efficacy when using more flexible, symptom-titrated dosing schedules currently under investigation (Lisanby *et al*, 2008).

When interpreting results of this meta-analysis, certain limitations should be borne in mind. Much of the available evidence comes from small, underpowered, predominantly observational studies. There was substantial variability between the included studies in design, quality, and patient selection criteria that appeared to influence outcomes. Very few RCTs of continuation therapies with long-term follow-up exist, with evidence particularly lacking for outcomes beyond 6 months. Data from prospective controlled studies are particularly lacking for certain important clinical outcomes such as suicide and indeed all-cause mortality in this severely ill and treatment-resistant patient population.

In summary, our review found that up to half of all patients who respond to ECT relapse within the first year, the period of highest risk being the first 6 months. Continuation pharmacotherapy or C-ECT significantly reduces the risk of relapse. However, many questions remain unanswered. Future studies should clarify which patient characteristics might predict relapse and what the optimal post-ECT continuation treatment or combination thereof entails. More focus is required on treatments other than TCAs, including psychotherapy and indeed optimization of treatment schedules for C-ECT, preferably in conjunction with concomitant pharmacotherapy. Such research is required to keep ECT patients in remission for as long as possible and with the fewest side-effects.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

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Review

When less is more – microRNAs and psychiatric disorders

Kolshus E, Dalton VS, Ryan KM, McLoughlin DM. When less is more – microRNAs and psychiatric disorders.

Objective: MicroRNAs are small non-coding RNA molecules that regulate gene expression, including genes involved in neuronal function and plasticity that have relevance for brain function and mental health. We therefore performed a systematic review of miRNAs in general adult psychiatric disorders.
Method: Systematic searches in PubMed/MEDLINE and Web of Science were conducted to identify published clinical articles on microRNAs in general adult psychiatric disorders. We also reviewed references from included articles.
Results: There is mounting evidence of microRNAs' regulatory roles in a number of central nervous system processes, including neurogenesis and synaptic plasticity. The majority of clinical studies of microRNAs in psychiatric disorders are in schizophrenia, where a number of specific microRNAs have been identified in separate studies. There is some evidence of marked downregulation of some microRNAs in affective disorders. Treatment with antidepressants appears to upregulate microRNA levels. There is currently little evidence from human studies in anxiety, addiction or other psychiatric disorders.
Conclusion: MicroRNA research in psychiatry is currently in a nascent period, but represents an emerging and exciting area, with the potential to clarify molecular mechanisms of disease and identify novel biomarkers and therapeutic agents.

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Summations

- A single microRNA can regulate hundreds of genes, making it a particularly appealing target for many psychiatric disorders, where no single gene underlies heritability.
- microRNA levels are frequently altered in psychiatric disorders.
- microRNA levels are affected by psychotropic medications.

Considerations

- The exact nature and extent of dysregulation of microRNAs in psychiatric disorders is yet to be determined.
- Current studies are heterogeneous, and even studies of same source material have delivered disparate findings.
- Considerable obstacles remain before microRNAs can be used as therapeutic targets in psychiatric disorders.

Introduction

Current aetiological models of the majority of psychiatric illnesses have moved beyond simple neurotransmitter models or notions of 'chemical imbalance'. Given the heterogeneity of psychiatric illnesses, pathogenesis is likely to involve dynamic processes such as neurogenesis and synaptic plasticity (1). Gene–environment interactions are thought to underlie the majority of psychiatric disorders. However, with the exception of specific entities like fragile X syndrome and some dementing disorders, no specific gene variant has been shown conclusively to have a large effect. This is despite heritability estimates of around 40% for depression to above 80% for bipolar affective disorder (BPAD) and schizophrenia (2). Genomewide association studies (GWAS) indicate that most psychiatric disorders are the result of multiple genes of small effect, with single gene variations insufficient to produce a neuropsychiatric phenotype (3). Epigenetics has recently emerged as a potential mechanism by which the combination of an accumulation of these gene variants and specific environmental stimuli may lead to the onset of illness. In recent years, the discovery of microRNAs (miRNAs) and other epigenetic modifications of gene expression have led to exciting new findings that may ultimately bridge the current gap in our knowledge of the biology and treatment of these disabling disorders.

MicroRNAs

Background and function

The first miRNA, lin-4, was discovered in 1993, but it took another 7 years until a second miRNA was identified (4). Since then, miRNAs have rapidly emerged as important regulators of post-transcriptional gene expression. They are endogenous non-protein-coding RNA molecules of approximately 21–23 bases. Over 2000 mature human miRNA sequences have been reported to date (5), and over 50% of mammalian messenger RNA (mRNA) species are potential targets for miRNAs (6). A single mRNA may be regulated by multiple miRNAs, and conversely, some miRNAs have the potential to target hundreds of mRNAs. Typically, miRNAs suppress target gene expression; however, reciprocal relationships between miRNAs levels and their targets are emerging (7). They are expressed during development and in adulthood, with the expression of some subsets being organ specific (8) and region specific within organs such as the brain (9). Functional studies indicate that miRNAs are involved in the control of cellular processes including neurogenesis, synaptic plasticity, cell fate decision and apoptosis (10–14). Furthermore, Dicer, one of the key miRNA-processing enzymes, has been shown to be necessary for correct embryonic brain development in zebra fish and for maintenance of neuron survival in the mature brain (15).

Synaptic plasticity refers to the ability of synapses to make adaptive changes in response to activity in their neuronal circuits. These changes include altered neurotransmitter release, altered receptor expression and alteration of dendritic density and size. These adaptive changes are thought to be key parts of the molecular mechanisms underlying higher functions such as learning and memory and, when dysregulated, may be involved in the pathogenesis of psychiatric disorders. Altered dendritic spine density and volume have been found to be associated with specific miRNAs, including miR-132 and miR-134 (16, 17). Target genes of these miRNAs include those encoding for: brain-derived neurotrophic factor (BDNF), widely expressed throughout the brain where it promotes neuronal survival and maturation, synaptic plasticity and synaptic function; cAMP response element-binding protein (CREB), a transcription factor involved in synaptic plasticity and memory formation; and LIM domain kinase-1 (LimK-1), an enzyme that regulates dendrite size. miRNA suppression of LimK-1 can be reversed by BDNF (17). miRNAs are also key regulators of postnatal neurogenesis, and specifically, miR-124 has been shown to dictate adult neurogenesis in mice (18). Antidepressant therapies stimulate neurogenesis, whereas stress and depression inhibit neurogenesis. Given miRNAs' role in neurogenesis, there is therefore added impetus to their study in psychiatric disorders (19).

miRNA biogenesis

Two pathways from which miRNAs arise have been described to date. In the canonical pathway, primary miRNAs (pri-miRNA) are transcribed by RNA polymerase II or III from independent miRNA genes and typically form a stem and terminal loop structure with flanking segments (see Fig. 1). Within the nucleus, a microprocessor complex that is generally made up of Drosha, an RNase type III endonuclease, and a cofactor for activation, such as DiGeorge syndrome critical region 8 protein (DGCR8), removes the flanking segments and stem region to generate a precursor miRNA (pre-miRNA) (20). Pre-miRNAs are between 70–110 nucleotides in length and form a hairpin secondary

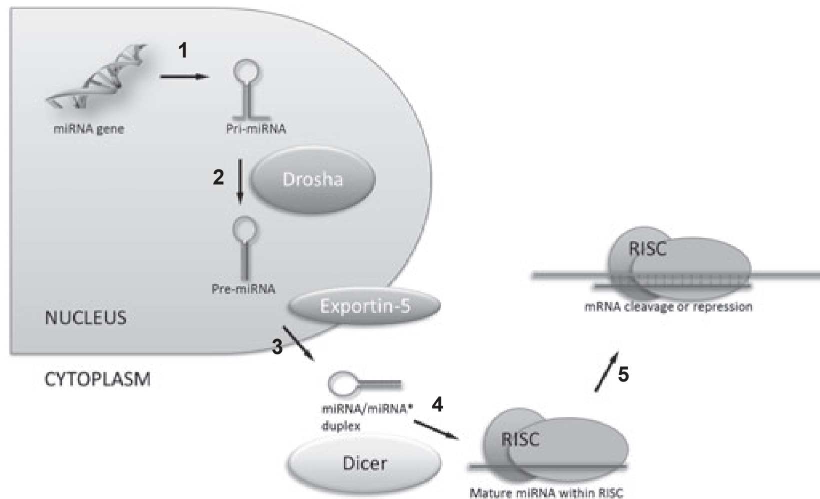

Fig. 1. MicroRNA biogenesis. Mature microRNAs (miRNAs) are formed following a series of steps involving (1) RNA transcription to generate a primary miRNA (pri-miRNA), followed by (2) Rnase modification by Drosha to form a precursor miRNA (pre-miRNA) which is (3) exported from the nucleus to the cytoplasm by exportin-5 and cleaved to form a 22 base pair miRNA/miRNA* duplex by Dicer (4). From the duplex, one miRNA is then preferentially loaded into a *RNA-induced silencing complex* (*RISC*) where it (5) binds to a target messengerRNA (mRNA) transcript, preventing translation.

structure. In the second Drosha/DGCR8-independent pathway, pre-miRNAs are derived from introns as a result of splicing and debranching by nuclear splicing machinery (21). In both cases, pre-miRNAs are then exported from the nucleus to the cytoplasm via exportin 5, a double-stranded RNA-binding protein that mediates nuclear export (22). In the cytoplasm, the pre-miRNA hairpin loop is cleaved to form a approximately 22 base pair duplex by the RNase III enzyme Dicer in combination with a cofactor such as TAR RNA-binding protein (TRBP) (23). One strand from the duplex is then preferentially loaded into the RNA-induced silencing complex (RISC) in a sequence of steps regulated by Dicer, Argonaute homologue proteins, TRBP and other regulatory proteins.

Once loaded into the RISC, the miRNA guides the complex to target sequences known as miRNA response elements (MRE), generally located within the 3′ untranslated region of mRNA transcripts. The mechanism of miRNA-induced regulation of mRNA translation depends on complementarity between the mRNA target and miRNA. Degradation of mRNA usually occurs when the miRNA–RISC binds with near-exact complementarity. On the other hand, when the miRNA binds with incomplete complementarity, mRNA translation to protein can also be prevented due to steric hindrance (24).

miRNA dysregulation

Dysregulation of miRNAs can be through altered expression or changes to miRNA targets. Deletions, amplifications or single nucleotide polymorphisms (SNPs) in a miRNA can lead to its over- or underexpression with ensuing changes to its targets. Alternatively, alterations in the miRNA-processing machinery or SNPs in the target region for a miRNA could lead to inappropriate targeting of mRNAs (25).

The discovery of miRNAs and their role in regulation of gene expression has led to a revision of the prevailing ‘central dogma’ of genetics. Recent findings have highlighted the regulatory role of miRNAs and other non-coding genetic material (26). It is therefore no surprise that dysregulation of miRNAs has been linked with human pathologies such as major depressive disorder (MDD), schizophrenia and cancer (27–29). It is hoped that further understanding of the role of miRNAs will elucidate molecular mechanisms of psychiatric illness, act as biomarkers and potentially become novel therapeutic targets (19). In other medical fields, miRNAs have already been identified as biomarkers (30) and therapeutic targets (31).

miRNA analysis

The techniques used to characterise miRNAs are similar to those used to study RNA or DNA, with some modifications. Studies investigating the role and distribution of miRNAs may be undertaken using cell culture, animal models and human samples, including postmortem brain specimens, cerebrospinal fluid (CSF) and peripheral blood. The study of postmortem specimens has the advantage of direct access to human brain, but has limitations

in terms of variations in pre- and postmortem conditions, diagnostic uncertainty and usually small sample sizes (32). CSF sampling has the advantage of the ability of tracking dynamic changes, but requires an invasive procedure that may not be acceptable to patients, especially for repeated sampling, and may limit ethical approval; therefore, studies using CSF are relatively rare. In contrast, peripheral blood sampling provides the benefit of easy and acceptable access, and miRNAs are present in a remarkably stable form in human blood. There is considerable communication between the central nervous system (CNS) and the immune system, and lymphocytes express several of the same neurotransmitter receptors seen in the CNS (33). Interestingly, miRNAs specific to brain diseases can be detected in peripheral blood, and peripheral blood levels can correlate with brain levels (34).

To analyse miRNA levels in these sample, researchers may use deep sequencing technology, microarrays or quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) techniques (35). Deep sequencing (also known as next-generation sequencing) is a relatively recent technological advance that allows for quantification of every miRNA in a sample. Such analysis enables robust quantification of known miRNAs as well as potential novel miRNA discovery. It is, however, a costly procedure, which limits its widespread use. Microarrays are less costly, but rely on *a priori* knowledge of sequences of interest. These consist of chips with probes for known miRNAs, usually covering hundreds of miRNAs. miRNAs in a sample will then hybridise to the probes, allowing for absolute or relative quantification. Typically, miRNAs identified by sequencing or array approaches are then confirmed using qRT-PCR. Because miRNAs are too short to accommodate standard primer pairs, they are first lengthened by stem–loop reverse transcription (36). An essential element of miRNA analysis is the use of bioinformatic approaches and databases to link identified miRNAs with their target genes and biological function. This '*in silico*' approach is crucial due to the ability of miRNAs to target hundreds of genes and complex pathways (35). A separate approach includes genotyping patients and controls to identify structural variants in miRNAs, miRNA targets or miRNA biosynthesis machinery that may be associated with psychiatric phenotypes.

miRNA nomenclature

Names are assigned to experimentally confirmed miRNAs using the following nomenclature: the prefix 'miR', indicating a mature miRNA, is followed by a dash and a number, usually indicating the order of naming. pre-miRNAs refer to precursor miRNAs and use the prefix 'mir'. miRNAs with nearly identical sequences except one or two nucleotides are annotated with an additional lower case letter; for example, miR-26a and miR-26b would be very closely related. Some of the first miRNAs to be discovered were the let family of miRNAs. At that time, the existence of the amount of miRNAs now discovered was not known, and they therefore do not follow the numerical system. If two miRNAs originate from opposite arms of the same pre-miRNA (which is in a hairpin loop, see Fig. 1), they are denoted with a −3p or −5p suffix. If relative expression levels of these opposite strands are known, an asterisk (*) following the miRNA denotes a miRNA expressed at low levels relative to its opposite arm of the hairpin loop. It is proposed that the use of the asterisk denomination be replaced by consistent use of the −3p or −5p suffix (5).

Aims of the study

In the following review, we aim to give a brief overview of the history and biogenesis of microRNAs (miRNAs). We then expand on this through a systematic review of the clinical evidence for the role of miRNAs in general adult psychiatric disorders, with an emphasis on human studies. Given space limitations and the overlap with neurological disorders, we did not include the dementias or other neurodegenerative diseases in this review.

Material and methods

Relevant clinical studies were identified using searches of PubMed/MEDLINE and Web of Science with the following terms cross-referenced with 'microRNA': 'depression', 'bipolar', 'schizophrenia', 'psychosis', 'anxiety', 'panic', 'obsessive–compulsive disorder (OCD)', 'addict*', 'anorexia', 'bulimia' and 'psychiatr*'. No language limit was used. We also reviewed the references from included articles.

Results

We retrieved 544 potentially relevant records of which 312 were duplicates, leaving 232 abstracts for screening. Seventy-five abstracts were removed on screening leaving 157 full-text records for review. Of these, 42 full-text records, which were either non-miRNA, non-clinical or non-psychiatry related, were removed. A final list of 115 records was therefore included in the

systematic review, the results of which are presented in Tables 1–4 and briefly described in the following sections.

miRNAs and schizophrenia

The heritability rate for schizophrenia is estimated to be about 80%, but to date, susceptibility genes have been of small effect (3). Given miRNAs' roles in brain development, from synaptic plasticity, neurogenesis and modulating neuronal function (8, 37, 38), there has been major interest in their potential role in the abnormal brain development seen in schizophrenia. Further support has come from the fact that patients with DiGeorge 22q11.2 deletion have a 30-fold increase in the risk of schizophrenia as well as deletions that affect *DGCR8*, a key miRNA-processing gene (39). A 'hot spot' for neuropsychiatric disorders, including autism and schizophrenia, has also been described on chromosome 8. This area includes several miRNAs, lending support to their role in schizophrenia (40). Currently, schizophrenia is one of the areas in psychiatry where miRNAs have been most widely studied. These include postmortem studies, CSF studies, peripheral blood studies and genotyping-based studies. These are summarised in Table 1.

Postmortem studies. Although several investigators have analysed miRNA levels in postmortem brains of patients with schizophrenia, there is a lack of consistent findings. In part, this can be explained by different approaches, with some investigators focusing on one or two miRNAs (41–44) and others on hundreds of miRNAs (45–52). Sample sizes varied from 20 to 74, and different regions of the brain were examined, although most focused on the prefrontal cortex (PFC). Gender differences have also been described (43). For details and results from individual studies, see Table 1. Some miRNAs were identified in more than one study, which might indicate their importance in schizophrenia. This includes members of the miR-15 family (including miR-195 and closely related miR-107) that were upregulated in the frontal cortex (42, 47, 48). The functional targets of these miRNAs include a number of genes that have been implicated in schizophrenia, such as *BDNF*, the dopamine receptor *DRD1*, the synaptic protein neuregulin 1 (*NRG1*) and early growth response gene 3 (*EGR3*). *EGR3*, apart from having been identified as a susceptibility gene for schizophrenia (53), has been identified as a possible regulator for a miRNA transcription factor network in schizophrenia (54). In the PFC, miR-195 levels were inversely associated with BDNF protein levels that were in turn correlated with dysregulation of GABAergic genes (42).

Of note, even when the brain tissues were the same, different studies showed disparate results, as seen in four brain expression studies that included BPAD subjects either as comparisons or controls (44, 49–51). All four studies investigated expression of miRNA from the same patients from the same brain bank (Stanley Medical Research Institute – SMRI). Three of the studies examined expression in the dorsolateral PFC (DLPFC) Brodmann's area (BA) 46 [Moreau et al. (51) used samples from BA 9]. Despite this, there were major differences in findings from the four studies, with different miRNAs identified as significantly altered, and in some cases, miRNAs that were significantly downregulated in one study (49) were significantly upregulated in another (50).

Cerebrospinal fluid and peripheral blood studies. The search for a peripheral biomarker in schizophrenia has led several investigators to describe miRNA profiles in CSF (55) and peripheral blood (56–58). In one study, seven miRNAs had a high discriminating accuracy for schizophrenia (miR-34a, miR-449a, miR-564, miR-432, miR-548d, miR-572, miR-652) (56). Elsewhere, a large cluster of miRNAs on chromosome 14q32 were reported to have the potential to serve as biomarkers in schizophrenia (57).

Genotyping and rare variants. The largest GWAS to date in schizophrenia, with over 40 000 individuals, found that the strongest association of any SNP with schizophrenia lied within the intron of miR-137 (59). There were also SNPs found in a number of miR-137 targets such as *TCF4*. Variation in miR-137 was subsequently found to be associated with brain activation responses in a schizophrenia high-risk group, but not in controls or a group with BPAD (60), and miR-137 has also been associated with schizophrenia using a combination of GWAS and fMRI/quantitative traits (61) as well as grade of membership (GoM) analysis (62). GoM is a mathematical model using multivariate analysis and was used to find a specific association with a subtype of schizophrenia with severe cognitive impairment. miR-137 has been found to regulate neurodevelopment and neurogenesis (63), and microdeletions in the chromosomal region harbouring miR-137 has also been associated with intellectual disability (64).

A phenotype-based genetic association study (PGAS) approach, which examines the role of genetic variants on schizophrenia phenotypes rather than schizophrenia as a whole, was used to

Table 1. Clinical studies of miRNAs in schizophrenia

Author	Patients	Tissue	Analysis	Main findings
Postmortem case–control brain studies				
Burmistrova et al. (2007) (41)	Scz = 12 Controls = 12	Parietal neocortex (BA 7)	Microarray	No difference in expression of miR-130b
Perkins et al. (2007) (45)	Scz = 13 SA = 2 Controls = 21	PFC (BA 9)	Microarray qRT-PCR	miR-26b, miR-30b, miR-92, miR-24 and miR-30e downregulated Targeted genes/gene pathways: regulators of actin cytoskeleton, focal adhesion, MAPK signalling, phosphatidylinositol signalling, all regulators of synaptic plasticity at dendritic spines
Beveridge et al. (2008) (46)	Scz = 21 Controls = 21	STG (BA 22)	Microarray qRT-PCR	miR-181b upregulated Targeted genes/gene pathways: VSNL1, GRIA2 LTP, MAPK signalling pathway, axon guidance and neurodegenerative disorders
Zhu et al. (2009) (44)	Scz = 35 BPAD = 35 Controls = 34	DLPFC (BA 46)	qRT-PCR	miR-346 downregulated Target genes/gene pathways: CSF2RA, GRID1 Cytokine, glutamate transmission
Mellios et al. (2009) (42)	Scz = 20 Controls = 20	PFC (BA 10)	qRT-PCR	miR-195 and GABAergic transcripts downregulated, BDNF inversely associated with miR-195 levels Targeted genes/gene pathways: BDNF, GRIN1, GABRA1, HTR2C, glutaminergic, dopaminergic and serotonergic signalling
Kim et al. (2010) (50)	Scz = 35 BPAD = 35 Controls = 35	DLPFC (BA 46)	Microarray qRT-PCR	miR-132, miR-132*, miR-154*, miR-212, miR-34a, miR-544, miR-7 upregulated Targeted genes/gene pathways: Tyrosine hydroxylase, GRM3, PGD
Beveridge et al. (2010) (47)	Scz = 21/15 Controls = 21/15 (STG/DLPFC)	STG (BA 22) DLPFC (BA 9)	Microarray qRT-PCR	Global increase in miRNA biogenesis; miR-107, miR-15a/b, miR-16, miR-195, miR-181b, miR-19a, miR-20a, miR-26b and let-7e upregulated in both STG/DLPFC Target genes/gene pathways: *RGS4*, GRM7, GRIN3A, HTR2A, RELN, VSNL1, DLG4, DRD1, PLXNA2 Neural connectivity and synaptic plasticity, such as axon guidance, long-term potentiation, Wnt, ErbB and MAPK signalling
Santarelli et al. (2011) (48)	Scz = 37 Controls = 37	DLPFC (BA 46)	Microarray qRT-PCR	miR-17, miR-107, miR-134, miR-328, miR-382, miR-652 upregulated Targeted genes/gene pathways: glutamate, serotonin, GABA receptors. NRG1/2, BDNF Axon guidance, LTP, nervous system development, neurogenesis, neuron differentiation
Moreau et al. (2011) (51)	Scz = 35 BPAD = 35 Controls = 35	PFC (BA 9)	qRT-PCR	miR-193b, miR-545 upregulated
Mellios et al. (2012) (43)	Scz = 30/11 Controls = 42/12 (PFC/parietal)	PFC/Parietal cortex (BA 10)	qRT-PCR	miR-30b downregulated in PFC of female patients only Target genes/gene pathways: Glutamate receptors 3/5
Miller et al. (2012) (49)	Scz = 35 BPAD = 31 Controls = 34	DLPFC (BA 46)	Microarray qRT-PCR	mIR-132 downregulated Target genes/gene pathways: DNMT3, GATA2, DPYSL3, CREB, ERK, p250GAP synaptic plasticity, LTP

Table 1. (Continued)

Author	Patients	Tissue	Analysis	Main findings
Wong et al. (2013) (52)	Scz = 37 Controls = 97	DLPFC	Microarray qRT-PCR	miR-17 upregulated Target genes/gene pathways: NPAS3
CSF/Peripheral blood studies				
Lai et al. (2011) (56)	Scz = 90 Controls = 90	WBC	Microarray qRT-PCR	miR-34a, miR-449a, miR-564, miR-432, miR-548d, miR-572, miR-652 form biomarker panel Target genes/gene pathways: Cdk5, notch signalling, nervous system development and function
Gardiner et al. (2012) (57)	Scz = 112 Controls = 76	PBMCs	Microarray qRT-PCR	miR-107, miR-134, miR-31, miR-431, miR-433, miR-487b, miR-99b downregulated Target genes/gene pathways: axon guidance, LTP, focal adhesion, neurotrophin, ErbB, calcium and MAPK signalling
Shi et al. (2012) (58)	Scz = 115 Controls = 40	Serum	Deep sequencing qRT-PCR	miR-181b, miR-219-2-3p, miR-1308, let-7g upregulated, miR-195 downregulated
Genotyping studies				
Hansen et al. (2007) (66)	Scz = 840 Controls = 1476	DNA (blood)	miR-SNP	miR-198, miR-206 Target genes/gene pathways: CCND2, PTPN1, CREB5
Feng et al. (2009) (68)	Scz = 193 Controls = 191	DNA	miR-SNP	let-7f-2, miR-18b, miR-505, miR-502, miR-188, miR-660, miR-509-3 Targeted genes/gene pathways: NGR1, DISC1, RGS4
Potkin et al. (2009) (61)	Scz = 92 Controls = 74	DNA	GWAS and quantitative trait imaging	miR-448, miR-218, miR-137 genes were significantly enriched for association
Xu et al. (2010) (67)	Scz = 456 Controls = 453	DNA	miR-SNP	Pre-miR-30e, miR-30e, miR-24 associated with schizophrenia. miR-30e also showed increased expression in PBMCs in Scz Target genes/gene pathways: MAPK14, NOTCH1, Ubc9
Begemann et al. (2010) (65)	Scz/SA = 1071 Controls = 1079	DNA	PGAS	Six SNPs in CPLX2 gene were highly associated with cognitive functioning in Scz Target genes/gene pathways: presynaptic regulatory proteins
Caputo et al. (2011) (69)	Scz = 70 Controls = 176	DNA	miR-SNP	SNPs in BDNF gene prevent binding of translational repressors miR-26a/miR26-b, but are not associated with Scz. Target genes/gene pathways: BDNF
Ripke et al. (2011) (59)	Scz = 17 791 Controls = 33 859	DNA	GWAS	Strongest finding for Scz association in SNP in miR-137. Four other significant loci contain targets for miR-137 Target genes/gene pathways: adult neurogenesis, neuronal maturation
Green et al. (2012) (62)	Scz/SA = 617 Controls = 764	DNA	GoM	SNP in miR-137 associated with cognitive deficits in Scz patients with severe negative symptoms

Up/downregulation in all studies refer to cases (schizophrenia) compared with controls.
BA, Brodmann's area; BDNF, brain-derived neurotrophic factor; BPAD, bipolar affective disorder; CREB, cAMP response element-binding protein; CSF, cerebrospinal fluid; DLPFC, dorsolateral prefrontal cortex; GoM, grade of membership; GWAS, genomewide association studies; PBMCs, peripheral blood mononuclear cells; PFC, prefrontal cortex; PGAS, phenotype-based genetic association study; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; SA, schizoaffective disorder; Scz, schizophrenia; SNP, single nucleotide polymorphism; STG, superior temporal gyrus; WBC, white blood cells.

look for SNPs in a gene (*complexin 2*) involved in synaptic regulation. Six SNPs were found to be associated with reduced cognitive performance in schizophrenia, and one of these SNPs was in miR-498 (65).

Elsewhere, SNPs in miRNAs such as miR-206, miR-198 and miR-30e that were associated with schizophrenia have been identified in Scandinavian (66), Chinese (67) and American samples (68), whereas studies of SNPs in miR-26 (69) and miR-130b (41) found no difference between schizophrenia and control groups.

Antipsychotic treatment. The interaction between antipsychotic treatment and miRNAs is also of interest, especially as many of the postmortem

studies include individuals who have been treated with these medications. Haloperidol was found to upregulate the level of a set of miRNAs in rats (45), and these were subsequently also found to be upregulated in the cortex of haloperidol-treated individuals with schizophrenia (47). Animal studies have shown that a reduction in miR-219 mediates schizophrenia-like effects of an NMDA (N-Methyl-D-aspartate) antagonist such as hyperlocomotion and stereotypy (70). Antipsychotics prevented this knockdown of miR-219 function. Of note, miR-219 has been found to be upregulated in the DLPFC of individuals with schizophrenia (47).

miRNAs and bipolar affective disorder

Bipolar affective disorder shares genetic risk factors with schizophrenia and has heritability estimates of 70–80% (2, 71). Several genes of modest effect have been identified, such as *CACNA1C*, *ANK3* and genes involved in circadian rhythm (72, 73). However, as in schizophrenia, the problematic issue of numerous genes of small effect has led to a growing interest in transcriptional regulators such as miRNAs in BPAD. To date, miRNA studies in BPAD have been mostly limited to postmortem studies, many of which included schizophrenia subjects. Others have focused on the impact of mood stabiliser treatment on miRNA levels. The studies are summarised in Table 2.

Postmortem studies. The majority of these studies used samples from the SMRI brain bank, which included healthy controls as well as individuals with a history of BPAD or schizophrenia. Several of these studies found an overlap in miRNA levels between BPAD and schizophrenia subjects (50, 51), but this did not survive correction for multiple

Table 2. Clinical studies of miRNAs in bipolar affective disorder (BPAD)

Author	Patients	Tissue	Analysis	Main findings
Postmortem case–controlbrain studies				
Zhu et al. (2009) (44)	Scz = 35 BPAD = 35 Controls = 34	DLPFC (BA 46)	qRT-PCR	Non-significant reduction in miR-346 levels in patients with BPAD. Significantdownregulation of GRID1 gene Target genes/gene pathways: CSF2RA,GRID1, cytokine, glutamate transmission
Kim et al. (2010) (50)	Scz = 35 BPAD = 35 Controls = 35	DLPFC (BA 46)	Microarray qRT-PCR	miR-504, miR-145/145*, miR-22*, miR-133b, miR-154*, miR-889 upregulated miR-454*, miR-29a, miR-520c-3p, miR-140-3p, miR-767-5p, miR-874, miR-32, miR-573 downregulated Targeted genes/gene pathways: GRIN, DRD1, DLG3/4, ITPR1, CEP290, HTT, SHANK3
Moreau et al. (2011) (51)	Scz = 35 BPAD = 35 Controls = 35	PFC (BA 9)	qRT-PCR	miR-330, miR-33. miR-193b, miR-545, miR-138, miR-151, miR-210, miR-324.3p, miR-22, miR-425, miR-181a, miR-106b, miR-193a, miR-192, miR-301, miR-27b, miR-148b, miR-338, miR-639, miR-15a, miR-186, miR-99a, miR-190, miR-339 downregulated
Miller et al. (2012) (49)	Scz = 35 BPAD = 31 Controls = 34	DLPFC (BA 46)	Microarray qRT-PCR	No significant differences in BPAD group
Mood stabiliser treatment studies				
Rong et al. (2011) (79)	BPAD = 25 Controls = 21	Whole blood	qRT-PCR	Plasma miR-134 levels decreased in mania patients vs. controls. Treatment with lithium led to increase in miR-134 levels Targeted genes/gene pathways: Limk-1, dendritic spine size regulation

Up/downregulation in all studies refer to cases (BPAD) compared with controls.
BA, Brodmann's area; DLPFC, dorsolateral prefrontal cortex; PFC, prefrontal cortex; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; Scz, schizophrenia.

testing in some studies (44, 49). These results do, nonetheless, support the hypothesis of a partially shared molecular basis to BPAD and schizophrenia (3). Elsewhere, a SNP in the dopamine transporter gene that was associated (OR 1.65–2.1) with BPAD has been identified (74). This SNP was identified *in silico* as being the target of several miRNAs, but further research is needed to clarify this association.

Mood stabiliser treatment. Preclinical studies have shown the effects of mood stabilisers (lithium and valproate) on miRNA levels in rats. This includes an upregulation of several miRNAs (let-7b, let-7c, miR128a, miR-24a, miR-30c, miR34a and miR-221) and downregulation of miR-144 in response to mood stabiliser treatment (75). miR-34a regulated levels of *GRM7*, a glutamate receptor coding gene that has been identified as a possible candidate gene for BPAD (76). Let-7b has also been shown to suppress muscarinic receptor function in a rat model, which was reversed by lithium treatment (77). Further evidence of the effect of lithium on miRNAs came from research using lymphoblastoid cell lines, which found three miRNAs upregulated (miR-221, miR-152 and miR-15a) and one downregulated (miR-494) in a small BPAD sample ($n = 10$, unaffected siblings $n = 10$) in response to 4–16 days of lithium treatment (78). Elsewhere, miR-134 has been suggested as a useful blood marker of clinical status in BPAD. In an initial study of 21 drug-free BPAD individuals with mania and 21 controls, miR-134 levels were significantly decreased in manic subjects (79). Although numbers were low and treatment was open label, this study supports miR-134 as a potential peripheral biomarker in BPAD.

miRNAs and depression

Depression has an estimated heritability of 37% (2). Initial evidence for the interaction between depression and miRNAs came from two important studies of the selective serotonin reuptake inhibitor (SSRI) fluoxetine (80, 81). Treatment with SSRIs typically takes weeks before symptomatic relief is achieved, suggesting that changes to serotonin signalling and downstream cascades are necessary for antidepressant action. miR-16 was identified as a regulator of the serotonin transporter (SERT) through computer analysis. Using human neuroectodermal cell lines (IC11) and in vivo study of the raphe nuclei of mice, the authors showed that fluoxetine increased levels of miR-16, which previously had been blocked by Wnt signalling pathways (80). Increased miR-16 levels in turn led to decreased SERT levels, which would result in increased serotonin signalling at the synapse. In addition, miR-16 induced an adaptational change in locus coeruleus neurons, from noradrenergic type to serotonergic type in IC11 cells. The authors also found that miR-16 could alter behaviour in depression models in mice. Following on from this study, the same group examined the role of miR-16 in hippocampal neurogenesis in mice (81). Although fluoxetine increases miR-16 maturation in the raphe nuclei, it decreased miR-16 levels in the locus coeruleus and hippocampus. These changes were mediated by BDNF, Wnt2 and the prostaglandin 15d-PGJ2. Furthermore, in nine patients with MDD, 12 weeks of fluoxetine treatment led to increased levels of these signalling molecules in CSF. miR-16 was therefore placed as a 'micromanager' of these changes (81). Apart from fluoxetine, the effect of paroxetine on human glioblastoma–astrocytoma cell lines has shown that miR-30 was overexpressed following 6 and 12 h of incubation (82).

Despite this early promising work, there have been only a handful of clinical studies so far investigating the role of miRNAs in depression, and these are summarised in Table 3.

Although the studies vary in their findings, there is preliminary evidence of a marked downregulation of numerous miRNAs in depression (83). In contrast, antidepressant treatments have been shown to be associated with an increase in the levels of several miRNAs (84, 85). Both miR-335 and miR-494 levels were significantly altered (in opposite directions) in suicide victims and escitalopram responders. Interestingly, this postmortem study also found evidence for the presence of correlated networks of miRNAs in suicide victims compared with controls. miRNAs in these networks were not necessarily significantly downregulated, but formed highly significant correlated pairs with other miRNAs.

Two studies have characterised miRNA profiles from peripheral blood (84, 85). The first study utilised peripheral blood samples from a small sample of 10 patients, all of whom were responders to escitalopram (84). Following 12 weeks of escitalopram, 28 miRNAs were significantly upregulated and two downregulated compared with drug-free baseline levels. Many of these miRNAs are important gene expression regulators in the brain and have been implicated in other psychiatric disorders. Of particular interest, miR-132 has repeatedly been identified as dysregulated in schizophrenia (49, 50). miR-128 was upregulated by both haloperidol and mood stabiliser treatment in previous studies (45, 75) and was upregulated by

Table 3. Clinical studies of miRNAs in depression

Author	Patients	Tissue	Analysis	Main findings
Postmortem case–control brain studies				
Smalheiser et al. (2012) (83)	Suicides = 18 Controls = 17	PFC (BA 9)	qRT-PCR	miR-142-5p, miR-137, miR-489, miR-148b, miR-101, miR-324-5p, miR-301a, miR-146a, miR-335, miR-494, miR-20a/b, miR-376a, miR-190, miR-155, miR-660, miR-130a, miR-27a, miR-497, miR-10a, miR-142-3p downregulated Targeted genes/gene pathways: CDK6, ELF1/6, NCOA2, DNMTB3, EZH2, MYCN, ICOS, SOX4, PTPRN2, MERTK, VEGFA, SLC16A1, SFRS11, TTK, AGTR1, BACH1, LDOC1, MATR3, TM6SF1, TAC1, CSF1, MAFB, MEOX2, HOXA1/5, SP1/3/4, RUNX1
Antidepressant treatment studies				
Launay et al. (2011) (81)	MDD = 9	CSF	qRT-PCR	Following fluoxetine administration, miR-16 targeting molecules BDNF, Wnt2, 15d-PGJ2 levels increased in CSF Targeted genes/gene pathways: miR-16: SERT, Bcl-2
Bocchio-Chiavetto et al. (2012) (84)	MDD = 10	Whole blood	qRT-PCR	miR-130b*, miR-505*, miR-29-b-2*, miR-26a/b, miR-22*, miR-664, miR-494, let7d/e/f/g, miR-629, miR-106b*, miR-103, miR-191, miR-128, miR-502-3p, miR-374b, miR-132, miR-30d, miR-500, miR-589, miR-183, miR-574-3p, miR-140-3p, miR-335, miR-361-5p upregulated miR-34c-5p, miR-770-5p downregulated Targeted genes/gene pathways: BDNF, NR3C1, NOS1, IGF1, FGF1, FGFR1, VEGFa, GDNF, CACn41C, CACNB4, SLC6A12, SLC8A3, GABRA4, 5HT-4. Neuroactive ligand–receptor interaction, axon guidance, LTP, signalling pathways
Genotyping studies				
Xu et al. (2010) (86)	MDD = 1088 Controls = 1102	DNA	miR-SNP	Positive association between SNP in miR-30e precursor and MDD
He et al. (2012) (89)	MDD = 314 Controls = 252	DNA	miR-SNP	SNP in miRNA-processing gene *DGCR8* increased frequency SNP in miRNA-processing gene *AGO1* decreased frequency. Associated with suicide risk and treatment response
Other studies				
Belzeaux et al. (2012) (85)	MDD = 9 Controls = 9	PBMCs	Microarray qRT-PCR	miR-941 and miR-589 upregulated

Up/downregulation in all studies refer to cases (depressed) compared with controls.

BA, Brodmann's area; BDNF, brain-derived neurotrophic factor; CSF, cerebrospinal fluid; LTP, long-term potentiation; MDD, major depressive disorder; PBMCs, peripheral blood mononuclear cells; PFC, prefrontal cortex; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; SNP, single nucleotide polymorphisms.

escitalopram here (84). A recent study investigating transcriptional signatures at different time points in a depressive episode found two miRNAs upregulated at remission compared with healthy controls (85). This was in a sample of nine patients, who all responded to a range of antidepressant treatments. Several mRNA candidates were also identified that may have the potential to serve as biomarkers in depression.

A number of studies have searched the human genome for SNPs that may be associated with depression. One such large study found a positive association between miR-30 and MDD (86). This was carried out in an ethnically homogenous Han Chinese population, in which an earlier study had found SNPs in miR-30 to be associated with schizophrenia (67). Depression has been linked with disruption to circadian rhythms, and sleep disturbance is a key symptom in affective disorders (87). A SNP in miR-182 was found to be associated with late insomnia in patients with MDD. Patients with this SNP had downregulated expression of genes linked to circadian rhythm like *Clock* that previously had been associated with affective disorders (88). Finally, SNPs in miRNA-processing genes like *DGCR8* and *AGO1* were associated with increased risk of suicidal tendency and antidepressant treatment response in a sample of 314 patients and 252 controls (89). Numbers were relatively low for genotyping studies, however, and need replication.

miRNAs and anxiety disorders

Anxiety disorders are a heterogeneous collection of disorders with varying prevalence and presentation. The role miRNAs play in these disorders has not been much studied in a clinical setting. Most of the work to date has come from animal models of stress or anxiety. These studies have implicated miRNAs in mediation of the glucocorticoid response to stress (90, 91), the amygdalar response to stress (92, 93), frontal cortex changes (94) and fear extinction (95). Stress response and resilience are implicated in both depression and anxiety disorders. In a learned helplessness (LH) model, rats

that did not display LH behaviour in response to inescapable shocks had a marked change in miRNA levels, predominantly showing a downregulation of several miRNAs, whereas LH rats displayed a blunted miRNA response (96). Early-life stressors such as maternal separation appear to be predictive of hippocampal volume in humans (97). Given miRNAs' roles in neurogenesis and brain maturation, they may play a role in modulating the response to such stressors. Initial evidence for this in an animal model showed that maternal separation led to an increase in miR-132, miR-124, miR-9 and miR-29a (98).

In humans, only two studies to date have investigated the role of miRNAs in anxiety disorders (see Table 4). Both studies, which looked for SNP associations, were from the same research group. In the first study, a SNP in the target site of miR-485-3p was associated with the hoarding phenotype in OCD. There was also an association between two newly identified SNPs and panic disorder (99). The targets for these new SNPs include the neurotrophic tyrosine kinase receptor 3 gene (*NTRK3)*, which has been implicated in animal models of anxiety (100). In a further study from three European countries, the authors found several SNPs in miRNAs associated with panic disorder, but these did not stand up to correction for multiple testing (101).

miRNAs and other psychiatric disorders

Studies investigating the role of miRNAs in other general adult psychiatric disorders are either isolated to initial exploration studies or have not so far moved beyond animal models. Several animal studies in addiction disorders exist as well as one postmortem miRNA brain expression study (102) (see Table 4). Animal models have also shown upregulation of miRNAs in response to alcohol, and this may be a mechanism for neuronal adaptation (103). Cocaine addiction and response have been the target of a number of animal studies, but no human studies have yet been published. The data from animal studies implicate miR-212 (104, 105), miR-124, miR-181 and let-7d in the response to cocaine (106–108).

Other disorders where miRNAs have been implicated include Fragile X (16), autism (109, 110) and Tourette's syndrome (111).

Discussion

Investigating the role of miRNAs in psychiatric disorders should help improve our currently limited understanding of the biology of psychiatric disorders and their treatments. Excitingly, they also have the potential to act as biomarkers and novel therapeutic targets. In psychiatry, we are still very much at an early stage in the investigation of miRNAs and so time and more in-depth study will tell which findings will hold true. To date, most of the evidence comes from postmortem brain case–control studies. More recent studies are focusing on peripheral sources of miRNAs such as blood and to a lesser extent CSF. This is not surprising given blood's clinical utility as a readily accessible source of biomarkers.

Schizophrenia is the disorder most studied to date regarding miRNA (112). The clinical studies outlined in Table 1 implicate a number of miRNAs, but most of these have yet to be replicated. It

Table 4. Clinical studies of miRNAs in other psychiatric disorders

Author	Patients	Tissue	Analysis	Main findings
Genotyping studies				
Muinos-Gimeno et al. (2009) (99)	OCD = 153 PD = 212 Controls = 324	DNA	miR-SNP	Target site of miR-485-3p associated with hoarding phenotype in OCD Targeted genes/gene pathways: NTRK3
Muinos-Gimeno et al. (2011) (101)	PD = 626	DNA	miR-SNP	No SNPs associated with PD following correction for multiple testing
Post-mortem case–control brain studies				
Lewohl et al. (2011) (102)	AD = 14 Controls = 13	Prefrontal cortex	Microarray qRT-PCR	let-7g, miR-15b, miR-152, miR-7 upregulated in patients with AD Targeted genes/gene pathways: CXXR4, DICER1, BIRC6. Cellular component morphogenesis, cell development, fatty acid biosynthesis, cell cycle arrest, central nervous system myelination and axon ensheathment

AD, alcohol dependence disorder; OCD, obsessive–compulsive disorder; PD, panic disorder; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; SNP, single nucleotide polymorphisms.

is not yet clear whether changes in miRNA levels are due to global or individual expression changes. Nonetheless, some miRNAs have been repeatedly identified across several studies of schizophrenia, including in different tissues and patient cohorts, and these are worth further investigation and replication. The miR-15 family, targeting genes implicated in schizophrenia such as *BDNF*, *DRD1*, *NRG1* and *EGR3,* was upregulated in the frontal cortex in three separate studies (42, 47, 48). Genotyping studies have identified variations in miR-137 as strongly associated with schizophrenia (59–62). Other miRNAs of interest include miR-132, miR-30 and miR-219. Treatment with other antipsychotics also needs to be examined, including highly effective treatments such as clozapine.

The majority of studies of miRNA expression in BPAD (see Table 2), although from a single brain bank, remain contradictory. There is an overlap with miRNAs implicated in schizophrenia. miRNAs also appear to be making a contribution to the actions of lithium and sodium valproate, and studies replicating these findings as well as on other mood-stabilising treatments are indicated. Further analyses of miRNA changes in the different mood states of BPAD and unipolar depression are required to discriminate between trait and state effects.

There are only a handful of studies investigating miRNAs in depression (see Table 3). A combination of preclinical and clinical research has revealed information on critical role of miR-16 on the mechanism of action of fluoxetine, including a possible mechanism for its delayed onset of action (80, 81). miRNA profiling studies have found preliminary evidence for an upregulation of a number of miRNAs in patients treated with antidepressants. Additional miRNA profiling studies in larger well-characterised cohorts are required. Additionally, studies that examine the effect of powerful antidepressant treatments such as electroconvulsive therapy (ECT) would be helpful to identify relevant miRNAs that have a role in either the mechanism of antidepressant action or the neurobiology of depression itself.

Work in the area of anxiety disorders (Table 4) is at a preliminary level compared with schizophrenia and affective disorders. Additional studies of the whole spectrum of anxiety disorders are needed, as well as studies designed to characterise the effects of anxiety treatments on miRNA levels.

Overall, studies in new, larger and well-phenotyped cohorts are required as some of the findings to date are contradictory. For example, different authors have found varying results using the same samples from the Stanley brain bank (44, 49–51). Although blood is commonly used to investigate psychiatric disorders, there is still a lack of data on the origin of miRNAs in blood and also whether they accurately reflect miRNA activity in the brain. Studies that establish the distribution of miRNAs across different brain regions, various blood compartments and CSF in healthy controls and cases will help clarify some of these questions. Technological methods also need to be standardised to ensure data quality and facilitate replication. For example, some commonly used reagents have been shown to distort results in cell cultures, leading to a retraction of one published report (113). There are also concerns over potentially high false discovery rates and difficulties in correctly identifying miRNAs with similar base pair sequences using primer-based technologies. It is expected that next-generation deep sequencing should help address some of these issues (114).

There have been few truly novel psychiatric therapeutic developments in recent years, and some pharmaceutical companies have abandoned research efforts in the psychiatric field altogether (115). miRNAs harbour the possibility of opening up a genuinely new approach to therapeutics and are already in development in other fields. For example, miravirsen, a miR-122-targeting locked nucleic acid antisense drug has been developed for hepatitis C. In a recent phase 2 clinical trial, miravirsen was well tolerated and resulted in a dose-dependent reduction in hepatitis C mRNA levels (31). Existing drug treatments for psychiatric disorders have already been shown to alter miRNA expression. Targeting miRNAs may therefore represent a more direct route to treatment. Although delivering miRNA-targeted therapies to the CNS poses a considerable challenge, they have been identified as therapeutic targets of great potential in other CNS disorders such as glioblastoma (116). One challenge is that, as postmortem studies have shown, different regions of the brain express miRNAs at different, even opposing, levels. Techniques to target specific areas of the brain and not others, including outside the CNS, would therefore need to be developed to minimise inappropriate mRNA targeting. However, it is the ability of a single miRNA to target whole network of proteins that may make it fruitful for psychiatric disorders where it is the sum of many genes of small effect that is thought to underlie heritability. Viral and non-viral vectors are being investigated for their potential to deliver miRNAs to the CNS, but a non-invasive method that has also shown some promise in this regard is intranasal delivery (117).

miRNAs implicated in disease or therapeutic effects that are identified in clinical populations

should be further investigated in animal and other preclinical models, despite the drawbacks of extrapolating from an animal model to a clinical setting (118). This could involve overexpression or knockdown of a candidate miRNA in cell lines and measuring the target gene's protein expression levels as well as behavioural phenotype in animal models. Likewise, miRNAs identified in preclinical settings should be followed up in clinical samples. Identifying new rare variants through sequencing as well as genotyping larger cohorts for more common miRNA disease risk variants should also be explored. Studies correlating miRNA profiles with clinical outcomes would be helpful in the development of biomarkers.

In summary, there is a growing realisation that miRNAs are central to the control of gene expression in the CNS and are altered in the development, presentation and response to treatment of psychiatric disorders. Although in its infancy, investigating these small molecules may lead to big developments in biomarkers and therapeutic targets for psychiatric disorders.

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Declaration of interest

None.

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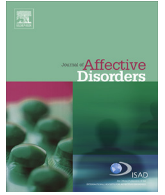

Review

Epigenetics and depression: return of the repressed

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ABSTRACT

Introduction: Epigenetics has recently emerged as a potential mechanism by which adverse environmental stimuli can result in persistent changes in gene expression. Epigenetic mechanisms function alongside the DNA sequence to modulate gene expression and ultimately influence protein production. The current review provides an introduction and overview of epigenetics with a particular focus on preclinical and clinical studies relevant to major depressive disorder (MDD).
Methods: PubMed and Web of Science databases were interrogated from January 1995 up to December 2012 using combinations of search terms, including “epigenetic”, “microRNA” and “DNA methylation” cross referenced with “depression”, “early life stress” and “antidepressant”.
Results: There is an association between adverse environmental stimuli, such as early life stress, and epigenetic modification of gene expression. Epigenetic changes have been reported in humans with MDD and may serve as biomarkers to improve diagnosis. Antidepressant treatments appear to reverse or initiate compensatory epigenetic alterations that may be relevant to their mechanism of action.
Limitations: As a narrative review, the current report was interpretive and qualitative in nature.
Conclusion: Epigenetic modification of gene expression provides a mechanism for understanding the link between long-term effects of adverse life events and the changes in gene expression that are associated with depression. Although still a developing field, in the future, epigenetic modifications of gene expression may provide novel biomarkers to predict future susceptibility and/or onset of MDD, improve diagnosis, and aid in the development of epigenetics-based therapies for depression.
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1. Introduction

The causes of major depressive disorder (MDD) remain unclear, posing a major gap in our knowledge and an obstacle to improving therapies. Although the heritability of MDD is between 31–42% (Edvardsen et al., 2009; Middeldorp et al., 2005), human genome-wide association studies (GWAS) have failed to demonstrate reproducible gene loci that contribute to the disease (Wray et al., 2012). Furthermore, the high discordance rate of 50% between monozygotic twins suggests factors other than genetics contribute to disease genesis (Haque et al., 2009). Environmental stressors have been identified as risk factors for depression, although individual variability in the susceptibility to adverse environmental stimuli has been reported. Since MDD cannot be attributed to a single genetic mutation or exposure to one specific environmental cue, it has been proposed that MDD arises from genetic variation which is moderated by external influences (Kendler et al., 2002, 2006).

Epigenetics has recently emerged as a potential mechanism by which adverse environmental stimuli, such as stress encountered in early life, can result in stable, persistent alterations in gene expression (Onishchenko et al., 2008; Roth et al., 2009). The prefix "epi" means above or alongside and the term epigenetics refers to modulatory mechanisms that operate above or alongside the nucleotide bases that comprise a gene's deoxyribonucleic acid (DNA) sequence. Epigenetic mechanisms are thus responsible for alterations in the expression and function of a gene that are not encoded by the gene's DNA sequence itself. Epigenetic modifications in gene expression exert lasting changes without altering the genetic code (Schroeder et al., 2012). They are modifiable by environmental factors and are also potentially heritable (Franklin et al., 2010; Roth et al., 2009). This review aims to summarise current evidence of the role that epigenetic mechanisms, such as DNA methylation, histone acetylation and microRNA dysregulation, may play in depression, as well as what future directions this field may take.

2. Methods

Although the field of epigenetics is well developed in cancer and developmental biology, evidence for epigenetic modification of gene expression in mental disorders is only now emerging (Schroeder et al., 2012). Epigenetics offers an exciting new avenue for improving diagnosis and developing novel therapies and biomarkers for MDD. Given the novelty of the field and therefore the reasonably small number of relevant studies, we have conducted a narrative review of preclinical and clinical investigations. PubMed and Web of Science databases were interrogated from January 1995 up to December 2012 using combinations of search terms, including "epigenetic", "microRNA" and "DNA methylation" cross referenced with "depression", "early life stress" and "antidepressant". A total of 1576 potentially relevant articles were identified and 72 records removed when they did not meet the criteria of publication in a peer-reviewed journal and in the English language. Duplicate records were removed ($n=371$). A total of 1133 titles were screened followed by 251 abstracts. After abstract screening, and also review of references from identified articles, a total of 77 full text papers were then reviewed for the current article. Our aims are to provide a scientific background to epigenetics and inform academic and clinical psychiatrists, and those in related disciplines, about epigenetics research in depression.

3. Epigenetic mechanisms modulating gene expression

3.1. Introduction to gene expression and protein synthesis

Genomic DNA constitutes the total genetic information of an organism and is made up of four different nucleotide bases – guanine (G), cytosine (C), arginine (A) and thymine (T) – arranged to form a DNA double helix. The double helix consists of two chains of nucleotide bases organised in a specific order or "sequence" around a sugar–phosphate backbone. Bases that comprise the sequences are arranged in a complementary manner and attached to the backbone sugars to form base pairs such that C is found opposite to G and T is found opposite to A (Fig. 1). Genomic DNA comprises regions of gene sequences, which ultimately give rise to proteins, along with other non-protein coding regions that are important in the control of gene expression or have an unknown function. Indeed, this non-coding DNA, previously considered to be "junk" DNA, is now attracting great interest. The Encyclopedia of DNA Elements (ENCODE) project, for example, has enabled researchers to assign biochemical functions for 80% of the human genome, particularly in non-protein coding regions that contain regulatory elements important in the control of gene expression (Encode Project Consortium, 2012).

Gene expression is the synthesis of a gene product (e.g. a protein) from the DNA sequence of a particular gene. Epigenetic factors can regulate gene expression at a number of levels (Fig. 1). During protein synthesis, first a gene is expressed within the cell nucleus as a ribonucleic acid (RNA) copy of its genomic DNA sequence, known as messenger RNA (mRNA) (Clancy and Brown, 2008). mRNA synthesis is activated by the binding of specific enzymes, known as mRNA transcription initiating factors, to a regulatory region of the DNA sequence called the promoter region (Fig. 1). It is at this transcriptional level that two modes of epigenetic regulation of gene expression may exert an effect in the form of chromatin remodelling and DNA methylation.

Genomic DNA is packaged as chromatin within the cell nucleus (Fig. 1). Chromatin can be remodelled and exists in open or closed functional states, which regulate enzymatic accessibility (Fig. 2B). When chromatin is in its open state, genomic DNA is accessible to the mRNA transcription initiating factors and facilitates gene expression. Chromatin in its closed state limits DNA accessibility for mRNA transcription initiating factors, thus suppressing gene expression. The mechanisms underlying chromatin remodelling are discussed in more detail below (Section 3.3).

DNA methylation is the second epigenetic factor that may have an effect on gene expression at the transcriptional level. It can suppress gene expression directly and indirectly by preventing the binding of enzymes important in RNA synthesis at the promoter region (Figs. 1 and 2D) (Hervouet et al., 2009; Szyf, 2006; Weber and Schubeler, 2007) and is discussed in more detail below (Section 3.2).

Provided that transcription factor binding is unhindered by the epigenetic modifications described above, the genomic DNA is then unwound and an enzyme known as RNA polymerase makes a

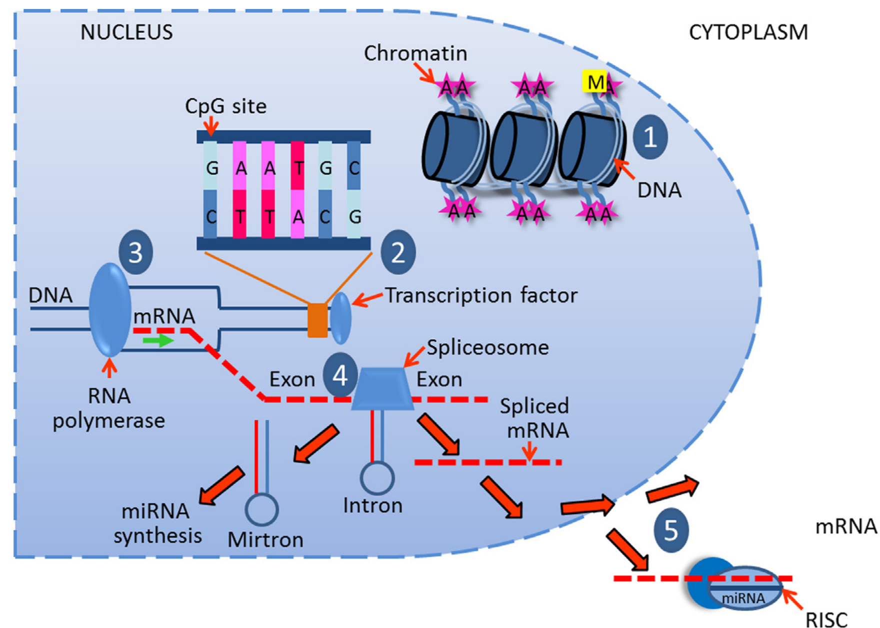

Fig. 1. Overview of gene expression and protein synthesis. (1) Within the cell nucleus, genomic DNA (shown as thin, pale blue/gray strands) is packaged as chromatin wrapped around a histone core. Epigenetic regulation of gene expression arises through the control of chromatin activation status. Active, open chromatin promotes gene expression by allowing access of mRNA transcription initiating factors to the genomic DNA. However, chromatin in its closed state suppresses gene expression by hindering transcription factor binding. The chromatin is shown in an open state. (2) Messenger RNA (mRNA) synthesis is initiated at the promoter region of a gene (represented by orange/black box) by the binding of enzymes known as mRNA transcription initiating factors (small blue/gray oval). The DNA sequence contained within the box is expanded above to show an example of the complementary arrangement of the four nucleotide bases – guanine (G), cytosine (C), arginine (A) and thymine (T) – that comprise the DNA double helix. Promoters usually contain multiple cytosine–guanine nucleotide pairings known as cytosine–phosphate–guanine dinucleotide (CpG) sites (example indicated by arrow). A second epigenetic mechanism known as DNA methylation can have an effect at the transcriptional level. DNA methylation involves the addition of methyl groups at CpG sites within the promoter. It can suppress gene expression directly and indirectly by preventing the binding of enzymes important in RNA synthesis at the promoter region. The DNA sequence shown is unmethylated therefore mRNA transcription can proceed. (3) After transcription is initiated, an enyzme called RNA polymerase makes a mRNA copy (broken red/gray line) of the DNA sequence. The direction of mRNA synthesis is shown by the white/green arrow. (4) Non-protein coding regions known as introns are removed from the mRNA sequence by a collection of enyzmes known as the spliceosome. Some introns may serve as templates for the synthesis of microRNAs (miRNAs) and are known as mirtrons. The protein coding exons are then spliced together and the mRNA is transported from the nucleus to the cytoplasm. (5) The mRNA is translated to protein by protein/enzyme complexes called ribosomes. Epigenetic modulation of gene expression may take place at the translational level due to the activity of miRNAs loaded into the RNA-induced silencing complex (RISC), which can suppress mRNA translation to protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mRNA copy of the DNA by creating a chain of nucleotides specified by the DNA gene sequence. Non-protein coding regions called introns are first removed from the synthesised mRNA by a process known as splicing. The processed mRNA is then transported outside the nucleus. It is at this point that microRNAs (miRNAs), another group of epigenetic modulators, may have an effect on gene expression. At this stage post-transcription, the mRNA sequence is usually translated into protein by complexes known as ribosomes. However, this process may be hindered by the presence of miRNAs thus leading to a decline in protein synthesis (discussed in more detail below in Section 3.3.2, see Figs. 1 and 3). Translation of mRNA to protein by ribosomes involves the linking of amino acids, the basic building blocks of proteins found within the cytoplasm, to form an amino acid chain. The amino acids that comprise the protein are determined by the mRNA sequence. Post-translation, the amino acid chain then undergoes structural changes and folds in a specific manner to give rise to a protein.

3.2. DNA methylation

The most studied epigenetic modification to date is DNA methylation. Gene promoter regions usually contain multiple cytosine–guanine nucleotide pairings distributed repeatedly throughout, known as cytosine–phosphate-guanine dinucleotide (CpG) sites (Fig. 2D). DNA methylation generally entails the addition of a methyl group at CpG sites within the promoter region of a target gene by enzymes known as DNA methyltransferases (Fig. 2D) (Szyf, 2006; Weber and Schubeler, 2007). Methylation of CpG sites can suppress gene expression directly by preventing the binding of enzymes important in RNA synthesis at the promoter region (Hervouet et al., 2009; Szyf, 2006; Weber and Schubeler, 2007). CpG methylation can also hinder gene expression indirectly by recruiting enzymes and other proteins that promote gene-suppressive chromatin remodelling (discussed in detail below), ultimately leading to a decrease in protein production. Originally it was thought that the pattern of DNA methylation was laid down before birth and was irreversible in later life. Evidence has now emerged though that DNA methylation is responsive to, and alterable by, environmental signals (Weber and Schubeler, 2007).

3.3. Histone acetylation and methylation

To allow the storage of highly condensed genomic information within the cell nucleus, genomic DNA is organised in the form of chromatin. The basic unit of chromatin is called the nucleosome and it consists of approximately 147 nucleotide base pairs (bp) of negatively charged DNA sequence wrapped around a core of histone proteins (Fig. 2A) (Hayes and Hansen, 2001; Khorasanizadeh, 2004). Histones make up the major protein constituents of the nucleus. The nucleosome histone core contains two copies of four histone proteins: H2A, H2B, H3 and H4. Each core histone protein possesses an outward facing tail consisting of amino acids such as lysine (Fig. 2A). A fifth histone protein, H1, acts as a linker between nucleosome units.

Modifications, such as the addition of acetyl or methyl groups at lysine residues on the histone tails (Fig. 2B and C), can change

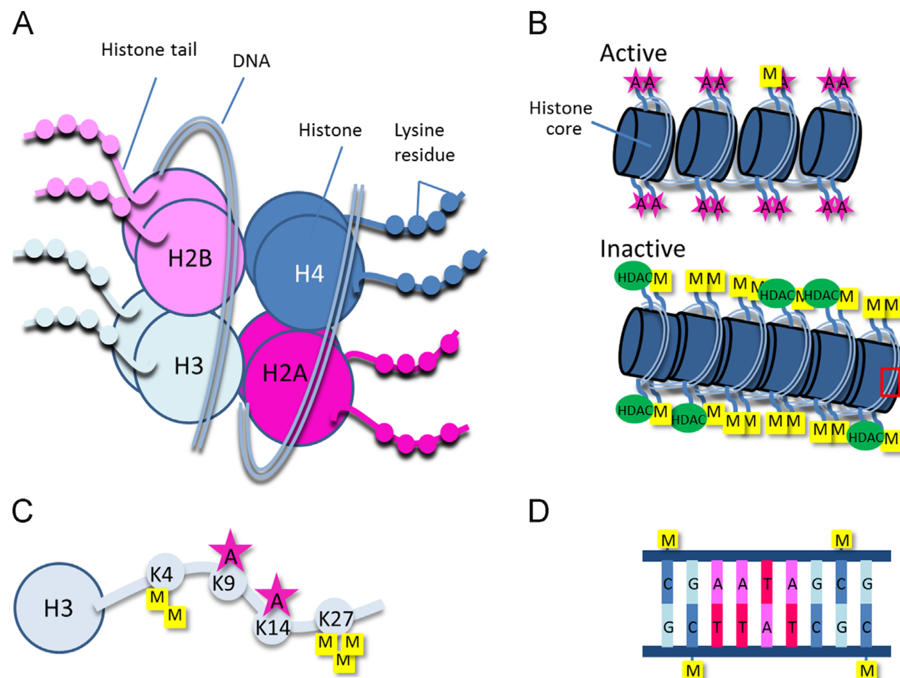

Fig. 2. Histone modifications. (A) A representation of the nucleosome showing double stranded DNA wrapped around the core comprising two copies of histones H2A, H2B, H3 and H4. Outward facing histone tails with lysine residues are shown. (B) Chromatin in its active open state and closed inactive state is shown. Acetylation of histone tails is associated with active chromatin. Acetyl (A) groups are represented by light grey/pink stars. Histone deacetylase (HDAC) enzymes, represented by the dark grey/green circles, remove acetyl groups and are associated with inactive chromatin. Methyl groups are represented by boxed Ms. The boxed region is expanded in D. (C) Histone 3 (H3) showing acetylated lysine (K) residues K9 and K14. Lysine acetylation generally promotes gene expression. Lysine methylation can promote or suppress gene expression depending on the lysine residue and the number of methyl groups added. The presence of two methyl groups at K4 and three methyl groups at K27 has been shown to promote and suppress gene expression, respectively. (D) Expansion of boxed region in B showing methylated genomic DNA which is often associated with inactive chromatin. The addition of methyl groups, shown by the boxed Ms, to cytosine–phosphate–guanine dinucleotide (CpG) sites within a gene promoter region can prevent the binding of transcription factors and hinder protein synthesis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

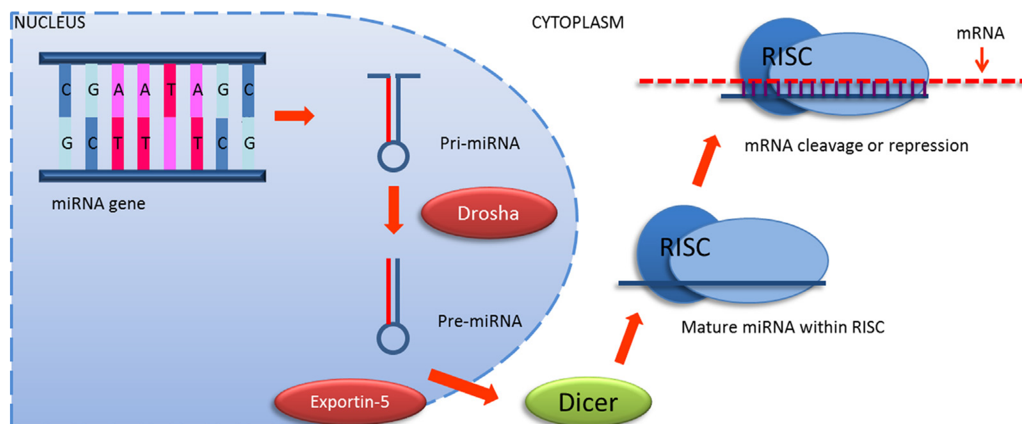

Fig. 3. miRNA biogenesis. Mature microRNAs (miRNAs) are formed following a series of steps involving RNA transcription to generate a primary miRNA (pri-miRNA). This is followed by RNase modification by Drosha to form a precursor miRNA (pre-miRNA) which is exported from the nucleus to the cytoplasm by Exportin-5. Pre-miRNA is cleaved by Dicer to form a 22 base pair duplex. From the duplex, one miRNA is then preferentially loaded into a *RNA-induced silencing complex* (*RISC*) where it binds to a target messenger RNA (mRNA) transcript, preventing translation.

the interaction between the genomic DNA and histone core, and are involved in control of gene expression (Berger, 2007; Hayes and Hansen, 2001). Chromatin enters its active state after addition of acetyl groups (hyperacetylation) to the histone tail by enzymes called histone acetyltransferases (HATs). This in turn promotes unfolding of the histone unit and decondensation of chromatin (Fig. 2B), allowing access of mRNA transcription initiating factors to the genomic DNA and promotes gene expression (Crosio et al., 2003; Eberharter and Becker, 2002; Grunstein, 1997; Hebbes et al., 1988; Korzus et al., 2004). On the other hand, removal of acetyl groups by enzymes known as histone deacetylases (HDACs) causes chromatin condensation (Fig. 2B) and decreases protein production (Grunstein, 1997; Pile et al., 2003). Alterations in the acetylation pattern of lysine residues comprising amino acid tails of histones 3 (Fig. 2C) and 4 have been implicated in depression (Iga et al., 2007; Levine et al., 2012; Onishchenko et al., 2008; Tsankova et al., 2006) and antidepressant treatment (Belzeaux et al., 2012; Dyrvig et al., 2012; Tsankova et al., 2006, 2004).

Alternatively to acetyl groups, methyl groups can be added to lysine residues on the histone tail (Fig. 2C). Chromatin activation status then depends on the particular lysine residue methylated as well as the number of methyl groups added to the residue (Fig. 2C)

(Dillon et al., 2005; Morgunkova and Barlev, 2006). The addition of three methyl groups at lysine 27 to the amino acid tail of histone 3 is associated, for example, with silencing of gene transcription in depressed patients (Lopez et al., 2013) and in an animal model of depression (Onishchenko et al., 2008). On the other hand, the addition of two methyl groups to lysine 4 of histone 3 has been shown to promote gene expression in a depression model following antidepressant treatment (Tsankova et al., 2006).

Chromatin activation status and DNA methylation often work in concert to control gene expression. Chromatin status can usually be correlated with the DNA methylation pattern of a gene. Active chromatin is partnered with unmethylated DNA whereas inactive chromatin is associated with methylated DNA (Szyf, 2006; Tachibana et al., 2008).

3.3.1. microRNAs

In the past 10 years, miRNAs have emerged as another important group of epigenetic modulators of gene expression. They are endogenous non-protein coding RNA molecules of approximately 21–23 nucleotide bases that bind to complementary regions at target mRNAs and regulate mRNA translation to protein (Kolshus et al., 2013). Over 1500 mature miRNA sequences have been reported in the human to date (Kozomara and Griffiths-Jones, 2011) and it appears that over 50% of mammalian mRNA species are potential targets for miRNAs (Friedman et al., 2009). A single mRNA may be regulated by multiple miRNAs and, conversely, some miRNAs have the potential to target hundreds of mRNAs. Typically, miRNAs suppress target gene expression; however, reciprocal relationships, whereby mRNA targets can control the level and function of miRNAs, are emerging (Pasquinelli, 2012). miRNAs are expressed during development and in adulthood, with the expression of some subsets being organ-specific (Sempere et al., 2004) and region-specific within organs such as the brain (Olsen et al., 2009). Functional studies indicate that miRNAs are involved in the control of many cellular processes including neurogenesis, synaptic plasticity, cell fate decision, and apoptosis (Bredy et al., 2011; Kloosterman and Plasterk, 2006; Luikart et al., 2012; Magill et al., 2010; Saba and Schratt, 2010). It follows therefore that dysregulation in their expression has been linked with human pathologies such as major depressive disorder, schizophrenia and cancer (Esteller, 2011; Im and Kenny, 2012).

3.3.2. microRNA biogenesis and regulation of protein synthesis

Biogenesis of miRNAs can occur through various different pathways (see Figs. 1 and 3) (Havens et al., 2012). In the canonical miRNA biogenesis pathway, primary miRNAs (pri-miRNA) are enzymatically transcribed from independent miRNA genes located within the genomic DNA (Fig. 3). Within the nucleus, a processing complex made up of multiple proteins then removes regions of the pri-miRNA to generate the double-stranded precursor miRNA (pre-miRNA), which is generally between 70 and 110 nucleotides in length. Pre-miRNAs can also be derived via alternative means. They can be directly transcribed, for example, from introns and are known as mirtrons (Fig. 1) (Chong et al., 2010; Krol et al., 2010). Following synthesis, pre-miRNAs are then exported from the nucleus to the cytoplasm (Yi et al., 2003), where the pre-miRNA is processed to form a 22 bp molecule made up of two complementary RNA strands (Fig. 3) (Chendrimada et al., 2005). One strand from the duplex is then preferentially loaded into a protein complex called the RNA-induced silencing complex (RISC) (Kawamata and Tomari, 2010). Once loaded into the RISC, the miRNA guides the complex to the target mRNA transcripts (Figs. 1 and 3). Depending on the number of sequence mismatches between the miRNA and its mRNA target, miRNA induced regulation of the mRNA then occurs due to enzymatic degradation of the target or prevention of mRNA translational to protein due to steric hindrance of protein synthesis machinery (Carthew and Sontheimer, 2009; Krol et al., 2010; Wu et al., 2006).

4. Epigenetics and environmental risk factors for depression

Epigenetics provides a potential mechanism by which adverse stimuli can result in lasting changes in gene expression. Preclinical and clinical studies have recently focused on the epigenetic effects of various environmental stressors that have been identified as risk factors for poor mental health outcome. Epigenetic modifications of gene expression have been reported in rodent models after acute stress (Fuchikami et al., 2009), chronic stress (Sterrenburg et al., 2011; Tsankova et al., 2006; Uchida et al., 2011; Wilkinson et al., 2009), fear conditioning (Bredy et al., 2007; Mizuno et al., 2012) and in a rat model of post-traumatic stress disorder (Roth et al., 2011). In addition to these studies, the epigenetic changes elicited by exposure to early life stress (ELS) have been a major focus of research into the epigenetic mechanisms involved in the pathogenesis of MDD in rodent models (Table 1) and humans. Evidence suggests that adverse external stimuli encountered in early life can affect adult behaviour and predispose individuals to lifelong health problems, including increased risk of mental illnesses such as depression (Heim and Binder, 2012; Markham and Koenig, 2011; Talge et al., 2007). Epigenetic mechanisms such as DNA methylation, histone modifications and dysregulation of microRNA expression represent plausible means by which ELS may cause lifelong changes in gene expression.

4.1. Epigenetics and early life stress (ELS)

In animal models of depression, evidence exists for an association between ELS and epigenetic modulation of a range of genes previously implicated in depression. Alterations in DNA methylation and histone modifications at promoters of genes, such as brain derived neurotrophic factor (*Bdnf*), as well as changes in miRNA expression patterns, have been reported in the rodent brain after exposure to prenatal and/or perinatal stress (see Table 1 for a summary). Exposure to methylmercury from gestational day 7 to postnatal day 7, for example, has been shown to result in depressive-like behaviour in mice (Onishchenko et al., 2008). Methylmercury-exposed pups show a decrease in hippocampal *Bdnf* mRNA that is accompanied by increases in gene-suppressing methylation of DNA and histone 3 at lysine 27, and a decrease in expression-promoting acetylation of histone 3 at a *Bdnf* promoter region. Postnatal stressors such as maternal separation and/or exposure to low levels of maternal care or attention have also been linked with changes in DNA methylation and chromatin activation status of various genes expressed in the rodent brain (Table 1). Maltreatment of rat pups by stressed caretakers in early development (between postnatal days 1 and 7) resulted in decreased *Bdnf* mRNA expression in the prefrontal cortex that was accompanied by an increase in expression-suppressing DNA methylation at *Bdnf* promoter regions in adulthood, an effect that could be reversed by the application of the DNA methyltransferase inhibitor, zebularine (Roth et al., 2009).

Altered DNA methylation patterns of genes implicated in depression such as *BDNF* (Toledo-Rodriguez et al., 2010), the glucocorticoid receptor (Mulligan et al., 2012; Oberlander et al., 2008; Radtke et al., 2011) and serotonin transporter (Devlin et al., 2010), have been reported in children following exposure of mothers to prenatal stressors such as smoking, maternal depression, partner violence and war. Adversities experienced in early childhood have also been

Table 1
Studies of epigenetic modification of gene expression following exposure to early life stress in rodent models.

Reference	Species/ strain	Stressor	Region/ age assessed	Epigenetic modification	Molecular and behavioural changes
Bai et al. (2012)	SD rats	MS from PD 1-13	HC/ > PD 91	↑miR16	↓Total BDNF mRNA inversely correlated with miR16 expression Depression-like behaviour
Chen et al. (2012)	SD rats	MS from PD 2-13	PVN and AMY/PD 60	↓DNA methylation of CRH in PVN	↑Heteronuclear RNA (mRNA precursor) for CRH following acute stress ↑Plasma CORT in response to stress
Jiao et al. (in press)	BALB/cJ &C57BL/ 6J mice	Strain-specific effects of gestational environment and maternal care	HC/PD 84-112	↑DNA methylation for CRP in BALB/cJ -gestated mice, no effect of maternal care on CRP DNA methylation	↑CRP mRNA in C57BL/6J gestated mice No effect of maternal care on CRP expression C57BL/6J gestated mice showed increased depression-like behaviour
Levine et al. (2012)	BALB/cJ &C57BL/ 6J mice	Strain-specific effects of MS from PD 2-15	Cortex/ PD 21, 28 and 60	↑H4K12 acetylation in Balb/cJ mice after MS	↓HDAC mRNA in Balb/cJ mice after MS Depression-like behaviour in Balb/cJ mice after MS
Matrisciano et al. (2013)	ND4 mice	Prenatal restraint stress	FC/PD 60	↑DNA methylation of GAD67 and reelin	↑GAD67 and reelin protein, ↑mRNA expression and protein levels of DNA methyltransferase enzymes Hyperactivity and deficits in social interaction, PPI and fear conditioning
Mueller and Bale (2008)	C57BL/ 6:129 mice	Chronic variable PS from GD 1-7	HYP, AMY, HC/PD 120	DNA methylation of CRH↓in HYP and AMY, and of GR↑in HYP	↑CRH mRNA in HYP and AMY, ↓GR in HC Depression-like behaviour
Murgatroyd et al. (2009)	C57BL/ 6N mice	MS from PD 1-10	PVN /PD 42, 84 and 1 year	↓DNA methylation of arginine vasopressin	↑Arginine vasopressin mRNA ↑Serum CORT response to stress and memory deficits
Mychasiuk et al. (2011)	LE rats	Mild or high PS from GD12-16	FC, HC/ PD 21	Global DNA methylation↑with mild PS and↓with high PS	Mild PS = ↓locomotion High PS = ↑locomotion
Novikova et al. (2008)	CD1 mice	Maternal cocaine (20 mg/kg) from GD8-19	HC/PD 3 and 30	Global DNA methylation—↓PD3 and ↑PD 30	Expression of selected genes was linked to DNA methylation
Onishchenko et al. (2008)	C57BL/ 6/Bk1 mice	Dams exposed to methylmercury from GD7-PD7	HC/PD 84 and 14 months	↑H3K27 tri-methylation, ↓H3K9K14 acetylation and↑DNA methylation at BDNF promoter	↓Total BDNF mRNA Depression-like behaviour
Roth et al. (2009)	LE rats	Abusive caretaker exposure from PD 1-7	FC/PD 8, 30 and 90	↑DNA methylation at BDNF promoters	↓Total BDNF mRNA
Uchida et al. (2010)	SD rats	MS from PD 2-14	FC/PD 14 and 58-60	PD14:↑in precursor miR-132, -124-1, -9-1, -9-3, -212, and -29a and ↑in mature miR132, -124, miR9 and -29a; PD 58-60:↑precursor miR-124, -212 and -132	↑mRNA for GluR2, NMDA receptor 1 subunit, CRH, CAM kinase II, cell adhesion molecule L1, adenylate cyclase 5, 5HT1aR and voltage-gated potassium channel subunit Kv3.1, ↓mRNA for neuron navigator 1 MS rats showed a greater HPA axis response to stress than controls.
Weaver et al. (2004)	LE rats	Effects of high and low levels of maternal care PD 1-7	HC/PD 90	↑DNA methylation and↓H3K9 acetylation of GR after low level maternal care	↓GR protein ↑Plasma CORT in response to stress

Abbreviations: 5HT1a-serotonin 5HT1a receptor, AMY-amygdala, BDNF-brain derived neurotrophic factor, CAM-calcium/calmodulin-dependent protein, CORT-corticosterone, CRH-corticotropin releasing hormone, CRP-calcitonin gene-related peptide, FC-frontal cortex, GAD67-Glutamate decarboxylase 67, GD-gestational day, GluR2-AMPA glutamate receptor 2 subunit, GR-glucocorticoid receptor, H-histone, HC-hippocampus, HDAC-histone deacetylase enzyme, HPA-hypothalamic-pituitary-adrenal, HYP-hypothalamus, K-lysine, mRNA-messenger RNA, LE-Long Evans, MS-maternal separation, NC-no change, NE-not examined, PD-postnatal day, PPI-prepulse inhibition, PS-prenatal stress, PVN-paraventricular nucleus of hypothalamus, SD-Sprague Dawley.

linked to epigenetic modulation of gene expression. Variations in DNA methylation status of the glucocorticoid receptor and ribosomal RNA promoter have been reported in the postmortem brains of suicide victims with a history of childhood abuse (McGowan et al., 2009, 2008). Additionally, early life poverty was associated with DNA methylation in an analysis of > 14,000 genes in peripheral blood mononuclear cells in a community-based cohort stratified for early-life socioeconomic status (Lam et al., 2012).

Taken together, these studies indicate that exposure to a range of pre- and post-natal stressors are associated with epigenetic modifications in depression-related genes in both rodent models of ELS (Table 1) and in humans. Epigenetics may thus be one of the underlying mechanisms by which ELS exerts long-term effects on gene expression in individuals and their offspring. Epidemiological studies have shown that the offspring of individuals exposed to ELS may display behavioural/developmental disturbances similar to their parent even if they themselves have not experienced the trauma. Furthermore, these disturbances can be passed on to the subsequent generations (Brand et al., 2010; Harper, 2005; Kim et al., 2009). Persistent epigenetic modification of gene expression induced by an environmental stimulus, e.g. ELS, has been suggested as one of the mechanisms underlying transgenerational phenotypic transmission (Harper, 2005). Preclinical research in rodent models supports this hypothesis (Franklin et al., 2010; Morgan and Bale, 2011; Roth et al., 2009). However, a considerable amount of further study is required to fully understand the range of genes epigenetically regulated by exposure to early life stress and the implications for future generations.

5. Epigenetics and major depressive disorder

Altered DNA methylation and chromatin activation status have been reported in humans with depression (see Table 2). Researchers have examined genome-wide DNA methylation patterns as well as epigenetic modification of individual depression-related genes such as the γ-aminobutyric acid (GABA) receptor subunits, synapsins and serotonin transporter as well as mRNA expression of enzymes involved in epigenetic modifications in patients with MDD (Table 2). Given the alterations in BDNF and its receptor Trk B observed in individuals with MDD (Dwivedi et al., 2003; Kim et al., 2007; Lee and Kim, 2010; Thompson Ray et al., 2011), particular attention has focused on epigenetic modification of the BDNF pathway in depression (Boulle et al., 2012). Alterations in DNA methylation of promoter regions of *BDNF* have been reported in the blood of patients with MDD (Fuchikami et al., 2011). Keller et al. (2010) observed an increase in DNA methylation at a *BDNF* promoter that was associated with decreased *BDNF* mRNA levels in Wernicke's area in brains of suicide victims, some of whom had a diagnosis of MDD (Keller et al., 2010). In the same cohort however, mRNA expression and DNA methylation of the BDNF receptor, *Trk B*, and its truncated form, *Trk B-T1*, in Wernicke's area did not correlate with suicidal behavior (Keller et al., 2011). On the other hand, decreased *Trk B-T1* mRNA in the frontal cortex was associated with increased methylation of CpG sites (Ernst et al., 2009b) and histone 3 at lysine 27 (Ernst et al., 2009a) in the promoter region of *Trk B-T1* in suicide completers, some of whom had MDD.

The few studies conducted to date in human subjects also provide evidence for alterations in miRNA directed translational regulation of coded proteins in major depression (Table 2). Recently, for example, Smalheiser et al. (2012) found that global miRNA expression was decreased and reorganised in a depressed suicide cohort compared to controls. The expression levels of 365 miRNAs were compared in the prefrontal cortex (Brodmann area 9) in 18 antidepressant-free depressed suicide subjects with 17 controls. The authors observed a global decrease in miRNA expression in the suicide group and identified 21 miRNAs that showed a statistically significant decrease of 30% or more. Approximately half of the downregulated miRNAs shared putative mRNA targets, including some that have been implicated in depression such as vascular endothelial growth factor and B-cell-CLL/lymphoma 2, and in the epigenetic control of gene expression including DNA methyltransferase 3b (Smalheiser et al., 2012). miRNAs exert their influence by binding to target mRNAs and ultimately suppressing protein synthesis according to sequence complementarity. Polymorphisms, which are naturally occurring variations in nucleotide sequences, have the potential to affect miRNA binding potential, and ultimately, miRNA-directed regulation of protein synthesis. In addition to changes in miRNA levels, polymorphisms in miRNAs and their precursors, their target mRNAs and in genes that regulate miRNA biogenesis have also been reported in MDD (Table 2).

Collectively, these studies suggest that alterations in DNA methylation, histone modifications and miRNA expression play an important role in dysregulation of gene expression in MDD. Clearly more research in epigenetic alteration of gene expression in individuals with depression is required, particularly into the role of chromatin activation status and miRNA changes in MDD given the paucity of literature on these topics. With further understanding, epigenetic modifications may serve as potential biomarkers to improve diagnosis and better understand the pathophysiology of MDD.

6. Epigenetics and antidepressant treatments

As well as its role in the pathogenesis of depression, research in animal models and humans suggests that epigenetic modulation of gene expression is also involved in the mechanisms of action of antidepressants (see Tables 3 and 4). Alterations in DNA

Table 2
Clinical studies of epigenetic modifications in mood disorders.

Reference	Tissue	Diagnosis	Epigenetic modification, molecular changes and other outcomes
Cruceanu et al. (2013)	Prefrontal cortex (BA 10)	MDD or BP	↑H3K4 methylation at *SYN1* promoter in MDD ↑mRNA for *SYN1a* in BP and MDD ↑mRNA for *SYN1b* in MDD only ↑H3K4 methylation at *SYN2* promoter in BP ↑mRNA for *SYN2a* in BP and ↑mRNA for *SYN2b* in MDD
Ernst et al. (2009a), Ernst et al. (2009b)	Frontal cortex	Suicide completers, some of whom had MDD	↑DNA and H3K27 methylation of *Trk B-T1* ↓Trk B-T1 mRNA
Fuchikami et al. (2011)	Blood	MDD	Altered BDNF DNA methylation pattern in MDD
He et al. (2012)	Blood	MDD	SNPs in microRNA processing genes in DGCR8 and AGO1 DGCR8 SNP associated with ↑suicide risk and improved antidepressant response and AGO1 SNP associated with ↓suicide risk
Iga et al. (2007)	Blood	MDD	↑HDAC 5 mRNA
Keller et al. (2011), Keller et al. (2010)	Wernicke's area	Suicide completers, some of whom had MDD	↑DNA methylation and ↓mRNA for BDNF but no correlation between *Trk B* and *Trk B-T1* DNA methylation and suicide
Philibert et al. (2008)	Lymphoblast cell lines	MDD	Females showed higher levels of DNA methylation of SERT compared to males Trend for association between ↑SERT DNA methylation and MDD
Poulter et al. (2008)	Frontal cortex	Suicide completers with MDD	↑DNA methylation of $GABA_A$ receptor subunit α1
Rahman et al. (2010)	Buccal epithelial cells	MDD or BP	Association between a polymorphism in P2X7 purinergic receptor gene and target site for miR-625 and -1302
Saus et al. (2010)	Blood	MDD	Association between a polymorphism in the circadian clock modulator pre-miR-182 and late insomnia
Smalheiser et al. (2012)	Prefrontal cortex (BA 9)	Suicide completers with MDD	Global ↓and reorganisation in miRNA expression in MDD ↓21 miRNAs
Uddin et al. (2011)	Blood	MDD	Altered DNA methylation pattern in MDD
Xu et al. (2010)	Blood	MDD	Polymorphism in pre-miR30e associated with MDD and difficulties with the speed of perception and processing of auditory stimuli

Abbreviations: AGO1, Argonaute 1 regulatory protein; BA-Brodmann area; BDNF, brain derived neurotrophic factor; BP, bipolar disorder; DGCR8, DiGeorge syndrome critical region gene 8; H-Histone; HDAC, histone deacetylase; K, lysine; MDD, major depressive disorder; mRNA, messenger RNA; NE, not examined; SERT, serotonin transporter; SNPs, single nucleotide polymorphism; SYN, synapsin; Trk B-T1, truncated form of tyrosine kinase B receptor.

methylation and chromatin activation status have been reported after antidepressant administration in preclinical and clinical investigations. In addition, antidepressants also appear to act by modifying miRNA expression, as evidenced by in vitro studies and research in animal models and humans.

Electroconvulsive therapy (ECT) is the most acutely effective treatment available for severe depression (Eranti et al., 2007; UK Ect Review Group, 2003). The precise mechanisms of action of ECT remain unknown but evidence in animal models suggests that treatment with ECS (electroconvulsive stimulation), the animal model equivalent of ECT, results in epigenetic modification of gene expression (Table 3). Tsankova et al. (2004), for example, examined histone modifications of *Bdnf* at 30 min, two hours and 24 h after acute and chronic ECS in the rat brain hippocampus. Their results indicated that histone modifications controlling the expression of *Bdnf* after ECS administration are dependent on treatment duration, post-treatment time and gene promoter region. ECS also results in histone modifications and accompanying alterations in mRNA for *CREB and c-Fos* (Dyrvig et al., 2012; Tsankova et al., 2004) as well as an increase in DNA methylation at the *Arc* promoter (Dyrvig et al., 2012). Taken together, these findings imply that epigenetic modulation of gene expression is important

Table 3
Antidepressant treatment associated epigenetic modifications in in vitro and preclinical studies.

Reference	Species	Tissue	Treatment	Treatment associated epigenetic modification, molecular changes and other outcomes
Angelucci et al. (2011)	Human	Cell line	Paroxetine	Rapid ↑in *BDNF* mRNA expression and protein synthesis followed by ↑in miR-30a-5p, a BDNF inhibitor, at six and 12 h after treatment
Baudry et al. (2010), Launay et al. (2011)	Mouse	RN LC HC	Fluoxetine applied at RN for 3 days	↓miR-16 and serotonin transporter in RN ↓miR-16 and↑serotonin transporter in LC and HC Anti-miR-16 applied at HC results in anti-depressant behaviour
Dyrvig et al. (2012)	SD rat	HC	ECS, 1 day	↑H4 acetylation at *C-fos* promoter 1 hr post-ECS, ↑DNA methylation of *Arc* 24 hr post-ECS
Melas et al. (2012)	FSL rat	PFC	Escitalopram, 3 weeks	Pre-treatment: ↑DNA methylation,↓mRNA and protein of P11 membrane trafficking protein Post-treatment: *P11* DNA methylation returned to control levels,↑ *P11* mRNA, ↓mRNA for DNA methyltransferase enzymes, *Dnmt1* and *Dnmt3a*
Onishchenko et al. (2008)	Mouse	HC	Fluoxetine, 21 days from PND 63	↑H3 acetylation at *Bdnf* promoter and ↑*Bdnf* mRNA compensating for epigenetic effects of perinatal methylmercury exposure (Table 1)
Oved et al. (2012)	Human	Cell line	Paroxetine, 3 days	Paroxetine sensitive cells had higher basal expression levels of miR-151-3p, associated with↓mRNA for miR-151-3p effector, *CHL1*, a neural cell adhesion molecule Basal levels of miR-212, mir-132, miR-30b*, let-7b and let-7c also differed significantly by more than 1.5 fold between the high and low sensitivity cell groups
Rodrigues et al. (2011)	Human	Cell lines	Fluoxetine, 24 h	↓miR124a in SH-SY5Y and BE(2)–M17 cell lines ↑miR-27b in BE(2)–M17 cell line
Tsankova et al. (2006)	Mouse	HC	Imipramine 4 weeks	Pre-treatment: ↑methylation of H3K27 at *Bdnf* promoters, ↓*Bdnf* mRNA after social defeat stress Post-treatment: ↑H3 acetylation at *Bdnf* promoters, ↑*Bdnf* mRNA, ↓HDAC5 mRNA
Tsankova et al. (2004)	SD rat	HC	ECS 1 or 7 days	↑ H3 and H4 acetylation at *Bdnf* promoter II 2 h after 1 and 7 days ECS, ↑*Bdnf* mRNA ↑H3 acetylation at *Bdnf* promoters III and IV 24 h after 7 days ECS, ↑*Bdnf* mRNA ↑ H3 and H4 acetylation at *C-fos* promoter 2 h after 1 and 7 days ECS,↑*C-fos* mRNA ↓H3 acetylation at *Creb* promoter 2 h after 1 and 7 days ECS, ↓H4 acetylation 24 h after 7 days ECS with ↓*Creb* mRNA
Zhou et al. (2009)	Wistar rat	HC	Lithium, VPA, 4 weeks	37 miRNAs altered by lithium, 31 miRNAs altered by VPA, nine miRNAs regulated by both drugs: ↓let-7b and let-7c, miR-105, miR-128a, miR-24, miR-30c, miR-34a, miR-221 and miR-136, and↑miR-144

Abbreviations: BDNF-brain derived neurotrophic factor, ECS-electroconvulsive stimulation, FSL-Flinders Sensitive Line, H-histone, HC-hippocampus, HDAC-histone deacetylase, h-hours, K-lysine, LC-locus coeruleus, mRNA-messenger RNA, PFC-prefrontal cortex, PND-postnatal day, RN-raphe nucleus, SD-Sprague Dawley, VPA-valproic acid.

Table 4
Antidepressant treatment associated effects on miRNA expression in in vitro, preclinical and clinical studies.

Reference	Species	Tissue	Treatment	Treatment associated epigenetic modification, molecular changes and other outcomes
Angelucci et al. (2011)	Human	Cell line	Paroxetine	Rapid ↑in *BDNF* mRNA expression and protein synthesis followed by ↑in miR-30a-5p, a BDNF inhibitor, at six and 12 hours after treatment
Baudry et al. (2010), Launay et al. (2011)	Mouse	RN LC HC	Fluoxetine applied at RN for 3 days	↓miR-16 and serotonin transporter in RN ↓miR-16 and↑serotonin transporter in LC and HC Anti-miR-16 applied at HC results in anti-depressant behaviour
Belzeaux et al. (2012)	Human, MDD	Blood	Various antidepressants 8 weeks	↑miR-20b-3p, miR-433, miR-409-3p, miR-410, miR-485-3p, miR-133a, miR-145 ↓miR-331-5p
Bocchio-Chiavetto et al. (2013)	Human, MDD	Blood	Escitalopram, 12 weeks	↑28 miRNAs including *BDNF*-related miR-132 ↓ miR-34c-5p and miR-770-5p
Oved et al. (2012)	Human	Cell line	Paroxetine, 3 days	Paroxetine sensitive cells had higher basal expression levels of miR-151-3p, associated with↓mRNA for miR-151-3p effector, *CHL1*, a neural cell adhesion molecule Basal levels of miR-212, mir-132, miR-30b*, let-7b and let-7c also differed significantly by more than 1.5 fold between the high and low sensitivity cell groups
Rodrigues et al. (2011)	Human	Cell lines	Fluoxetine, 24 h	↓miR124a in SH-SY5Y and BE(2)–M17 cell lines ↑miR-27b in BE(2)–M17 cell line
Zhou et al. (2009)	Wistar rat	HC	Lithium, VPA, 4 weeks	37 miRNAs altered by lithium, 31 miRNAs altered by VPA, nine miRNAs regulated by both drugs: ↓let-7b and let-7c, miR-105, miR-128a, miR-24, miR-30c, miR-34a, miR-221 and miR-136, and↑miR-144

Abbreviations: BDNF-brain derived neurotrophic factor, HC-hippocampus, LC-locus coeruleus, MDD-major depressive disorder, miRNA-microRNA, RN-raphe nucleus, VPA-valproic acid.

in the mechanism of action of this powerful antidepressant treatment. However, the epigenetic effects of ECS in animal models of depression and ECT in humans are still to be reported.

Epigenetic modification of gene expression has been reported following administration of antidepressant drugs in various models of depression (Table 3). In adult mice, epigenetic modifications of *Bdnf* have been reported after social defeat stress, which results in phenotypic behaviours that mimic depressive symptoms in humans (Tsankova et al., 2006). In a study by Tsankova et al. (2006), social defeat stress was associated with decreased *Bdnf* mRNA in the hippocampus of mice and was accompanied by an increase in gene-suppressing methylation of histone 3 at lysine 27 at *Bdnf* gene promoter regions. Antidepressant treatment with imipramine in this model resulted in increased mRNA levels for *Bdnf*. Although methylation of histone 3 at lysine 27 was not reversed by imipramine, compensatory *Bdnf* gene expression enhancing epigenetic modifications were observed, including hyperacetylation of histone 3 at *Bdnf*-promoters, and a decrease in mRNA levels for histone deacetylase 5 (HDAC5), an enzyme that decreases gene expression by decreasing histone acetylation (Tsankova et al., 2006). In addition, HDAC5 overexpression prevented the ability of imipramine to reverse the depressive phenotype in stressed animals, highlighting the importance of histone acetylation in the antidepressant effect.

Studies examining the expression of miR-16, which targets the serotonin transporter, have provided insight into the mechanism of action of fluoxetine in the mouse brain (Baudry et al., 2010; Launay et al., 2011). Infusion of fluoxetine into the raphe nucleus, a major source of ascending serotonergic projections in the brain, resulted in a decrease in miR-16 and a two-fold reduction in binding of serotonin transporter (Baudry et al., 2010). Fluoxetine applied at the raphe, also appears to affect miRNA expression in the locus coeruleus (Baudry et al., 2010) and hippocampus (Launay et al., 2011) causing a reduction in miR-16 and a subsequent increase in serotonin transporter levels in both brain regions. Furthermore, neutralisation of miR-16 in the hippocampus by application of anti-miR16 results in antidepressant-like behavioural effects highlighting the importance of its role in the downstream effects of fluoxetine (Launay et al., 2011).

Few studies to date have reported on the epigenetic effects of antidepressant treatment in humans (Belzeaux et al., 2012; Bocchio-Chiavetto et al., 2013; Chen et al., 2011; Lopez et al., 2013; Sharma et al., 2006) (Table 4). Chen et al. (2011) reported an increase in *BDNF* expression in the prefrontal cortex postmortem that was associated with a decrease in gene-repressing methylation of histone 3 at lysine 27 in patients taking a variety of antidepressants (Chen et al., 2011). In a follow-up study, citalopram treatment for eight weeks induced an increase in BDNF protein levels in peripheral blood of treatment-naïve MDD patients (Lopez et al., 2013). This was accompanied by a decrease in methylation of histone 3 at lysine 27 primarily in blood plasma in patients that were drug responders. miRNA changes following antidepressant treatment have been reported in two studies (Belzeaux et al., 2012; Bocchio-Chiavetto et al., 2013). miRNA changes were examined in blood from 10 patients with MDD following 12 weeks of administration of the SSRI escitalopram. After treatment, 28 miRNAs were upregulated, including the *BDNF*-related miR-132, while two miRNAs, miR-34c-5p and miR-770-5p, were robustly downregulated (Bocchio-Chiavetto et al., 2013). Predicted target genes included growth factors such as *BDNF* and vascular endothelial growth factor, calcium channels and neurotransmitter receptors. Further analysis indicated that miRNA expression was enriched for pathways important in brain function such as axonal guidance and long-term potentiation.

Collectively, the results of studies in humans and animal models suggest that antidepressant treatment reverses or elicits compensatory epigenetic changes such as histone hyperacetylation and DNA hypomethylation that promote previously decreased gene expression. The research highlighted above also provides evidence of miRNA regulation following antidepressant treatment both in vitro and in vivo. The miRNAs altered by antidepressants are important in neuronal processes that are affected in MDD such as neurogenesis and long-term potentiation which is important in memory formation. Epigenetics-based treatments have shown promising results in clinical trials for cancer (O'Rourke et al., 2013; Qiu et al., 2013; Tan et al., 2010), hepatitis C (Janssen et al., 2013) and provide an exciting new field for the development of therapies for MDD. Gaining a greater understanding of the epigenetic mechanisms underlying antidepressant mechanisms may guide the development of novel treatments for MDD, e.g. targeted HDAC and DNA methyltransferase inhibitors, and inhibition of miRNAs with small molecules. Additionally, miRNA expression profiles may serve as useful biomarkers to predict treatment response in individuals.

7. Limitations

Given the novelty of the field, a narrative approach was taken with regard to this review which has its limitations in terms of subjective bias. This review is meant to give an overview of an emerging field and does not lend itself to meta-analysis. Many of the included studies were carried out in small samples and have not been validated or replicated. Caution should also be used in extrapolating findings from pre-clinical settings to patient populations. The technical analyses of epigenetic alterations are still under development and in some instances there has been failure to replicate findings in the field of neuropsychiatric epigenetics (Houston et al., 2013). For example, inconsistent results have been reported regarding hypermethylation of the REELIN gene promoter in brains of patients with schizophrenia (Abdolmaleky et al., 2005; Tochigi et al., 2008). The current methods available to study epigenetic factors can be crude and imprecise, and researchers face a number of challenges when studying epigenetic changes, particularly in humans, some of which are detailed below.

Disease aetiology and varying exposure to environmental factors, including alcohol and psychostimulants, can alter epigenetic marks such as DNA methylation. Variations in glia to neuron ratio can introduce a high degree of heterogeneity and thus affect reproducibility of epigenetic studies examining cohort-based effects in relatively small samples (Houston et al., 2013).

In addition, the techniques for analysis of chromatin activation or DNA methylation status typically require relatively large amounts of input material and lack single-cell resolution (Akbarian and Huang, 2009). This can pose a problem when working with brain tissue, particularly in preclinical studies due to the relatively small brain size of rodents. One solution is to use whole-brain lysates. However, epigenetic changes can vary between brain areas (Roth et al., 2009) and researchers run the risk of neglecting subtle, region-specific changes. Although region-specific studies give a more precise view of epigenetic changes in the brain, tissue-homogenates comprise a heterogeneous set of neurons, glia and other cells, making identification of specific neuronal subtype (e.g. GABAergic interneuron) epigenetic alterations difficult (Houston et al., 2013). Purification techniques for separating neuronal and non-neuronal cells are now available and may improve the precision of post-mortem brain analyses.

Alternatively to post-mortem brain tissue, peripheral blood offers a relatively accessible biological fluid that can be sampled multiple times from the same individual. Using peripheral blood, changes in miRNA expression and epigenetic modulation of genes such as *BDNF* have been reported in patients with MDD before and

after antidepressant treatment (Tables 2 and 4; (Lopez et al., 2013)). It remains to be seen to what degree changes in blood represent changes in the brain (Peedicayil, 2008). Furthermore, it is not clear if changes in blood reflect the pathological state or compensatory alterations in response to the pathological condition and/or treatment (Fass et al., 2013). Nonetheless, alterations in peripheral blood may serve as useful biomarkers in the diagnosis and treatment of psychiatric conditions including MDD (Cheng et al., 2013; Lopez et al., 2013).

Alterations in chromatin remodelling and/or DNA methylation can be measured globally or at a specific gene promoter. Promoter specific studies usually involve an isolation step to separate the epigenetically modified DNA (e.g. chromatin immunoprecipitation or bisulfite conversion), followed by analysis using PCR amplification (Fuchikami et al., 2011; Roth et al., 2009; Tsankova et al., 2004). Researchers examining changes in this manner must take into account multiple splice variants of genes of interest and design primers and PCR assays accordingly. For example, transcription of *BDNF* can arise from 11 different exons in humans and eight in rats, giving rise to multiple transcripts, known as splice variants, which can be individually epigenetically regulated (Boulle et al., 2012; Tsankova et al., 2004). As mentioned above, heterogeneity at the subject and tissue levels can also affect reproducibility in target-gene focused investigations. Furthermore, recent research indicates that in some conditions (e.g. autism), chromatin remodelling affects gene expression in subjects on an individual basis, a caveat which may limit findings in certain gene-specific studies (Houston et al., 2013).

Methodologies to assess epigenetic alterations on a global, genome-wide basis may help to overcome some of the limitations of target specific studies and provide the opportunity to identify previously unknown mechanisms involved in the pathology and treatment of MDD (Vialou et al., 2013). In terms of chromatin remodelling for example, genome-wide analysis was initially carried out using a technique known as ChIP-chip. Chromatin immunoprecipitation (ChIP) is a procedure whereby DNA bound to epigenetically modified lysine residues on histone tails (e.g. histone 3 at lysine 9, see Fig. 2) can be precipitated using targeted antibodies. Sequences of interest are then identified by hybridising bound DNA to a chip (microarray) containing a number of known DNA sequences. Wilkinson et al. (2009) used ChIP-chip with an antibody for histone 3 lysine 9 to identify genes that were upregulated or downregulated in the nucleus accumbens in mice one month after exposure to chronic stress, before and after imipramine treatment.

ChIP-chip is now being superseded by ChIP-seq (Houston et al., 2013). ChIP-seq analysis, which combines ChIP with next generation sequencing (NGS) technologies involving high-throughput sequencing of all enriched DNA sequences, provides the opportunity for increased sensitivity, higher resolution and more comprehensive screening of genomic profiles whilst requiring less input material than ChIP-chip (Park, 2009). NGS analysis is now also being combined with other techniques to identify epigenetic marks on a genomic scale. For instance, methods for characterising DNA methylation in human and rodent brain tissue have been combined with NGS analysis to create a database of genome-wide DNA methylation profiles (Xin et al., 2012). NGS also has the potential to examine miRNA alterations in psychiatric conditions and has already been used to study miRNA changes in the hippocampus of Alzheimer's patients (Lau et al., 2013). With the recent development of more widely available and affordable sequencing platforms, as well as databases containing epigenetic profiles of the human and rodent brain allowing cross-study and -species comparisons, NGS will enhance precision and resolution in pre-clinical and clinical epigenetic studies (Fass et al., 2013; Wang et al., 2013; Xin et al., 2012).

8. Summary and conclusions

Epigenetic mechanisms operate alongside the DNA sequence and promote or suppress gene expression, ultimately influencing protein production. Epigenetic modifications of gene expression include alterations in DNA methylation, chromatin remodelling and miRNA expression patterns. Epigenetics is an important new area in the field of MDD research and provides a mechanism for understanding the link between the long-term effects of adverse life events and the changes in gene expression that are associated with depression. Indeed, risk factors for depression, such as early life stress, are associated with epigenetic changes in gene expression in both preclinical and clinical studies. Persistent epigenetic modification of gene expression induced by environmental stimuli may also play a role in the transgenerational transmission of behavioural disturbances such as depression. In individuals with MDD, epigenetic modification of genes such as *BDNF* and its receptor *Trk B* as well as synapsins and GABA receptor subunits have been observed. Alterations in miRNA levels and polymorphisms in miRNAs, their precursors, their target mRNAs and in genes that regulate miRNA biogenesis have also been noted in MDD. Although still a developing field, in the future, epigenetic modifications of gene expression may provide novel biomarkers to predict future susceptibility and/or onset of MDD, to improve diagnosis and to better understand the pathophysiology of depression.

Epigenetics also appears to be important in the mode of action of antidepressant treatments. In preclinical studies, ECS, the animal model equivalent of the most acutely effective treatment for depression, causes alterations in chromatin activation status and DNA methylation of depression-related genes. Antidepressant drug treatments appear to reverse or bring about compensatory changes in gene expression in an animal model of depression and in clinical studies of MDD. Furthering our knowledge of the epigenetic modifications of gene expression elicited after antidepressant treatment may aid in the development of epigenetics-based therapies for depression and to predict treatment outcome.

Conflict of interest

All authors declare that they have no conflicts of interest.

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Mapping of Nervous System Diseases via MicroRNAs

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Preface

Molecular and cellular neurobiological studies of microRNA (miRNA)-mediated gene silencing in the nervous system represent the exploration of a new frontier of miRNA biology and the potential development of new diagnostic tests and genetic therapies for neurological disease. Over recent years our understanding of microRNA (miRNA) biogenesis, molecular mechanisms by which miRNAs regulate gene expression, and the functional roles of miRNAs has been expanded. MiRNAs are ≈22 nucleotide-long double-stranded RNAs. One strand of these small noncoding RNA molecules operates as guide for RISC (RNA-induced silencing complex) to produce either the block of translation or the decay of target mRNAs. In mammalian cells, miRNA action is mediated by an imperfect pairing between the 3′UTRs of the mRNA targets and nucleotides 2–8 from the 5′ of the miRNA.

Recently, microRNAs are emerging as important players in posttranscriptional regulation in the brain. Several studies have shown spatially and/or temporally restricted distribution of miRNAs, suggesting that they may control the fine-tuning regulation of neuronal gene expression.

Individual microRNAs can reduce the production of a hundred proteins and miRNA-mediated posttranscriptional regulation is involved in neuronal differentiation, dendritic spine development, and synaptic plasticity.

Increasing evidence suggests that miRNAs are dysregulated in several neurological disorders. The collection of data on the association between human brain diseases and miRNAs has focused on expression profiles of miRNAs and their quantitative modulation (i.e., upregulation versus downregulation), according to age, gender, phase of the disease, and specific brain area.

Depending on the role of the miRNA, the goal of the treatment will be to either increase or reduce miRNA function. Given the importance that miRNAs might play in neuropathology, several strategies to manipulate miRNA activity and expression are being pursued. Two main strategies may be applied to target miRNA expression in the brain: directly, by using oligonucleotides or virus-based constructs, and indirectly, by using drugs to modulate miRNA expression at the transcriptional and/or processing level. To date, all delivery strategies have been important for identifying a suitable way to generate microRNA-based therapies for neurological diseases related to the perturbation of miRNAs. The main challenge for miRNA therapeutics in neurology, beyond stability and safety, is delivery to the appropriate tissue and neurons. Molecules modulating miRNA action must reach the cells and must function at the site of the disease.

Due to the ever-expanding knowledge of miRNAs as fine tuners of gene expression in all aspects of biology and medicine, and to the emerging impact of sequence-specific posttranscriptional gene silencing mediated by miRNA as a potential therapeutic approach directed to the nervous system, we believe that this book in the series Frontiers in Neurotherapeutics will be of great interest to a broad scientific audience. This book, titled *Mapping of Nervous System Disease via MicroRNAs*, consists of nine chapters, and opens a window on our current understanding of the

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microRNAs involved in neurological diseases. This is an exciting work because the collection of selected chapters provides insight into the full range of concepts and a snapshot of the current status of this dynamic field.

The book is divided into four sections. Section I gives an overview of the landscape of miRNAs biology and function in the nervous system. This is followed by Chapters 2 through 4 under Section II, which focuses on discovery, regulatory functions, and molecular mechanism of miRNAs associated with neuropsychiatric disease. These chapters provide an excellent account of the miRNAs in anxiety and depression, and in mental disorders characterized by abnormal social behavior, such as schizophrenia, or important affective disorders with a high risk for suicidal behavior. These chapters are particularly important because they offer a new point of view to identify novel insights into brain/behavioral diseases.

Section III provides the state of the art in neurological diseases, which have been little explored from a "microRNA research view." The authors of these chapters illustrate how microRNAs could be involved in the prion pathogenesis, or in molecular mechanisms of epilepsy and pain processing and conditions, showing a new frontier of neuroscience research.

The "Applications" Section IV includes Chapters 8 and 9, which analyze the advancement of the study of miRNAs in biofluids in both physiological and pathological conditions. In addition, miRNAs are described as biomarkers for different neurodegenerative diseases, such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis or for CNS development and during postnatal life, an emerging field of research.

Mapping of Nervous System Diseases via MicroRNAs serves as an introduction to the miRNAs in the nervous system, and their increasingly important role in neurological diseases. In addition, in each chapter of the book, readers and scholars will find material on the future diagnostic and therapeutic advances of microRNA in selected neurological diseases.

We thank all the authors who have contributed excellent chapters to this book and reviewers for their critical comments to improve the quality and integrity of the chapters. We are grateful to Laura Berliocchi for the invitation to initiate this book in the Frontiers in Neurotherapeutics Series and for her continuing support and commitment in making this book a reality and to staff members involved in the production of the book.

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2 MicroRNAs in Mood and Anxiety Disorders

Karen M. Ryan, Erik Kolshus, and Declan M. McLoughlin

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2.1 INTRODUCTION TO MOOD AND ANXIETY DISORDERS

Mood and anxiety disorders are among the most common mental health disorders, with a huge individual, societal, and economic burden (Wittchen et al., 2011). Affective disorders are characterized by episodes of abnormally depressed or elated

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mood. Depression is frequently recurrent with lifetime prevalence rates in the region of 10%–20% (Kessler et al., 2012; Kessler and Bromet, 2013). It can be difficult to treat and is associated with increased suicide rates (Bostwick and Pankratz, 2000). Bipolar affective disorder (BPAD) is less common, with lifetime prevalence rates around 2.5% (Kessler et al., 2012). Anxiety disorders is an umbrella term for a number of disorders, including specific phobias, social phobias, posttraumatic stress disorder (PTSD), generalized anxiety disorder, panic disorder, agoraphobia, and obsessive-compulsive disorder (OCD). The overall lifetime prevalence of any anxiety disorder is estimated at 31.6% (Kessler et al., 2012). Both mood and anxiety disorders are often life-long conditions, which is reflected in the WHO's latest ranking of top 10 causes of Global Years Lived with Disability with depression ranking second and anxiety disorders seventh (Murray et al., 2012).

Unipolar depression is characterized by a clear period of low mood and/or anhedonia accompanied by a variable number of other symptoms, including "biological" symptoms such as disturbed sleep, appetite, activity levels and energy, as well as "psychological" symptoms such as poor concentration, feelings of guilt, worthlessness, and suicide (American Psychiatric Association, 2013). BPAD is characterized by alternating episodes of depression (as described above) and hypomanic or manic episodes that are defined by a period of persistently elevated, expansive, or irritable mood accompanied by a variable number of other symptoms, including increased self-esteem, racing thoughts, pressure of speech, distractibility, risk-taking behavior, and goal-directed behavior. Anxiety disorders all share a common theme of a heightened sense of arousal accompanied by physical and psychological symptoms that can be either generalized or linked to specific triggers. Physical symptoms include shortness of breath, palpitations, chest pain or discomfort, and choking sensations, while psychological symptoms include fear of dying, fear of losing control, and apprehension. Diagnosis of mood and anxiety disorders currently depends on clinical judgement, with no laboratory test to date having any practical utility.

There is considerable genetic influence on both mood and anxiety disorders. Heritability is ~39%–42% for unipolar depression (Flint and Kendler, 2014), 70%–80% for BPAD (Craddock and Sklar, 2013), and 30%–67% for anxiety disorders (Domschke and Deckert, 2012). Candidate genes for unipolar depression include *BDNF*, *5HTT*, *FKBP5*, *TPH2*, and *HTRA2* (Flint and Kendler, 2014). While no single gene with a large effect has been found for BPAD, genes such as *CACNA1C* and *ANK3* and genes involved in circadian rhythm have been associated with it (McCarthy et al., 2012). There has been little progress in identifying candidate genes for specific anxiety disorders. Therefore, although the evidence-base points to considerable genetic influence on both mood and anxiety disorders, current thinking is that genetic influences arise from many genes with small effect.

2.2 TREATMENT OF MOOD AND ANXIETY DISORDERS

Major breakthroughs in the 1950s and 1960s resulted in the development of tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) for depression and benzodiazepines for anxiety disorders. However, there have been few new major pharmacological breakthroughs since (Spedding et al., 2005).

The mainstay of pharmacological therapy for depression remains selective serotonin reuptake inhibitors (SSRIs), serotonin–noradrenaline reuptake inhibitors (SNRIs), TCAs, MAOIs, and others, which all aim to increase the amount of monoamines, such as serotonin or noradrenaline, at the synapse. However, the most acutely effective treatment for severe depression remains electroconvulsive therapy (ECT), which has been used since the 1930s (Group, 2003). Treatment of BPAD can involve the use of antidepressant medications during a depressive episode, but typically involves the use of mood stabilizers such as lithium and anticonvulsants for continuation therapy, and mood stabilizers and antipsychotics or benzodiazepines in the acute phase. Pharmacological treatments for anxiety disorders primarily target the gamma-aminobutyric acid (GABA) (benzodiazepines or pregabalin) or serotonergic (SSRIs, SNRIs, and TCAs) systems.

2.3 MOLECULAR NEUROBIOLOGY OF MOOD AND ANXIETY DISORDERS

One of the great challenges facing neuroscience is improving our understanding of the molecular basis of psychiatric disorders, including mood and anxiety disorders.

The accidental discovery that some antitubercular medications had antidepressant effects led to the development of the TCAs in the 1950s (Berton and Nestler, 2006). This laid the premise for early models of major depression, which postulated a "chemical imbalance" in monoamine neurotransmitters. Subsequent models of depression, that are also relevant in BPAD and anxiety disorders, include hypothalamic–pituitary axis (HPA axis) dysregulation, inflammatory models, and neurotrophic models of affective disorders. **Q1**

The monoamine hypothesis of depression states that depression is caused by a deficiency of monoamines in the brain and that antidepressant treatment will normalize these levels (Berton and Nestler, 2006). However, although catecholamine levels at the synapse increase acutely in response to treatment, a clinical response can take weeks or months. A number of neurotransmitters have been implicated in BPAD, chiefly serotonin and the catecholamines. Drugs that cause an increase in serotonin or catecholamines (e.g., antidepressants, amphetamine, and cocaine) can induce hypomanic or manic episodes (Manji et al., 2003). Initial pharmacological treatment for anxiety targeted the GABA system; however, SSRIs are the current first-line treatment for many anxiety disorders. Thus, monoamine neurotransmitters and their pathways clearly play a part in the pathophysiology of mood and anxiety disorders, although it has become clear they are only one part of a more complex system.

Hyperactivity of the HPA axis has long been linked to anxiety and affective disorders, particularly depression. In response to stress, the hypothalamus secretes corticotropin-releasing hormone (CRH), which acts on the anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH in turn stimulates cortisol release from the adrenal glands (Pariante and Lightman, 2008). Circulating cortisol normally triggers a negative feedback loop, inhibiting further release of CRH and ACTH. In many depressed patients, this negative feedback system appears to be dysregulated leading to chronically high levels of CRH, ACTH, and cortisol. CRH

interacts with other neurotransmitter systems, including the catecholamines (Berton and Nestler, 2006). It is postulated that in patients vulnerable to developing depression, the glucocorticoid receptors (GRs) lose their inhibitory effect leading to loss of the negative feedback loop but the exact mechanism remains unclear. The HPA axis response to stress is also clearly involved in the pathophysiology of anxiety disorders.

Some of the first evidence linking the immune system to mood and anxiety disorders came from the observation that patients with immune system disorders had increased rates of psychiatric disorders and many of the key symptoms of depression are also seen in immune-related illnesses. In later years, patients undergoing interferon (a form of cytokine) therapy for cancers or viral infections were observed to have high rates of depression (Hoyo-Becerra et al., 2014). Elevated levels of some cytokines and other inflammatory markers have since been found in depressed patients (Dowlati et al., 2010). However, it is still not known how these cytokines and other inflammatory markers lead to psychiatric symptomatology.

In recent years, focus has shifted on to the neurotrophic model which suggests that drug treatments act by inducing neuroplastic changes, for example, hippocampal neurogenesis, synaptogenesis, increased dendritic spines, and dendrites. The importance of neuroplasticity is emphasized by evidence of loss of neurons and glia in the hippocampus and prefrontal cortex in depression. Brain-derived neurotrophic factor (BDNF) is widely expressed throughout the brain, promoting neuronal survival and maturation, synaptic plasticity, and synaptic function. Low levels of BDNF have been found in postmortem brains of depressed patients, and BDNF can exert antidepressant activity. Other potential therapeutic targets include vascular endothelial growth factor (VEGF) and the transcription factor CREB (cAMP response element–binding protein; Duman and Aghajanian, 2012).

There have been few truly new developments in the pharmacological treatment of mood and anxiety disorders and there is still a lack of understanding of the molecular basis of these disorders. Genetic and environmental factors both contribute to the development of these disorders, but how they do so, and how they may interact, remains unsolved. Recent findings implicate microRNAs (miRNAs) and other epigenetic changes as potential "micromanagers" of these changes (Dalton et al., 2014). MiRNAs may also address the problematic issue of numerous genes of small effect, as one miRNA can potentially regulate hundreds of genes (Kolshus et al., 2014). With diagnosis of psychiatric disorders still relying on clinical judgement, the search for a blood biomarker to aid diagnosis, prognosis, and response to treatment is very much on going. MiRNAs may ultimately offer one such possibility.

2.4 ROLE OF MicroRNAs IN THE BRAIN

About half of all known miRNAs are expressed in the brain (Landgraf et al., 2007; Shao et al., 2010), where they play a role in a variety of processes including cell proliferation (Delaloy et al., 2010; Liu et al., 2010; Niu et al., 2013), neurogenesis (Morgado et al., 2014; Rago et al., 2014), cell specification (Smirnova et al., 2005), and synaptic plasticity (Schratt et al., 2006; Gao et al., 2010), among other functions. MiRNAs show cell and tissue-specific expression (Landgraf et al., 2007) and play an

important role in neural cell-type specification. The various cell types in the brain, that is, neurons, astrocytes, oligodendrocytes, and microglia have distinct miRNA profiles (Jovicic et al., 2013) and various neuronal subpopulation, for example, glutamatergic and GABAergic neurons, also show distinct miRNA profiles (He et al., 2012a). Moreover, miRNAs are found to be localized in many different subcellular compartments such as axons and synapses (Lugli et al., 2008; Natera-Naranjo et al., 2010; Sasaki et al., 2014). MiRNA expression can occur in a temporal fashion in both the developing brain (Miska et al., 2004; Mineno et al., 2006) and after the induction of neuronal activity (van Spronsen et al., 2013). Thus, miRNAs can specifically alter local gene expression profiles indicating their potentially unique roles in the brain. Since miRNAs participate in such a variety of cellular processes, changes in miRNA levels can have profound and wide-ranging effects. Several lines of evidence exist to suggest that changes in miRNA levels are involved in the development and treatment of neuropsychiatric conditions such as mood- and anxiety-related disorders which will be discussed in the following sections.

2.5 SYSTEMATIC REVIEW OF MicroRNAs IN MOOD AND ANXIETY DISORDERS

2.5.1 Introduction

A number of reviews of miRNAs in psychiatric disorders have been published but this is a rapidly changing field with ongoing developments, and few of the existing reviews have been systematic. Here we offer an up-to-date systematic review of both preclinical and clinical evidences for the role of miRNAs in mood and anxiety disorders.

2.5.2 Methods

Relevant preclinical and clinical studies were identified using searches of PubMed/Medline and Web of Science up to July 2014 with the following terms cross-referenced with “miRNA”: “depression,” “antidepressant,” “bipolar,” “anxiety,” “panic,” “OCD,” “PTSD,” “phobia,” “psychiatr*.” No language limit was used. The references from included articles were also reviewed. The results are presented in Tables 2.1 through 2.6 and discussed in the following sections.

2.6 MicroRNAs IN MOOD DISORDERS

2.6.1 Preclinical Studies

A review of the literature identified 24 articles which were relevant for inclusion here. Preclinical studies assessing the role of miRNAs in mood disorders have so far focused on four major areas, that is, the role of miRNAs in the stress response, in depressive-like behaviors, and in the mechanism of action of antidepressants and mood stabilizers. The findings from these studies are outlined in Table 2.1 and summarized below.

TABLE 2.1
Preclinical Studies of MiRNAs in Stress

Author	Species	Tissue	Analysis	Main Findings
Uchida et al. (2008)	Fischer 344 rats; chronic RS; SH-SY5Y cells	PVN	RT-qPCR; northern blotting	• MiR-18a ↓ GR protein *in vitro* • ↑ pre-miR-18a and ↑ mature miR-18a in F344 rat PVN under nonstressed and chronic stress conditions • ↓ GR protein in F344 rat PVN
Vreugdenhil et al. (2009)	Long-Evans rats; NS1, A549, COS-1 cells	Frontal cortex, hippocampus	RT-qPCR	• MiR-18 and miR-124a ↓ GR-mediated events and ↓ GR protein levels • GC induced GILZ activity impaired by overexpression of miR-124a and miR-18 • ↑ miR-18 and miR-124a in frontal cortex and hippocampus during postnatal development
Kawashima et al. (2010)	Rat primary cortical cultures	–	RT-qPCR	• BDNF ↑ miR-132 *in vitro* • Exogenous ds-miR-132 ↑ postsynaptic proteins (NR2A, NR2B, GluR1) • Dexamethasone ↓ BDNF-induced ERK1/2 activation, miR-132 expression, and postsynaptic proteins
Meerson et al. (2010)	Male rats; acute/chronic RS	Amygdala, hippocampal CA1	Microarray; RT-qPCR	• Acute RS amygdala: ↑ miR-106b, miR-134, miR-183, miR-382; ↓ let-7a-1, miR-202, miR-361, miR-376b, miR-381, miR-9-1 • Acute RS hippocampus: ↑ miR-1-2, miR-376b, miR-182*, miR-424, miR-190, miR-19a, miR-208, miR-216, miR-32; ↓ let-7f-2, miR-124a-1, miR-138-1. miR-15b, miR-202, miR-422a, miR-9-1 • Chronic RS amygdala: ↑ miR-1-2, miR-15a, miR-190, miR-193, miR-208, miR-22, miR-322, miR-361, miR-369, miR-376b, miR-381; ↓ let-7a-1, let-7c, let-7f-1, let-7f-2, miR-103-1, miR-134, miR-138-1, miR-182, miR-216, miR-222, miR-298, miR-323, miR-34a, miR-368, miR-9-1, miR-96 • Chronic RS hippocampus: ↑ miR-132, miR-17-5p, miR-208, miR-23a, miR-369, miR-376b, miR-410; ↓ let-7c, let-7f-1, miR-100, miR-134, miR-148a, miR-16-1, miR-182*, miR-219-1, miR-22, miR-221, miR-30a-3p, miR-330, miR-376a, miR-9-1, miR-96 • MiR-134 and miR-183 altered by RS, target SC35 mRNA

(*Continued*)

TABLE 2.1 (*Continued*)
Preclinical Studies of MiRNAs in Stress

Author	Species	Tissue	Analysis	Main Findings
Rinaldi et al. (2010)	Male CD1 mice; acute/chronic RS	Frontal cortex	Microarray; northern blotting	• Acute RS: ↑ miR-9, miR-9*, miR-26b, miR-29b, miR-29c, miR-30b, miR-30c, miR-30e, miR-125a, miR-126-3p, miR-129-3p, miR-207, miR-212, miR-351, miR-487b, miR-690, miR-691, miR-709, miR-711, let7a-e; ↓ miR-423, and miR-494 • Repeated RS: ↑ miR-29b, miR-29c, miR-129-3p, miR-207, miR-212, miR-351, miR-423, miR-487b, miR-494, miR-690, miR-691, miR-709, miR-711; ↓ miR-9, miR-9*, miR-26b, miR-30b, miR-30c, miR-30e, miR-125a, miR-126-3p, let7 a-e
Uchida et al. (2010)	Sprague-Dawley rats; MD	Medial prefrontal cortex	RT-qPCR; northern blotting	• MD ↑ pre-miR-132 ,-124-1, 9-1, -9-3, -212, -29a • MD ↑ RE-1-containing genes—Glur2, Nr1, CamKIIα, L1, Adcy5, Kcnc1 • MD ↑ mature miR-132, -124, -9, and -29a (all have RE-1 site) • REST4 overexpression in neonatal mice ↑ pre-miR-132, -212, and -9-3 and ↑ CamKIIα, Glur2, Adcy5
Bai et al. (2012)	Male Sprague-Dawley rats; MD	Hippocampus	RT-qPCR	• MD ↓ BDNF mRNA and protein • MD ↑ miR-16 • BDNF positively correlates with depressive-like behaviors in FST and OFT • MiR-16 negatively correlates with depressive-like behaviors in FST and OFT
Rodgers et al. (2013)	C57BL/6:129 F1 hybrid mice; CUPS of sires during puberty or adulthood	Sperm	Microarray	• Paternal stress ↑ miR-29c, miR-30a, miR-30c, miR-32, miR-193-5p, miR-204, miR-375, miR-532-3p, miR-698 • mRNA targets: DNMT3a, Tnrc6b, Mtdh • Offspring have ↓ HPA axis stress responsivity
Zhang et al. (2013a)	Sprague-Dawley rats; MD, CUPS	Nucleus accumbens	RT-qPCR	• MD + CUPS ↑ miR-504 • ↑ miR-504 negatively correlates with DRD2 expression • DRD2 expression negatively correlates with immobility in FST

(*Continued*)

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TABLE 2.1 (*Continued*)
Preclinical Studies of MiRNAs in Stress

Author	Species	Tissue	Analysis	Main Findings
Zucchi et al. (2013)	Long-Evans rats; MS	Dams: frontal cortex; offspring: whole brain	Microarray; RT-qPCR	• Dams: ↑ 147 miRNAs, ↓147 miRNAs; ↓miR-329, miR-380, miR-20a, miR-500, let-7c, miR-23b, miR-181, miR-186; ↑ miR-24-1 • Offspring: ↑ 205 miRNAs, ↓ 131 miRNAs; ↓ miR-361, miR-17-5p, miR-425, miR-345-5p, miR-505, miR-103, miR-151, miR-145; ↑ miR-23a, miR-129-2, let-7f, miR-98, miR-9, miR-216-5p, miR-667, miR-219-2-3p, miR-323
Issler et al. (2014)	5HT neuronal cultures; male C57BL/6 mice	–	Microarray; RT-qPCR	• 5HT neurons: ↑ miR-375, miR-376c, miR-7a, miR-137, mghv-miR-M1-2, miR-709, miR-291b-5p, miR-1224, miR-1892, miR-702, miR-139-3p, miR-762, miR-671-5p, miR-483*; ↓ miR-691, miR-466l, miR-17, miR-376b, miR-124, miR-218, miR-128, miR-140*, miR-148a, miR-340-5p, miR-181c, miR-210, miR-135a, miR-27a, miR-452, miR-370, miR-300, miR-376a, miR-127, miR-15b, miR-101a, miR-16, miR-324-5p, miR-434-5p, miR-92a, miR-669i, • miR-135a ↑ by SSRI antidepressants • miR-135a overexpression in 5HT neurons ↓ anxiety- and depression-like behaviors in mice • Knockdown of miR-135a ↑ anxiety- and depression-like behaviors in mice

Note: PVN = paraventricular nucleus; GR = glucocorticoid receptor; RT-qPCR = real-time quantitative polymerase chain reaction; GILZ = glucocorticoid-induced leucine-zipper; BDNF = brain derived neurotrophic factor; ERK1/2 = extracellular signal-regulated kinase 1/2; RS = restraint stress; MD = maternal deprivation; LH = learned helplessness; FST = forced swim test; OFT = open field test; HPA = hypothalamic-pituitary-adrenal axis; MS = maternal separation; CUPS = chronic unpredictable stress.

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2.6.1.1 MicroRNAs and Stress

Stress is considered to be a precipitating factor for the development of many psychiatric illnesses. Studies examining the role of miRNAs in the stress response have focused primarily on the role of miRNAs in the regulation of the GR, the effects of early-life stress on miRNAs, and the impact of various psychological stressors on miRNAs.

Studies assessing the role of miRNAs in GR regulation indicate that miR-18 plays an important role in this process. Uchida et al. (2008), investigating the role of miRNAs in the stress response and vulnerability to repeated stress, were the first to demonstrate that GR mRNA translation is inhibited by miR-18a *in vitro*. MiR-18a was also found to be increased in the paraventricular nucleus of F344 rats, a strain hyperresponsive to stress, following repeated restraint stress. A subsequent *in vitro* study by Vreugdenhil et al. (2009) confirmed the role of miR-18 in GR regulation by showing that overexpression of miR-18 reduces GR protein levels, attenuates GR-mediated transactivation, and reduces the induction of glucocorticoid-induced leucine zipper, a GR target gene (Vreugdenhil et al., 2009). These findings suggest that miR-18-mediated downregulation of the GR may be important in susceptibility to stress-related disorders.

As previously mentioned, increased levels of glucocorticoids and decreased levels of BDNF are common features of depressive disorders. Interestingly, a study by Kawashima et al. (2010) demonstrated that treatment of primary cortical neuronal cultures with the synthetic glucocorticoid dexamethasone attenuates BDNF and the BDNF-induced upregulation of miR-132 and postsynaptic glutamate receptors (NR2A, NR2B, GluR1). Increased miR-132 is critical for BDNF-induced dendritic outgrowth (Vo et al., 2005). Chronic stress and exogenous glucocorticoids are known to induce dendritic atrophy (Woolley et al., 1990; Watanabe et al., 1992) and this study is the first to suggest a possible mechanism by which this may occur, although further studies are required to fully evaluate this.

The adverse effects of early-life stress are suggested to contribute to the development of depressive-like behavior but little is known about the molecular mechanisms underlying the vulnerability to stress. Epigenetic mechanisms, such as those induced by miRNAs, appear to play a role with evidence coming from a number of studies that have investigated changes in miRNA levels in prenatal stress models. The majority of studies have so far focused on gestational stress or maternal deprivation (MD) with only one study examining the effects of paternal stress on miRNAs. The various forms of early-life stress used in these studies all result in increased depressive- and anxiety-like behaviors in the offspring (Uchida et al., 2010; Bai et al., 2012; Rodgers et al., 2013; Zhang et al., 2013a; Zucchi et al., 2013) but no clear role for miRNAs in the development of these behaviors has yet emerged.

Using an MD model, where young rats were separated from the mother during the early postnatal period, Bai et al. (2012) demonstrated increased levels of miR-16 in the hippocampus of offspring, corresponding with a decrease in BDNF. Interestingly, miR-16 levels negatively correlated with depressive-like behaviors. This finding is noteworthy because, as discussed in detail later, miR-16 has been shown to be a critical mediator of antidepressant action (Launay et al., 2011). Uchida

et al. (2010) also investigated miRNA changes in the MD rat model and found increased levels of a number of brain-enriched precursor miRNAs and mature miRNA species in the medial prefrontal cortex (PFC; see Table 2.1). There was no overlap in the findings of this study and the one conducted by Bai et al. However, one issue with these studies is that they each examined miRNA levels in different brain regions and tissue types. As discussed earlier, different brain regions and cell types display different miRNA profiles and so this may account for the lack of consistency in the findings to date.

Two studies have examined the effects of parental stress on offspring, one a study of gestational stress in the mother (Zucchi et al., 2013) and the other a study of paternal stress (Rodgers et al., 2013). Both studies found that parental stress has a profound impact on offspring. Using a model of maternal stress (MS) in rats where dams were subjected to stress during late gestation, Zucchi et al. (2013) showed alterations in miRNA levels in the brains of both the mother and the offspring. MS disrupted antepartum maternal behavior and induced alterations in miRNA levels in the frontal cortex of dams (Table 2.1), a region of the brain involved in maternal care. MS also impacted on miRNA levels in the brains of offspring from stressed dams (Table 2.1) and these miRNAs were shown to theoretically target mRNA species implicated in apoptosis, brain pathologies, neurotransmission, neurodevelopment, angiogenesis, cell signaling, and the stress response. The perinatal period represents a period of particular vulnerability for the developing brain. Thus, the findings from this study suggest that stress during gestation can modify the epigenetic signature of both the mother and offspring during critical periods of fetal brain development, which may result in life-long consequences in the offspring. Examining the role of paternal stress on miRNAs, Rodgers et al. (2013) demonstrated that paternal stress induces robust changes in mouse sperm miRNAs as outlined in Table 2.1. Interestingly, four of these miRNAs (miR-29c, miR-30a, miR-30c, and miR-204) are found to target DNMT3a (DNA methyltransferase 3a), a critical regulator of *de novo* DNA methylation. Offspring from these mice, which were bred following exposure of sires to pubertal or adulthood stress, had a blunted HPA axis response to acute restraint stress. Importantly, this study is the first to suggest that paternal stress exposure may be transmitted to future generations by inducing changes in miRNA levels in sperm. Overall, these studies suggest that alterations in miRNAs during early life can have life-long consequences for the organism.

One of the predominant findings from the preclinical studies conducted to date is that different stressors act by different molecular mechanisms and have varying effects on miRNAs. For instance, Bai et al. compared the effects of MD on miRNAs with that of chronic unpredictable stress (CUPS) and found that while CUPS induced more pronounced depressive-like behaviors, the underlying molecular mechanisms were different, that is, MD-induced depression was associated with changes in miR-16 and BDNF while CUPS was not. Combining MD, an early-life stressor, with later exposure to CUPS results in more pronounced depressive-like behaviors in rats than either MD or CUPS alone (Zhang et al., 2013a), implying that early-life stress enhances the vulnerability to stress in later life. This was associated with an increase in miR-504 and a decrease in levels of the dopamine receptor D2 (DRD2) in the nucleus accumbens of rats.

Two studies by Meerson et al. (2010) and Rinaldi et al. (2010) found that restraint stress modulates miRNA levels quickly but the effects are not long lasting. There was overlap in a number of miRNAs identified by these studies (let-7a, miR-9) but the direction of change was in opposition. However, it must be noted that these studies used different species, rats (Meerson et al., 2010) and mice (Rinaldi et al., 2010), and analyzed different brain regions, amygdala and hippocampus versus frontal cortex. The findings from these studies suggest that the stress-induced effects on miRNAs are both temporally and regionally specific.

In summary, the preclinical studies conducted to date indicate that miRNAs play a role in modulating the response to stress. Various psychological stressors seem to act through different molecular mechanisms and induce differing changes in miRNA levels. In addition, gestational or early-life stress can significantly impact on miRNA levels which may result in life-long consequences for the organism.

2.6.1.2 Depressive-Like Behavior and Antidepressant Therapies

Preclinical miRNA studies on depression have so far focused on the role of miRNAs in the development of depressive-like behaviors and in the mechanism of action of antidepressants and mood stabilizers. These are outlined in Table 2.2 and summarized below.

2.6.1.2.1 MicroRNAs and Depressive-Like Behavior

Only three studies have so far directly examined the role of miRNAs in the development of depressive-like behaviors. Using the MD model, Bai et al. (2014) demonstrated that anhedonia is associated with upregulation of miRNA let-7a in the hippocampus of rats corresponding with downregulation of serotonin receptor 4 (Htr4). CUPS was again shown to induce similar depressive-like behaviors to MD but did not induce alterations in Htr4 or let-7a levels, once again suggesting that different psychological stressors have different neurobiological mechanisms. Notably, anhedonia is thought to act as a predictor of poor response to SSRI treatment in depressed patients. Thus, based on previous observations, it is postulated that upregulated let-7a and downregulated Htr4 may be linked to decreased hippocampal neurogenesis which may in turn contribute to SSRI resistance in depression although the evidence to support this is lacking at present.

The BDNF and other neurotrophins have been closely linked to depression (Duman and Aghajanian, 2012). Social defeat stress–induced depressive-like behavior was shown by Bahi et al. (2014) to be associated with decreased levels of BDNF and increased levels of hippocampal miR-124a, an miRNA known to target BDNF. Interestingly, the authors found that overexpression of miR-124a in the hippocampus exacerbated depressive-like behavior, whereas silencing hippocampal miR-124a reduced depressive-like behavior supporting a role for miR-124a in the development of depressive-like behaviors.

An individual's ability to cope with stress is critical in the development of major depressive disorder (MDD). Using the learned helplessness (LH) model, an animal model of stress-induced behavioral depression, Smalheiser et al. (2011) found that nonlearned helpless (NLH) rats show a normal response to inescapable shock and significant alterations in miRNA levels in the frontal cortex (Table 2.2). Half of

TABLE 2.2
Preclinical Studies of MiRNAs in Depression

Author	Species	Tissue	Analysis	Main Findings
Smalheiser et al. (2011)	Male Holtzman rats; LH	Frontal cortex	Microarray	• LH ↑ miR-200b, miR-300, miR-miR-184, miR-106b*, miR-297a*, miR-136*, miR-496, miR-211, miR-214*, miR-369-3p, miR-18a*, miR-466d-3p, miR-467a*, miR-376a*, miR-142-3p, SNORD65, miR-22, miR-181a-1*, miR-29c*, miR-376a; ↓ miR-384-5p, miR-350
Bahi et al. (2014)	Male Wistar rats; social defeat stress	Frontal cortex, hippocampus	RT-qPCR	• Social defeat stress ↓ BDNF and ↑ miR-124a in hippocampus but not frontal cortex • Overexpression of hippocampal miR-124a ↑ depressive-like behavior • Knockdown of miR-124a ↓ depressive-like behavior
Bai et al. (2014)	Male Sprague Dawley rats; MD vs. CUPS	Hippocampus	RT-qPCR; western blotting	• MD but not CUPS ↑ let-7a • Let-7a negatively correlates with sucrose preference rate • Let-7a correlates with hippocampal Htr4 mRNA and protein
				Antidepressants
Baudry et al. (2010)	1C11 neuroectodermal cell line; Male Swiss-Kumming mice; Flx treatment	–	RT-qPCR	• ↑ miR-16 in NA neurons but not 5HT neurons • ↓ miR-16 in NA neurons ↑ SERT • Flx ↑ miR-16 and ↓ SERT in raphe nuclei *in vivo* • S100β ↓ miR-16 and ↑ SERT in NA neurons and *in vivo* • Overexpression of miR-16 *in vivo* ↓ depressive-like behaviors following CUPS
Launay et al. (2011)	Male Swiss-Kumming mice; Flx treatment	Hippocampus	RT-qPCR	• Flx ↓ depressive-like behaviors following CUPS, ↓ hippocampal miR-16, ↑ SERT, ↑ neurogenesis • Knockdown of hippocampal miR-16 ↓ depressive-like behaviors • BDNF, Wnt2 and PGJ2 act synergistically to ↓ miR-16 and ↑ SERT in hippocampus

(*Continued*)

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TABLE 2.2 (*Continued*)
Preclinical Studies of MiRNAs in Depression

Author	Species	Tissue	Analysis	Main Findings
Huang et al. (2012)	Male C57BL/6J, B6.129-Kdr^{tm1Jrt}/J mice; primary hippocampal neurons; EE or CMS	Hippocampus	RT-qPCR	• EE ↑ antidepressant-like effects in TST in CMS mice • MiR-107 inhibits HIF-1α/VEGF/Flk-1 signaling and negatively regulates dendritic spine formation • MiR-107 overexpression blocks EE-induced antidepressant-like effects in the TST and HIF-1α
O'Connor et al. (2013)	Male Sprague-Dawley rats; MD; Flx, Ket or ECS treatment	Hippocampus	Microarray	• Flx, ECS and Ket ↑ miR-598-5p using microarray in nonstress animals • Flx and Ket ↑ miR-598 using RT-qPCR in nonstress animals • MD ↓ miR-451 using microarray and RT-qPCR • Flx, ECS and Ket reverse MD-induced ↓ miR-451 using microarray • Flx reverse MD-induced ↓ miR-451 using RT-qPCR • MiR-451 mRNA targets: CREB5, GABAa receptor associated protein, muscarinic cholinergic receptor 5
Ryan et al. (2013)	Male Sprague-Dawley rats; acute and chronic ECS	DG, hippocampus, frontal cortex, cerebellum, whole blood	RT-qPCR	• ↑ miR-212 in DG following both acute and chronic ECS • Positive correlation between miR-212 in DG and whole blood following chronic ECS
Smalheiser et al. (2014)	Male Sprague-Dawley rats; LH model; enoxacin treatment	Frontal cortex	RT-qPCR	• Enoxacin ↓ LH behavior following inescapable shock • Enoxacin ↑ let-7a, miR-124, miR-125a-5p and miR-132 in frontal cortex

(*Continued*)

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TABLE 2.2 (*Continued*)
Preclinical Studies of MiRNAs in Depression

Author	Species	Tissue	Analysis	Main Findings
Yang et al. (2014)	Male Sprague-Dawley rats; primary hippocampal neuronal cultures; Ket treatment	Hippocampus	Microarray	• Ket ↑ miR-30e-5p, miR-218a-5p, miR-181a-5p, miR-181c-5p, miR-136-5p, miR-487b-3p, miR-132-3p, miR-345-5p, miR-598-3p, miR-98-5p, miR-221-3p, miR-138-5p, miR-219a-5p, miR-495, miR-497-5p, miR-99a-5p, miR-29c-3p, miR-124-5p, let-7c-5p, miR-29a-3p, miR-488-3p, miR-365-3p; ↓ miR-150-5p, miR-344b-1-3p, miR-299a-5p, miR-206, miR-103-1-5p, miR-344b-2-3p, miR-935, miR-132-5p, miR-340-3p, miR-465-3p, miR-3557-5p, miR-22-5p, miR-485-3p, miR-1839-3p, miR-3568, miR-221-5p, miR-214-3p, miR-3596c • miR-206 ↓ following Ket • miR-206 overexpression attenuates Ket-induced ↑ BDNF
				Mood Stabilizers
Zhou et al. (2009)	Male Wistar-Kyoto rats; primary hippocampal neurons; Li or VPA treatment	Hippocampus	Microarray	• Li and VPA ↑ miR-144, miR-136 and ↓ Let-7b, let-7c, miR-105, miR-128a, miR-24, miR-30c, miR-34a, miR-221 • Predicted mRNA targets of these miRNAs: CAPN6, DPP10, ESRRG, FAM126A, GRM7, THRB • miR-34a regulates GRM7 protein *in vitro*
Creson et al. (2011)	Rat primary cortical neuronal cultures; Li treatment	–	Western blotting	• Let-7b negatively regulates M1 receptor protein • Lithium ↑ M1 receptor protein
Zhang et al. (2013b)	HEK293 cells; VPA treatment	–	Microarray	• VPA ↓ DICER mRNA and protein • VPA ↑ 49 miRNAs, ↓ 68 miRNAs

Abbreviations: Flx = fluoxetine; NA = noradrenergic; 5HT = serotonergic; SERT = serotonin transporter; Li = lithium; VPA = valproate; Ket = ketamine; RT-qPCR = real-time quantitative polymerase chain reaction; TST = tail suspension test; CMS = chronic mild stress; VEGF = vascular endothelial growth factor; BDNF = brain-derived neurotrophic factor; ECS = electroconvulsive stimulation; MD = maternal deprivation; EE = environmental enrichment; DG = dentate gyrus; CUPS = chronic unpredictable stress; Htr4 = serotonin receptor 4; LH = learned helplessness.

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these miRNAs were found to target Creb1. On the other hand, LH rats had a blunted response to inescapable shock compared to NLH rats and show aberrant miRNA levels. MiRNA alterations in NLH rats may be interpreted as a homeostatic response to minimize the effects of stress, in particular on Creb1, while the inability of LH rats to mount a homeostatic response to stress might be accounted for by their abnormal miRNA response.

No clear role for miRNAs in the development of depressive-like behaviors has emerged from the studies conducted to date. As mentioned previously, different psychological stressors appear to act by different neurobiological mechanisms. Thus, these studies highlight the difficulties in using various animal models to investigate the underlying molecular mechanisms of depression.

2.6.1.2.2 MicroRNAs and Antidepressants

The majority of the preclinical work conducted to date examining the role of miRNAs in depression has focused on miRNAs in the mechanism of action of antidepressant therapies. Initial evidence for the interaction between antidepressants and miRNAs came from two important studies of the SSRI fluoxetine (Baudry et al., 2010; Launay et al., 2011). Treatment with SSRIs typically takes weeks before symptomatic relief is achieved, suggesting that changes to serotonin signaling and downstream cascades are necessary for antidepressant action. MiR-16 was identified as a regulator of the serotonin transporter (SERT) through computer analysis. Using human neuroectodermal cell lines and *in vivo* study of the raphe nucleus (RN) of mice, the authors showed that fluoxetine increased levels of miR-16, which previously had been blocked by Wnt signaling pathways (Baudry et al., 2010). Increased miR-16 levels in turn led to decreased SERT levels, which would result in increased serotonin signaling at the synapse. In addition, miR-16 induced an adaptational change in locus coeruleus neurons, from noradrenergic to serotonergic type. MiR-16 could also alter behavior in depression models in mice. Following on from this study, the same group examined the role of miR-16 in hippocampal neurogenesis (Launay et al., 2011). Although fluoxetine increases miR-16 maturation in the RN, it decreased miR-16 levels in the locus coeruleus and hippocampus. These changes were mediated by BDNF, Wnt2, and the prostaglandin 15d-PGJ2.

Regulation of miRNAs by other antidepressant therapies has since been investigated by others. These studies have primarily focused on the role of miRNAs in the response to electroconvulsive stimulation (ECS) (O'Connor et al., 2013; Ryan et al., 2013), the animal model equivalent of ECT, and ketamine (O'Connor et al., 2013; Yang et al., 2014).

The first study to examine miRNAs in the mechanism of action of ECT showed that treatment of rats with ECS increases levels of the BDNF-associated miRNA miR-212 in the rat dentate gyrus (Ryan et al., 2013). A positive association was found between miR-212 levels in the dentate gyrus and in whole blood indicating that miRNA changes in the periphery can reflect changes occurring in the brain following antidepressant treatment. Subsequently O'Connor et al. (2013) investigated changes in miRNA levels in the MD model of depression following treatment with ECS, ketamine or fluoxetine. There was no overlap in the miRNAs altered by ECS in this study or the study by Ryan et al., but the differences in time points used

post-ECS in these studies might account for this. Treatment of MD rats with ECS, ketamine, or fluoxetine reversed MD-induced changes in miRNA levels (Table 2.2). MiR-451 was identified as a common target of all three antidepressants in MD rats. Interestingly, bioinformatic analysis revealed that miR-451 theoretically regulates genes involved in the CREB pathway and in GABAergic and cholinergic neurotransmission. The role of miRNAs in the mechanism of action of ketamine was further examined by Yang et al. (2014). Following treatment of rats with ketamine, an miRNA screen of the hippocampus identified changes in the levels of 40 miRNAs (Table 2.2); however, again there was very little overlap between the miRNAs identified in this study and that of O'Connor and colleagues. The authors went on to further investigate the role of one miRNA, miR-206. Ketamine downregulated levels of miR-206 both *in vivo* and *in vitro* leading to an upregulation of BDNF. This study suggests that miR-206 may underlie the antidepressive effects of ketamine, although a role for other miRNAs cannot be ruled out.

2.6.1.2.3 MicroRNAs and Mood Stabilizers

Only three studies have so far examined the role of miRNAs in the mechanism of action of mood stabilizers (Zhou et al., 2009; Creson et al., 2011; Zhang et al., 2013b). These studies have focused on two drugs in particular, namely lithium and valproate. The first study in this area was conducted by Zhou et al. (2009) who showed that lithium and valproate alter the levels of a number of shared miRNAs (Table 2.2). Interestingly, bioinformatic analysis revealed that these miRNAs target genes implicated in BPAD such as those involved in neurite outgrowth, neurogenesis, and signaling pathways. Notably, treatment of primary neuronal cultures with either lithium or valproate lowered levels of miR-34a and elevated levels of its target gene GRM7, a glutamate receptor encoding gene which has previously been identified as a candidate gene for BPAD.

Perturbations of the cholinergic system and alterations in muscarinic acetylcholine receptor levels have been implicated in mood disorders. Lithium is known to impact on the cholinergic system. Based on the findings of Zhou et al. (2009) which showed lithium-induced downregulation of let-7b, Creson et al. (2011) went on to further investigate the effects of chronic lithium treatment on let-7b and its target the presynaptic M1 muscarinic receptor (M1). They found that let-7b negatively regulates levels of M1 *in vitro* and that lithium significantly increases M1 levels *in vivo* in the frontal cortex. However, while there was an increase in M1 in the frontal cortex following lithium let-7b was not measured. Thus, while these results are interesting, there is a lack of evidence to support the idea that lithium-induced effects on let-7b underlies its therapeutic actions *in vivo* and further investigations are needed.

Zhang et al. (2013b) also investigated the effects of valproate *in vitro* and found that it induces the proteasomal degradation of Dicer and alters the levels of numerous miRNAs (Table 2.2) of which there was some overlap with the findings of Zhou and colleagues. Unexpectedly, the authors report an upregulation of 49 miRNAs, despite valproate-induced Dicer depletion, suggesting that these miRNAs may be activated via their hosting genes or may be generated by a Dicer-independent mechanism.

While interesting, the results from these studies have primarily been generated from *in vitro* work. A lot of details have yet to be determined about the effects of

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mood stabilizers on miRNAs. Moreover, whether such changes have any functional or behavioral consequences need to be examined in order to fully establish a role for miRNAs in the mechanism of action of mood stabilizers and to evaluate their potential as future therapeutic targets.

2.6.2 Clinical Studies

2.6.2.1 Clinical Studies in BPAD

Our systematic review identified 10 articles that were relevant for inclusion here. There is considerable evidence that BPAD and schizophrenia have at least a partially shared molecular basis and genetic risk (International Schizophrenia Consortium et al., 2009). It is therefore not surprising that the majority of clinical studies of miRNAs in BPAD have also included samples of schizophrenia patients. A series of postmortem brain expression studies (Zhu et al., 2009; Kim et al., 2010; Moreau et al., 2011; Miller et al., 2012; Smalheiser et al., 2014) as well as several studies (Whalley et al., 2012; Cummings et al., 2013; Guella et al., 2013) investigating a single nucleotide polymorphism (SNP) identified in a large genome-wide association study (GWAS; Schizophrenia Psychiatric Genome-Wide Association Study, 2011) make up the bulk of clinical studies in BPAD patients. One study has looked at the plasma levels of miRNAs following treatment for mania (Rong et al., 2011), and another study profiled miRNA expression in patients with postpartum psychosis (PP), which is closely related to BPAD (Weigelt et al., 2013). These studies are summarized in Table 2.3 and briefly outlined below.

2.6.2.1.1 Postmortem Studies

Six studies to date have investigated the expression of miRNAs in postmortem brain samples. They all included samples from the Stanley Medical Research Institute (SMRI) brain bank (Zhu et al., 2009; Kim et al., 2010; Moreau et al., 2011; Miller et al., 2012) or a subset of these found in the Stanley Neuropathology Consortium (Smalheiser et al., 2014). One study also utilized brain samples from the University of California, Irvine Brain Bank (UCI; Guella et al., 2013) alongside samples from the SMRI. The brain banks contain samples from patients with schizophrenia, BPAD, and healthy controls.

There is considerable variation in the results from these studies, although they differ in many methodological ways. Reflecting the technological advances in this field in a relatively short time frame, the first study in 2009 only looked at a single miRNA (Zhu et al., 2009). Although there were differences in patients with schizophrenia, these changes did not meet statistically significant criteria in the BPAD group. By 2010, researchers were able to investigate the expression of 667 different miRNAs, and successfully validated four upregulated miRNAs (Kim et al., 2010). In contrast, Moreau et al. (2011) investigating the expression of 435 miRNAs found 5.5% of these to be downregulated and Miller et al. (2012) failed to find any change in levels in 800 miRNAs in BPAD patients. The most recent miRNA profiling study found 9 miRNAs out of 377 to have altered levels (Smalheiser et al., 2014). A final study, based on the hypothesis that miR-137 is linked to BPAD failed to find any difference in miR-137 expression between BPAD or schizophrenia patients and controls (Guella et al., 2013).

TABLE 2.3
Clinical Studies of MiRNAs in Bipolar Affective Disorder (BPAD)

Author	Patients	Tissue	Analysis	Main Findings
				Postmortem Case-Control Brain Studies
Zhu et al. (2009)	Scz = 35 BPAD = 35 Controls = 34	DLPFC (BA 46)	RT-qPCR	• Nonsignificant reduction in miR-346 levels in BPAD patients • Target genes/gene pathways: CSF2RA, GRID1; Cytokine, glutamate transmission
Kim et al. (2010)	Scz = 35 BPAD = 35 Controls = 35	DLPFC (BA 46)	Microarray; RT-qPCR	• ↑ miR-145*, miR-133b, miR-154*, miR-889 • Targeted genes/gene pathways: GRIN, DRD1, DLG3/4, ITPR1, CEP290, HTT, SHANK3
Moreau et al. (2011)	Scz = 35 BPAD = 35 Controls = 35	PFC (BA 9)	RT-qPCR	• ↓ miR-330, miR-33, miR-193b, miR-545, miR-138, miR-151, miR-210, miR-324-3p, miR-22, miR-425, miR-181a, miR-106b, miR-193a, miR-192, miR-301, miR-27b, miR-148b, miR-338, miR-639, miR-15a, miR-186, miR-99a, miR-190, miR-339
Miller et al. (2012)	Scz = 35 BPAD = 31 Controls = 34	DLPFC (BA 46)	Microarray; RT-qPCR	• No significant differences in BPAD group
Guella et al. (2013)	Scz = 42 BPAD = 40 Control = 43	DLPFC	RT-qPCR	• No significant difference in groups
Smalheiser et al. (2014)	Scz = 15 BPAD = 15 Controls = 15	DLPFC (BA 10)	RT-qPCR	• ↑ miR-17-5p, miR-579, miR-106b-5p, miR-29c-3p; ↓ miR-145-5p, miR-485-5p, miR-370, miR-500a-5p, miR-34a-5p

(*Continued*)

TABLE 2.3 (*Continued*)
Clinical Studies of MiRNAs in Bipolar Affective Disorder (BPAD)

Author	Patients	Tissue	Analysis	Main Findings
				Genotyping Studies
Whalley et al. (2012)	High risk groups of: Scz = 44 BPAD = 90 Controls = 81	DNA (venous blood)	PCR; genotyping assay	• No significant difference between BPAD subjects and controls
Cummings et al. (2012)	Scz = 573 SA = 123 BPAD = 125	DNA	PCR; genotyping assay	• No association between SNP and diagnosis • Risk allele associated with fewer and mood-congruent symptoms
Guella et al. (2013)	Scz = 42 BPAD = 40 Control = 43	DNA (brain)	PCR; genotyping assay	• No association between SNP and diagnosis • Homozygous controls had lower miR-137 levels
				Other Studies
Rong et al. (2011)	BPAD = 25 Controls = 21	Whole blood	RT-qPCR	• ↓ plasma miR-134 in mania patients vs. controls. • Treatment with lithium led to increase in miR-134 levels. • Targeted genes/gene pathways: Limk1, dendritic spine size regulation
Weigelt et al. (2013)	PP = 20 Controls = 40	Monocytes	microarray; RT-qPCR	• MiR-146a ↓ • ↓ miR-212 in those with history of BPAD • Targeted genes/gene pathways: Inflammatory markers; IL-6, ADM, CD4+ T cell subsets

Note: ↑/↓ in all studies refer to cases (BPAD/PP) compared to controls.

Abbreviations: BA = Brodmann's area; DLPFC = dorsolateral prefrontal cortex; PFC = prefrontal cortex; qRT-PCR = quantitative reverse transcriptase polymerase chain reaction; Scz = schizophrenia; SA = schizoaffective disorder; PP = postpartum psychosis.

Some of these studies found an overlap between BPAD and schizophrenia patients (Kim et al., 2010; Moreau et al., 2011; Smalheiser et al., 2014), whereas others did not (Zhu et al., 2009; Miller et al., 2012; Guella et al., 2013). Only two miRNAs were identified in more than one study (miR-106b and miR-145-5p), although in opposite directions.

What does one make of this apparent lack of consistency in these results to date? Although the majority of brain samples are from the same source different brain regions were examined, and different brain regions may express different levels of miRNAs. There is still a lack of consensus as to the best statistical significance testing approach, correction for multiple testing, normalization strategy, and what endogenous controls are best suited to this type of analysis (Liu et al., 2014). The postmortem intervals differ considerably among the samples in the SMRI which may have influenced miRNA expression levels (Smalheiser et al., 2014). These findings emphasize the importance of basic scientific principles of confirmation and validation of findings using different experimental techniques and cohorts.

2.6.2.1.2 Genotyping and Rare Variants

One of the largest GWAS to date in schizophrenia, with over 40,000 individuals, found that the strongest association of any SNP with schizophrenia lies within the intron of miR-137 (Schizophrenia Psychiatric Genome-Wide Association Study, 2011). There were also SNPs found in a number of miR-137 targets such as *TCF4*. Those with the TT risk allele in rs1625579 are presumed to be at higher risk of schizophrenia and given the overlap between schizophrenia and BPAD it may also be a risk allele for BPAD (Guella et al., 2013). Three studies to date have examined this SNP in BPAD (Whalley et al., 2012; Cummings et al., 2013; Guella et al., 2013), but none of these genotyping studies supported an association between rs1625570 SNP and BPAD. This is in line with previous expression studies.

2.6.2.1.3 Other Studies

MiR-134 has been suggested as a useful blood marker of clinical status in BPAD. In an initial study of drug-free BPAD individuals with mania and controls, miR-134 levels were significantly decreased in manic subjects (Rong et al., 2011). Although numbers were low and treatment was open label, this study supports miR-134 as a potential peripheral biomarker in BPAD.

Postpartum psychosis is a rare, but very severe postpartum disorder that can present with manic and psychotic symptoms. There are strong links between BPAD and PP and underlying molecular mechanisms may well be shared between these disorders (Weigelt et al., 2013). An initial profiling experiment in eight PP patients subsequently led to the validation of miR-146a being significantly decreased in PP patients compared to controls. In further analysis, miR-212 was also associated with a past diagnosis of BPAD in PP patients. The levels of these miRNAs correlated with inflammatory genes such as IL-6 (miR-212) and subsets of CD4 T cells. However, these results should be treated with caution as multiple comparisons were carried out with no correction.

2.6.2.2 Clinical Studies in Depression

Our systematic review identified 15 articles that were relevant for inclusion. Research investigating the role of miRNA involvement in depressive disorder is rapidly

gathering pace with a number of clinical studies in recent years. A number of studies have investigated miRNA expression in the brain (Smalheiser et al., 2012, 2014; Issler et al., 2014; Lopez et al., 2014a,b). Others have focused on peripheral sources of miRNA, typically in the search of a biomarker for depression. These include studies investigating miRNA expression in cerebrospinal fluid (CSF; Launay et al., 2011), blood (Belzeaux et al., 2012; Bocchio-Chiavetto et al., 2013; Li et al., 2013; Issler et al., 2014; Lopez et al., 2014b), and dermal fibroblasts (Garbett et al., 2015). Searching for rare variants that may be associated with depression has also yielded interesting results (Saus et al., 2010; Xu et al., 2010b; He et al., 2012b; Guintivano et al., 2014; Jensen et al., 2014). These results are summarized in Table 2.4 and expanded upon below.

2.6.2.3 Postmortem Studies

The first study to examine miRNA expression levels in human brain was in a sample of 18 antidepressant-free suicide and 17 matched nonpsychiatric controls (Smalheiser et al., 2012). Using multiplex PCR, the authors found a downregulation of 21 miRNAs. Validated predicted targets of these miRNAs included VEGFA, BCL-2, and DMNT3B, but when their respective protein levels were measured in the same cohorts, only DMNT3B was significantly upregulated.

The same group went on to study the expression of miRNAs in a subset of SMRI brain samples, along with patients with schizophrenia, BPAD, and healthy controls (Smalheiser et al., 2014). MiR-508-3p and miR-152-3p were both significantly downregulated but no correction for multiple testing was carried out.

Building on previous work, identifying the polyamine genes SAT1 and SMOX as playing a role in suicidal behavior, Lopez et al. (2014a) investigated PFC levels of miRNAs predicted to target these genes. Four miRNAs were upregulated in suicide completers. Two of these, miR-34c-5p and miR-320c, had a significant negative correlation with mRNA levels of SAT1, and miR-139-5p and miR-320c had a significant negative correlation with SMOX mRNA levels. The protein products of these mRNA transcripts were not measured, and multiple comparison correction was omitted.

The same group went on to examine miRNA expression in a larger sample of brains from the Douglas-Bell Canada Brain Bank in Quebec (Lopez et al., 2014b). A microarray-based approach tested for 866 miRNAs and found miR-1202 to be significantly decreased in the PFC of 14 depressed subjects compared to 11 controls. This miRNA was brain enriched and is only present in humans and primates. These findings were further validated with qRT-PCR in a sample of depressed subjects with a history of antidepressant use and controls. Interestingly, miR-1202 expression in those who had a history of antidepressant use was significantly different from those not exposed to antidepressants, with expression levels more similar to healthy controls. *GRM4*, a glutamate receptor-encoding gene, was predicted in silico to be targeted by miR-1202. *GRM4* levels were increased in these brain samples and negatively correlated with miR-1202 levels.

A final study, which investigated the role of miR-135 in mouse models went on to investigate levels of miR-135 and miR-16 in various subnuclei of the raphe in the brains of depressed suicide victims and controls (Issler et al., 2014). Significantly

TABLE 2.4
Clinical Studies of miRNAs in Depression

Author	Patients	Tissue	Analysis	Main Findings
				Postmortem Case–Control Brain Studies
Smalheiser et al. (2012)	Suicides = 18 Controls = 17	PFC (BA 9)	RT-qPCR	• ↓ miR-142-5p, miR-137, miR-489, miR-148b, miR-101, miR-324-5p, miR-301a, miR-146a, miR-335, miR-494, miR-20a/b, miR-376a, miR-190, miR-155, miR-660, miR-130a, miR-27a, miR-497, miR-10a, miR-142-3p • Targeted genes/gene pathways: CDK6, ELF1/6, NCOA2, DNMTB3, EZH2, MYCN, ICOS, SOX4, PTPRN2, MERTK, VEGFA, SLC16A1, SFRS11, TTK, AGTR1, BACH1, LDOC1, MATR3, TM6SF1, TAC1, CSF1, MAFB, MEOX2, HOXA1/5, SP1/3/4, RUNX1
Smalheiser et al. (2014)	Scz = 15 BPAD = 15 Dep = 15 Controls = 15	PFC (BA 10)	RT-qPCR	• ↓ miR-508-3p and miR-152-3p
Lopez et al. (2014a)	Dep = 15 Controls = 16	PFC (BA44)	RT-qPCR	• ↑ miR-34c-5p, miR-139-5p, miR-195, miR-320c • Targeted genes/gene pathways: SAT1, SMOX
Lopez et al. (2014b)	Dep = 64 Controls = 40	PFC (BA44)	Microarray; RT-qPCR	• ↓ miR-1202 • Targeted genes/gene pathways: GRM4
Issler et al. (2014)	Dep = 6 Control = 11	Raphe nuclei	RT-qPCR	• ↓ miR-135a, miR-16
				Peripheral Tissue Studies
Launay et al. (2011)	MDD = 9	CSF	RT-qPCR	• Following fluoxetine administration, miR-16 targeting molecules BDNF, Wnt2, 15d-PGJ2 levels ↑ in CSF • Targeted genes/gene pathways: miR-16: SERT, Bcl-2

(*Continued*)

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TABLE 2.4 (*Continued*)
Clinical Studies of miRNAs in Depression

Author	Patients	Tissue	Analysis	Main Findings
Bocchio-Chiavetto et al. (2012)	MDD = 10	Whole blood	RT-qPCR	• ↑ miR-130b*, miR-505*, miR-29-b-2*, miR-26a/b, miR-22*, miR-664, miR-494, let7d/e/f/g, miR-629, miR-106b*, miR-103, miR-191, miR-128, miR-502-3p, miR-374b, miR-132, miR-30d, miR-500, miR-589, miR-183, miR-574-3p, miR-140-3p, miR-335, miR-361-5p • ↓ miR-34c-5p, miR-770-5p • Targeted genes/gene pathways: BDNF, NR3C1, NOS1, IGF1, FGF1, FGFR1, VEGFa, GDNF, CACn41C, CACNB4, SLC6A12, SLC8A3, GABRA4, 5HT-4. Neuroactive ligand–receptor interaction, axon guidance, LTP, signaling pathways
Belzeaux et al. (2012)	MDD = 9 Controls = 9	PBMCs	Microarray; RT-qPCR	• ↑ miR-941, miR-589
Li et al. (2013)	MDD = 40 Controls = 40	Serum	RT-qPCR	• ↑ miR-132, miR-182 • Targeted genes: BNDF
Lopez et al. (2014b)	MDD = 32 Controls = 18	Whole blood	RT-qPCR	• ↓ miR-1202
Issler et al. (2014)	MDD = 11 Controls = 12	Whole blood	RT-qPCR	• ↓ miR-135a
Garbett et al. (2014)	MDD = 16 Controls = 16	Dermal fibroblasts	PCR array	• ↑ miR-132, miR-421, miR-542, miR-450a, miR-16-2*, miR-424, miR-628-3p, miR-629-5p, miR-4293, miR-661, miR-3909, miR-33a*, miR-135b, miR-7, miR-4267, miR-548a-3p, miR-548d-3p, miR-613, miR-3714, miR-1294, miR-429 • ↓ miR-122, miR-32, miR-196b*, miR-377, miR-193a-3-, miR-337-5p, miR-675*, miR-3176, miR-21*, miR-22, miR-425*, miR-185, miR-296-5p, miR-103a, miR-107, miR-186, miR-887

(*Continued*)

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TABLE 2.4 (*Continued*)
Clinical Studies of miRNAs in Depression

Author	Patients	Tissue	Analysis	Main Findings
				Genotyping Studies
Xu et al. (2010)	MDD = 1088 Controls = 1102	DNA	MiR-SNP	• Positive association between SNP in miR-30e precursor and MDD
He et al. (2012)	MDD = 314 Controls = 252	DNA	MiR-SNP	• SNP in miRNA processing gene DGCR8 increased frequency • SNP in miRNA processing gene AGO1 decreased frequency • Associated with suicide risk and treatment response
Jensen et al. (2014)	6725 subjects	DNA	Microarray	• Association between SNP in target site of miR-330-3p in MDD
Guintivano et al. (2014)	PM brains MDD = 29 BPAD = 40 Scz = 29 Controls = 70 Blood MDD = 75 BPAD = 15 Controls = 308	DNA (PM brain, blood)	Microarray, pyrosequencing	• Association between SNP in SKA2 and suicide, possibly mediated by miR-301a

Note: ↑/↓ in all studies refer to cases (depressed) compared to controls.

Abbreviations: BA = Brodmann's area; BPAD = bipolar affective disorder; DLPFC = dorsolateral prefrontal cortex; LTP = long-term potentiation; MDD = major depressive disorder; PBMCs = peripheral blood mononuclear cells; PFC = prefrontal cortex; PM = postmortem; RT-qPCR = real-time quantitative polymerase chain reaction; Scz = schizophrenia.

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lower levels of miR-135 and miR-16 were found in the dorsal raphe and raphe magnus compared to controls.

As discussed in the BPAD section, postmortem studies have significant limitations and to date the results in depressed cohorts suffer from a similar lack of correlation with other postmortem studies.

2.6.2.4 Peripheral Tissue Studies

Correlating miRNA level changes before and after treatment and matching them to clinical outcomes offers an exciting potential for biomarkers as well as the molecular basis of depression. For biomarkers to be clinically relevant, they need to be easily accessible from sources such as peripheral blood. Initial work using CSF had highlighted that targets of miR-16, previously implicated in the action of the antidepressant fluoxetine (Baudry et al., 2010), were increased following fluoxetine treatment (Launay et al., 2011). This highlighted the translational potential of miRNAs.

The first study investigating the effect of blood miRNA changes following treatment involved 10 treatment-naive depressed patients who showed a good response to 12 weeks of the antidepressant escitalopram (Bocchio-Chiavetto et al., 2013). Thirty miRNAs were significantly altered, and many of these are important gene expression regulators in the brain and have been implicated in other psychiatric disorders.

A further study investigating transcriptional signatures at different time points in depression identified two miRNAs that were upregulated at remission compared to healthy controls (Belzeaux et al., 2012). A panel of eight miRNAs was also identified that matched clinical response.

Li et al. (2013) investigated levels of two miRNAs that are thought to regulate BDNF, miR-132 and miR-182. In depressed patients, compared to controls, BDNF serum levels were lower, and miR-132 and miR-182 were upregulated. However, only miR-132 was significantly negatively correlated with BDNF levels. Of note, there was a significant positive correlation between the levels of these miRNAs and scores on a self-rating scale of depression, but no correction for multiple testing was performed.

Previously, we presented the evidence for the involvement of miR-1202 in depression based on postmortem findings (Lopez et al., 2014b). In a further experiment, miR-1202 blood levels were measured before and after treatment with the antidepressant citalopram. Compared to controls, miR-1202 levels were downregulated, matching the results seen in postmortem brain samples. Furthermore, after classifying the patients into those who remitted, and those who did not, the lower miR-1202 levels were specific to remitters, indicating the potential of miR-1202 to identify those who will respond to citalopram treatment.

In a similar vein, Issler et al. (2014) built on work investigating the role of miR-135 and miR-16 in mouse models and measured their levels in brain samples and in human blood. Compared to controls, miR-135a levels were significantly decreased. Of note, after 3 months of cognitive behavioral therapy, there was a significant increase in blood miR-135a levels compared to patients receiving the antidepressant escitalopram.

Finally, both mRNA and miRNA levels in dermal fibroblasts were investigated using a PCR array examining 1008 miRNAs (Garbett et al., 2015). Thirty-eight

miRNAs were differentially expressed. Using miRNA-targeting software, 89% of the 38 miRNAs targeted at least one mRNA that was differentially expressed, indicating a close relationship between miRNA and mRNA networks. There was no correction for multiple testing, and culturing of fibroblasts represents a more time-consuming and delayed source of biomarkers in comparison to blood.

These studies in general need to be validated, and many may have a high false discovery rate. Some studies have investigated the role of specific miRNAs based on preclinical, in silico, or other tissues, such as miR-1202, miR-135a, and miR-132. MiR-132 was also found to be upregulated in dermal fibroblasts in depressed patients, whereas the others have not been identified in general profiling studies. Questions also arise over whether changes in miRNA levels in the periphery give any helpful information about what is happening in the brain (Kolshus et al., 2014). However, as any clinically useful biomarker for depression is likely to come from these peripheral sources, continued efforts in this field would be welcomed.

2.6.2.5 Genotyping and Rare Variants

A number of studies have searched the human genome for SNPs that may be associated with depression. One such large study of 1088 depressed patients and 1102 controls found a positive association between miR-30 and MDD (Xu et al., 2010b). This was carried out in an ethnically homogenous Han Chinese population (Xu et al., 2010a).

A key symptom in affective disorders is disturbed sleep, and disruption of circadian rhythms has been associated with depression (Germain and Kupfer, 2008). An SNP in miR-182 was found to be associated with late insomnia in 359 patients (341 controls) with MDD (Saus et al., 2010). Patients with this SNP had downregulated expression of genes previously associated with affective disorders and circadian rhythm such as *CLOCK* (Serretti et al., 2003).

SNPs in the genes involved in the miRNA processing machinery, like *DGCR8* and *AGO1*, were associated with increased risk of suicidal tendency and antidepressant treatment response in a sample of 314 patients and 252 controls (He et al., 2012b).

Using previous GWAS data and miRNA target prediction software, a recent publication identified a link between an miR-330-3p target site SNP (rs41305272) in mitogen-activated protein kinase 5 (MAP2K5) and restless leg syndrome (Jensen et al., 2014). This disorder is frequently comorbid with anxiety and depression, and this SNP was therefore investigated in a separate GWAS data set. This data set consisted of 6725 unrelated drug-dependent subjects and nondrug dependent subjects recruited for a genetic study of dependence. There was an association between rs41305272 and MDD (OR = 2.64, $p = 0.01$) in subjects of African-American descent. However, these results should be treated with caution, as comorbidity with other disorders was very common in this sample.

Finally, a study investigating epigenetic and genetic markers of suicide has identified a potential role of miR-301a in suicide etiology (Guintivano et al., 2014). Incorporating postmortem and blood samples in various diagnostic groups and patient cohorts, the authors found an association between rs7208505, an SNP in a CpG site in the SKA2 gene. In suicide cases, there was a higher level of DNA methylation of

Q2

SKA2, as well as lower levels of SKA2 gene expression, associated with the SNP. Further analysis of related regions identified a possible role for miR-301a in SKA2 gene expression, although not in the suicide phenotype itself. MiR-301a has previously been found to be associated with suicide (Smalheiser et al., 2012).

2.6.3 Summary

In summary, there has been a rapid growth in studies of miRNA involvement in depression as evidenced by expanding reviews in this field (Dwivedi, 2011; Mouillet-Richard et al., 2012; Kolshus et al., 2014; Maffioletti et al., 2014). Findings from preclinical studies are promising in terms of teasing out the contribution of miRNAs to depressive-like behaviors and the therapeutic antidepressant response. Moreover, the translational aspect of findings from preclinical studies is promising. However, despite a growth in miRNA studies in this field, there has been little progress in the way of validation and replication, and there are large variations in methodological approaches including normalization strategies (Liu et al., 2014). It is encouraging to see studies beginning to explore correlations between the brain and the periphery (Guintivano et al., 2014; Lopez et al., 2014b). However, a lot more work is needed in this area in order to fully evaluate the role of miRNAs in depression and their potential to act as biomarkers for diagnosis and treatment.

2.7 REVIEW OF MicroRNAs AND ANXIETY DISORDERS

2.7.1 Preclinical Studies

Anxiety disorders are a heterogeneous collection of disorders with varying prevalence and presentation. Investigations into the role miRNAs play in these disorders are at an early stage with only a small number of preclinical studies published to date. A review of the literature identified 8 studies that were relevant for inclusion here. These are outlined in Table 2.5 and summarized below.

The first preclinical indication of a role for miRNAs in anxiety came from a study by Parsons et al. (2008). The authors examined basal miRNA levels in the hippocampus of four inbred mouse strains and showed 11 miRNA species significantly altered between strains. Of these, miR-34c, miR-323, and miR-212 correlated with behavioral measures for anxiety. MiR-34c has emerged as a key miRNA in anxiety-like behavior with two subsequent studies reinforcing these early findings (Haramati et al., 2011; Parsons et al., 2012). Parsons et al. (2012) demonstrated that basal hippocampal miR-34c levels positively correlate with anxiety-like behaviors in C57BL/6 J X DBA/2 J (BXD) recombinant inbred mice, reinforcing the significance of their early findings. Haramati et al. (2011) also showed that miR-34c is significantly upregulated in the amygdala of C57BL/6 mice following exposure to restraint or social defeat stress. Overexpression of miR-34c in the central amygdala of normal mice induces anxiolytic effects that were more pronounced following a stressful stimulus, suggesting a role for miR-34c in stress-induced anxiety. The stress-related corticotrophin-releasing factor receptor type I (CRFR1) was identified as an mRNA target of miR-34c along with other stress-related proteins such as the

TABLE 2.5
Preclinical Studies of MiRNAs in Anxiety

Author	Species/Model	Tissue	Analysis	Main Findings
Parsons et al. (2008)	Male and female A/J, BALB/cJ, C57BL/6J, DBA/2J mice	Hippocampus	RT-qPCR	• Differential expression of miR-203, miR-451, miR-378, miR-195, miR-34a, miR-34c, miR-15b, miR-323, miR-301a, miR-212, miR-31 across strains • MiR-34c and miR-323 correlate with anxiety-like behavior in EPM • MiR-212 correlates with grooming duration in EPM, light-dark box, OFT
Haramati et al. (2011)	Dicer^{flox}/Dicer^{flox} mice and C57BL/6 or C57BL/6X129SvJ mice	amygdala	microarray; RT-qPCR	• Dicer ablation ↑ anxiety-like behavior • Acute restraint stress ↑ miR-34c, miR-100, miR-15b, miR-34a, miR-92a, miR-15a • Overexpression of miR-34c ↓ anxiety-like behavior in dark-light box test and EPM
Lin et al. (2011)	Male C57/Bl6 mice; auditory cued fear conditioning	ILPFC	RT-qPCR	• Fear extinction learning ↑ miR-128b • ↓ miR-128b impairs fear extinction memory, ↑ miR-128b enhances fear extinction memory • MiR-128b negatively regulates Rcs mRNA
Parsons et al. (2012)	Male C57BL/6J X DBA/2 J recombinant inbred mice (BXD)	Hippocampus	RT-qPCR	• MiR-31 correlates with anxiety-like behavior in light-dark box and OFT • MiR-34c correlates with anxiolytic behaviors in EPM
Ragu Varman et al. (2013)	Male Indian field mice; EE	Amygdala	RT-qPCR; semi-quantitative RT-PCR	• EE ↓ anxiety-like behavior in EPM and OFT • EE ↑ miR-183 • EE ↓ SC35 mRNA, ↓ AChE levels, ↑ AChE-S mRNA
Yoon et al. (2013)	Rat C6 astroglioma cultures	–	RT-qPCR; miR-16 binding assay	• hnRNPK regulates SERT expression by antagonizing miR-16 binding
Shaltiel et al. (2013)	Male C57Bl/6J mice; predator scent stress	Hippocampus	RT-qPCR	• Predator scent stress ↑ anxiety in EPM • Predator scent stress ↑ miR-132 ~220% • ↑ miR-132 correlates with ↓ AChE activity
Durairaj and Koilmani (2014)	Male Indian field mice; EE	Amygdala	RT-qPCR; semi-quantitative RT-PCR; western blotting	• EE ↓ anxiety-like behavior in light-dark box • EE ↑ Dicer, Ago-2 mRNA and protein, ↑ miR-124a and ↓ GR mRNA and protein

Abbreviations: EPM = elevated plus maze; OFT = open field test; ILPFC = infralimbic prefrontal cortex; EE = environmental enrichment; AChE = acetylcholinesterase; SERT = serotonin transporter; GR = glucocorticoid receptor; RT-qPCR = real-time quantitative polymerase chain reaction.

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5-hydroxytryptamine (serotonin) receptor 2C, $GABA_A$ receptor α1, and BDNF. It is postulated that miR-34c assists in stress recovery by downregulating stress-related proteins.

Environmental enrichment (EE) has been found in two studies to induce an anxiolytic phenotype in the Indian field mouse (Ragu Varman et al., 2013; Durairaj and Koilmani, 2014). However, no clear role for miRNAs in the anxiolytic effects of EE has yet emerged. Ragu Varman et al. (2013) showed that the anxiolytic behavioral effects of EE were accompanied by an increase in miR-183 levels and an miR-183-induced suppression of SC35, a serine–arginine protein implicated in the stress response, in the amygdala. Suppression of SC35 causes a shift in the splicing of AChE toward that of the AChE-S (synaptic) form and decreases stress and anxiety-like behaviors. Notably, anxiety has been linked to reduced AChE activity by another group (Shaltiel et al., 2013) who found that predator stress-induced anxiety is associated with reduced hippocampal AChE activity (~25%) and a long-lasting increase in hippocampal miR-132 levels (~220%). Transgenic mice overexpressing both miR-132 and AChE were shown to have an anxiogenic phenotype. Durairaj and Koilmani (2014) also went on to show that EE induces a reduction in anxiety-like behaviors. This anxiogenic effect was accompanied by increased levels of Dicer and Ago-2, members of the miRNA biogenesis pathway, and an upregulation of pre-miR-124a in the amygdala. These findings are notable as depletion of Dicer in the amygdala was previously shown by Haramati et al. (2011) to induce anxiety-like behaviors in mice. Of note, *in vitro* analysis revealed that miR-124a regulates levels of the GR, and the GR was downregulated in the amygdala of EE mice suggesting that these changes may contribute to the anxiolytic condition (Durairaj and Koilmani, 2014).

Another important finding regarding the role of miRNAs in anxiety comes from Yoon et al. (2013). In line with previous studies (Baudry et al., 2010; Launay et al., 2011), they showed that miR-16 regulates SERT levels. This was linked to heterogeneous nuclear ribonucleoprotein K (hnRNPK), a SERT distal polyadenylation element binding protein, which can regulate the expression of SERT by antagonizing miR-16 binding and depressing SERT translation. Serotonergic signaling plays a major role in anxiety-related behaviors and pharmacological therapies targeting the serotonergic system are often used to treat anxiety. Importantly, expression of SERT mRNA containing the distal polyadenylation element is known to be anxiolytic in humans and mice.

A number of other studies have also examined miRNAs in anxiety. Details of these are outlined in Table 2.5. Overall, no clear role for miRNAs has emerged from the preclinical studies conducted so far. MiR-34c holds promise as an anxiety-related miRNA; however, despite a number of studies having been conducted to date, further work is required in order to fully establish a role for it in anxiety.

2.7.2 Clinical Studies in Anxiety Disorders

Our systematic review identified eight articles that were relevant for inclusion here. Compared to the affective disorders, there is a paucity of clinical studies of miRNAs in anxiety disorders, despite them being common with a high burden of disease. As in other disorders, genotyping rare variants (Donner et al., 2008; Muinos-Gimeno

et al., 2009, 2011; Hanin et al., 2014; Jensen et al., 2014) has been a productive approach to understand the genetic basis of anxiety disorders. Other studies have focused on establishing a blood biomarker in anxiety disorders (Katsuura et al., 2012; Honda et al., 2013; Zhou et al., 2014). These are summarized in Table 2.6 and expanded on below.

2.7.2.1 Genotyping and Rare Variants

To date, there is still a lack of solid evidence identifying genes predisposing to anxiety disorders, and heritability rates are variable. Identifying SNPs can therefore be a challenge, and one of the first studies in this regard investigated SNPs based on 17 anxiety genes previously identified in mice (Donner et al., 2008). In a sample of 974 Finnish subjects and controls with varying anxiety disorders, 208 SNPs, including some in miRNA target-binding sites, were examined for association with diagnostic status. One SNP, rs817782 in the aminolevulinate dehydratase (*ALAD*) gene, is a putative target site for miR-211 and miR-204, and was associated with social phobia.

Other genotyping studies have identified an association between an SNP in the target site of miR-485-3p and the hoarding phenotype in OCD. There was also an association between two newly identified SNPs and panic disorder (Muinos-Gimeno et al., 2009). The targets for these SNPs included neurotrophic tyrosine kinase receptor 3 gene (*NTRK3*), which has been implicated in animal models of anxiety (Dierssen et al., 2006). In a further study from three European countries, the authors found several SNPs in miRNAs associated with panic disorder, but these did not stand up to correction for multiple testing (Muinos-Gimeno et al., 2011).

In a previously discussed study by Jensen et al. (2014), a target site for miR-330-3p was associated with MDD. This group also examined the association between this SNP and anxiety disorders, and found rs41305272 was associated with agoraphobia. However, these findings are complicated by the high rate of comorbidity between substance dependence, affective and anxiety disorders.

A final genotyping study examined the effect of an SNP in the target site of miR-608. This SNP (rs17228616) was in the acetylcholinesterase enzyme gene (AChE), previously implicated in anxiety (Shaltiel et al., 2013). The authors showed that rs17228616 bound less strongly to miR-608, and in postmortem brains, was associated with higher levels of AChE. Patients with this SNP had higher blood pressure and lower cortisol levels than those with the normal allele, both risk factors for anxiety-related disorders (Hanin et al., 2014).

Most of the current rare variant studies have examined miRNA target sites rather than miRNAs per se. These have supported the potential involvement of the *NTRK3* gene in anxiety disorders but, in general, sample sizes have been low and the diagnostic purity of patients has been low.

2.7.2.2 Expression Studies in Peripheral Blood

Although not a study of clinical anxiety, a study of 10 Japanese students before and after national examinations measured levels of miRNAs using a microarray (Katsuura et al., 2012). MiR-144/144* and miR-16 were significantly increased immediately after examinations and returned to normal after a week. Advancing this paradigm, the same group subsequently followed 25 medical students in a period

TABLE 2.6
Clinical Studies of MiRNAs in Anxiety Disorders

Author	Patients	Tissue	Analysis	Main Findings
		Genotyping Studies		
Donner et al. (2008)	PD = 108 GAD = 73 SP = 58 AGO = 31 Other = 58 Controls = 653	DNA	MiR-SNP	• An SNP in anxiety-related gene is a putative target for miR-211, miR-204
Muinos-Gimeno et al. (2011)	PD = 626	DNA	MiR-SNP	• No SNPs associated with PD following correction for multiple testing
Muinos-Gimeno et al. (2009)	OCD = 153 PD = 212 Controls = 324	DNA	MiR-SNP	• Target site of miR-485-3p associated with hoarding phenotype in OCD • Targeted genes/gene pathways: NTRK3
Jensen et al. (2014)	6725 subjects	DNA	Microarray	• Association between SNP in target site of miR-330-3p in agoraphobia
Hanin et al. (2014)	461 healthy volunteers	DNA	Various	• Target site of miR-608 associated with SNP in AChE. SNP associated with altered gene expression of AChE and anxiety-related changes in peripheral blood
		Expression Studies in Peripheral Blood		
Katsuura et al. (2012)	10 healthy volunteers	Whole blood	Microarray; RT-qPCR	• ↑ miR-144, miR-144*, miR-16 following exposure to exam situation
Honda et al. (2013)	25 healthy volunteers	Whole blood	Microarray; RT-qPCR	• ↑ miR-16, miR-20b, miR-26b, miR-29a, miR-126, miR-144, miR-144* in pre-examination period • Gene targets: WNT4, CCM2, MAK, FGFR1
Zhou et al. (2014)	PTSD = 30 Controls = 42	PBMC; lymphocytes	Microarray; RT-qPCR	• ↓ miR-125a, miR-181c downregulated • Gene targets: IFN-gamma, Il-23, IL-8

Abbreviations: AGO = agoraphobia; GAD = generalized anxiety disorder; OCD = obsessive compulsive disorder; PBMC; peripheral blood mononuclear cells; PD = panic disorder; PTSD = posttraumatic stress disorder; SP = social phobia; RT-qPCR = real-time quantitative polymerase chain reaction.

before and after national examinations (Honda et al., 2013). They found seven miRNAs elevated in the pre-examination period accompanied by a downregulation of their target mRNAs. Levels of these miRNAs were significantly reduced 1 month after exams. In contrast to their previous study, the authors also found a significant correlation between state anxiety levels and miR-16.

Finally, a study of 30 PTSD patients and 42 controls examined miRNA expression in peripheral blood (Zhou et al., 2014). The results highlight the role that miRNAs may play in altered immune function in these patients. MiR-125a and miR-181c were significantly downregulated in PTSD subjects. MiR-125a was subsequently found to downregulate interferon-gamma in normal subjects. This suggests that alterations in miRNA levels may contribute to a raised inflammatory tone, which has been linked to the pathophysiology of PTSD (Pace and Heim, 2011).

2.7.3 Summary

Despite some new studies in the area, there is still much to be done to clarify both what miRNAs might be involved in the molecular basis of anxiety and which might function as biomarkers. There is little overlap in miRNAs identified to date, but many studies have included either naturalistic models of anxiety or groups with multiple psychiatric disorders. Given the amount of "noise" inherent in epigenetic mechanisms like miRNAs that can target hundreds of genes, it would seem sensible to start with well-defined patient groups with little comorbidity, although these can be hard to find. Few of the traditional brain banks have samples of anxiety disorders, which limit this area of study. Studying the effect of anxiolytic treatment on miRNA levels could be a productive avenue.

2.8 CONCLUSIONS

In summary, we have carried out a systematic review of the evidence from preclinical and clinical studies of miRNA involvement in mood and anxiety disorders. The review has identified a number of studies in BPAD, MDD, and to a lesser extent anxiety disorders. Although the number of preclinical and clinical studies has rapidly increased in recent times, the psychiatric field is still behind other areas of medicine. Therefore, it is hoped that further understanding of miRNAs may help to elucidate the molecular mechanisms of psychiatric illnesses such as mood and anxiety disorders. MiRNAs play a key role in virtually all functions in the central nervous system and represent a truly novel therapeutic target for the treatment of psychiatric illnesses. In other medical fields, miRNAs have already been identified as biomarkers (Cho, 2010) and therapeutic targets (Janssen et al., 2013). The ability of miRNAs to target hundreds of genes is part of its attractiveness in polygenic disorders. Bioinformatic approaches can help to identify miRNA targets much more easily than experimental validation, but can potentially identify thousands of targets and it is inevitable that some of these targets might be of interest in psychiatric disorders. This puts an emphasis on validation and replication in well-defined samples, as the risk of false negatives is high. Future studies should measure not just miRNA levels but also downstream products (mRNA, protein) to better understand

the impact of miRNAs. MiRNAs have great potential to inform us of pathophysiology, act as biomarkers and even be therapeutic agents or targets. It is expected that miRNAs will contribute to a leap forward in our understanding and treatment of psychiatric disorders. This will therefore be a fascinating field to follow over the coming years.

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Bitemporal Versus High-Dose Unilateral Twice-Weekly Electroconvulsive Therapy for Depression (EFFECT-Dep): A Pragmatic, Randomized, Non-Inferiority Trial

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Objective: ECT is the most effective treatment for severe depression. Previous efficacy studies, using thrice-weekly brief-pulse ECT, reported that high-dose (6× seizure threshold) right unilateral ECT is similar to bitemporal ECT but may have fewer cognitive side effects. The authors aimed to assess the effectiveness and cognitive side effects of twice-weekly moderate-dose (1.5× seizure threshold) bitemporal ECT with high-dose unilateral ECT in real-world practice.

Method: This was a pragmatic, patient- and rater-blinded, noninferiority trial of patients with major depression (N=138; 63% female; age=56.7 years [SD=14.8]) in a national ECT service with a 6-month follow-up. Participants were independently randomly assigned to bitemporal or high-dose unilateral ECT. The primary outcome was change in the 24-item Hamilton Depression Rating Scale (HAM-D) score after the ECT course; the prespecified noninferiority margin was 4.0 points. Secondary outcomes included response and remission rates, relapse status after 6 months, and cognition.

Results: Of the eligible patients, 69 were assigned to bitemporal ECT and 69 to unilateral ECT. High-dose unilateral ECT was noninferior to bitemporal ECT regarding the 24-item HAM-D scores after the ECT course (mean difference=1.08 points in favor of unilateral ECT [95% CI=−1.67 to 3.84]). There were no significant differences for response and remission or 6-month relapse status. Recovery of orientation was quicker following unilateral ECT (median=19.1 minutes versus 26.4 minutes). Bitemporal ECT was associated with a lower percent recall of autobiographical information (odds ratio=0.66) that persisted for 6 months.

Conclusions: Twice-weekly high-dose unilateral ECT is not inferior to bitemporal ECT for depression and may be preferable because of its better cognitive side-effect profile.

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ECT is used to treat severe mental disorders in 1.4 million people annually worldwide, depression being the most common indication in Western countries (1). ECT is the most acutely effective treatment for treatment-resistant, sometimes life-threatening, depression (2, 3). Nevertheless, its use remains limited, mainly because of cognitive side effects (4), especially concerns about retrograde amnesia (5, 6).

Research on electrode placement has focused on preserving efficacy and minimizing side effects. Based on dosage, right unilateral ECT is less effective than bitemporal ECT (2), the most commonly used electrode placement worldwide (1), but causes less cognitive deficits (7). High-dose is more effective than low-dose ECT but more adversely affects memory (2, 7). However, efficacy trials (8–13) have demonstrated that unilateral ECT can be as effective as bitemporal ECT if delivered in high doses at multiples (e.g., 5×–8×) of seizure threshold, the minimum charge required to induce the generalized seizure needed for therapeutic effect.

Although unilateral ECT causes fewer cognitive side effects, the higher charges required to achieve comparable antidepressant efficacy might diminish its cognitive advantage. Relevant trials of brief-pulse (i.e., 1.0 msec–1.5 msec pulse width) ECT have obtained inconsistent results: some show comparable cognitive performance following high-dose (5×–8× threshold) unilateral ECT with reference to moderate-dose (1.0×–1.5× threshold) bitemporal ECT (9, 10, 13), while others demonstrate less cognitive decline following high-dose (6× threshold) unilateral ECT (8, 11, 12), although the latter studies mostly compared it with higher-dose (2.5× threshold) bitemporal ECT that increases cognitive side effects (7). None of these studies were designed to determine unilateral noninferiority for antidepressant effect, and most had very limited follow-up. All used thrice-weekly treatment,

common practice in the United States where most of these trials originated, even though this does not result in better outcomes than twice-weekly ECT (14) but is associated with increased cognitive side effects (15). This limits their generalizability for populations in which twice-weekly frequency is common practice, as occurs in many European countries (e.g., Belgium, Ireland, the Netherlands, United Kingdom) (16, 17). Additionally, none of the previous trials reflected routine practice in that antidepressants were discontinued before ECT, and all but one (9) required receiving at least eight ECT sessions unless response criteria were met.

To date, no randomized trial has tested whether twice-weekly high-dose (6× threshold) unilateral ECT is noninferior to reference (1.5× threshold) bitemporal ECT nor evaluated its superiority in terms of cognition and retrograde memory preservation over a prolonged follow-up period. We aimed to examine short- and long-term effectiveness and cognitive side effects of high-dose unilateral ECT compared with bitemporal ECT for severe depression in routine practice over 6 months.

METHOD

Study Design and Participants

EFFECT-Dep (Enhancing the Effectiveness of Electroconvulsive Therapy in Severe Depression) was a pragmatic, patient- and rater-blinded two-group parallel, randomized, noninferiority trial with a 6-month follow-up (18, 19). Participants were all in-patients recruited between May 2008 and October 2012 from St. Patrick's Mental Health Services (http://www.stpatricks.ie/), an independent nonprofit organization that provides national services and runs Ireland's largest ECT clinic, including referrals from public-sector hospitals. Eligible participants were ≥18 years old, referred for ECT, met diagnostic criteria for major depressive episode (unipolar or bipolar; Structured Clinical Interview for DSM-IV [20]) and scored ≥21 on the 24-item Hamilton Depression Rating Scale (HAM-D) (21). Exclusion criteria were conditions rendering patients unfit for general anesthesia or ECT; ECT in previous 6 months; history of schizophrenia, schizoaffective disorder, or neurodegenerative or other neurological disorder; alcohol/substance abuse in previous 6 months; involuntary status; and inability/refusal to consent. Treatment during the follow-up period was determined by patients in consultation with treating clinicians. This study was approved by the hospital's research ethics committee (approval reference 012/07), and written informed consent was obtained after procedures were fully explained.

Randomization and Masking

After baseline assessments and before the first ECT session, patients were allocated (1:1) to bitemporal or unilateral ECT using an online system by the Clinical Trials Unit, King's College London. Minimization with variable block sizes ensured that group allocation was balanced regarding three stratifiers: age >65 years (yes/no), previous ECT (yes/no), and referral site (St. Patrick's Mental Health Services/St. James's Hospital/other hospital). Recruiting researchers electronically submitted participants' identifying number, initials, birthdate, history of ECT, and referral site. Treating clinicians received e-mail notification of randomization but were not involved in outcome assessments. Allocation was concealed from patients (prepared for receiving both electrode placements), recovery staff, referring clinicians, assessors, and trial statistician until completion of final analyses. Success of masking was investigated after end-of-treatment assessments by asking patients and raters to guess the treatment used.

Interventions

Brief-pulse (1.0-msec pulse width; current amplitude 800 mA) ECT was administered twice weekly (Mecta 5000M device, Mecta Corp., Portland, Ore.; maximum 1200 mC) according to Royal College of Psychiatrists' guidelines, using methohexital (0.75 mg/kg–1.0 mg/kg) anesthesia, and succinylcholine (0.5 mg/kg–1.0 mg/kg) for muscle relaxation (16, 22). Seizure threshold was established by dose titration at the first session (see the data supplement accompanying the online version of this article). Subsequent treatments were 1.5×-threshold for bitemporal and 6×-threshold for unilateral (d'Elia placement) ECT. Stimulus charge was titrated upward as required during the treatment course (22, 23). In line with regular practice, the number of ECT sessions was determined by referring clinicians in consultation with patients, up to 12 sessions in accordance with recommendations of the Irish Mental Health Commission (http://www.mhcirl.ie/Mental_Health_Act_2001/Mental_Health_Commission_Codes_of_Practice/Use_of_ECT_for_Voluntary_Patients/), who publish annual national data on ECT (http://www.mhcirl.ie/Publications/Publications/). Patients continued regular antidepressant treatments. ECT characteristics were recorded: seizure threshold (millicoulomb [mC]); mean stimulus charge (mC) for all sessions and nontitration sessions; motor and EEG seizure durations (seconds); total number of sessions; and number of sessions to establish threshold. Time to recovery of orientation (i.e., ability to answer 4/5 simple orientation questions, such as person, place, age, birthdate, day), once breathing spontaneously post-ECT, was recorded after each session (24).

Common adverse physical effects (nausea, headache, muscle aches) were recorded for each session to measure occurrence (yes/no) within each course. Serious adverse events that required prolonged medical attention or were life-threatening were recorded.

Data were obtained at baseline, within, and soon after (2–4 days) completing the ECT course, as well as during the 6-month follow-up. Baseline assessments included the National Adult Reading Test (premorbid IQ), collection of demographic variables (age, gender, years in education, socioeconomic and marital status), and collection of the following clinical variables: referral reason, treatment resistance (yes/no; Antidepressant Treatment History Form

[25]), current psychotropic medications, number of previous depressive episodes, current episode duration, presence of psychosis, and depression polarity. Baseline Clinical Global Impression (CGI) severity was rated by referring clinicians.

Primary Outcome

The primary outcome was depression severity measured by the 24-item HAM-D after completing the ECT course (end of treatment). Interrater reliability for HAM-D scoring was assessed every 6 months; median intraclass correlation agreement was 0.96 (range: 0.89–0.98). To classify depression status, HAM-D scores were obtained after every second ECT session and 1 week after the final session if indicated.

Secondary Outcomes

Secondary depression outcomes included HAM-D scores at the 3- and 6-month follow-ups, end-of-treatment remission and response status, and relapse status for remitters during the 6-month follow-up. Remission was defined as ≥60% decrease from the baseline HAM-D score and a score ≤10 for two consecutive weeks; response was defined as ≥60% decrease from the baseline HAM-D score and a score ≤16; and relapse was defined as a HAM-D score ≥16 for two consecutive weeks. The majority of patients who relapse following successful ECT do so within 3 months (26). To monitor relapse, HAM-D scores were obtained after the end of treatment at 2, 4, 6, and 8 weeks plus 3, 4, and 6 months.

The post-ECT cognitive secondary outcome of main interest, retrograde amnesia measured by the Columbia University Autobiographical Memory Interview-Short Form (27), was prioritized for data collection. Further nonprioritized cognitive outcomes are summarized in Table S2 in the online data supplement and included standardized measures of global cognition (Mini-Mental State Examination [MMSE]), auditory attention and verbal working memory (digit spans forward and backward), psychomotor speed and executive function (Trail-Making Test, parts A and B), semantic memory (category fluency), verbal learning and delayed recall (Free and Cued Selective Reminding Test), and visuo-spatial functioning and memory (Complex Figures Test). Alternative versions were used where appropriate. Cognitive outcomes were collected at baseline, end of treatment, and 3- and 6-month follow-ups. These outcomes, as well as the HAM-D scores, were similar to ones used to establish efficacy and side effects of bitemporal ECT (7, 8, 11–13, 16).

Subjective symptoms attributable to ECT were assessed with the Columbia ECT Subjective Side Effects Schedule, including six items on memory, concentration, and orientation for self-rating of cognition (total=18) (28).

Sample Size

Based on a large bitemporal ECT series (29), we estimated that 69 patients were required per group to have 80% power to demonstrate, using a one-sided equivalence t test at 5% level, that the mean reduction in the 24-item HAM-D score following high-dose unilateral ECT was no more than 4 points (i.e., equivalent to 3 points on the 17-item HAM-D, deemed to be clinically relevant [30]) less than that achieved using bitemporal ECT.

Statistical Analyses

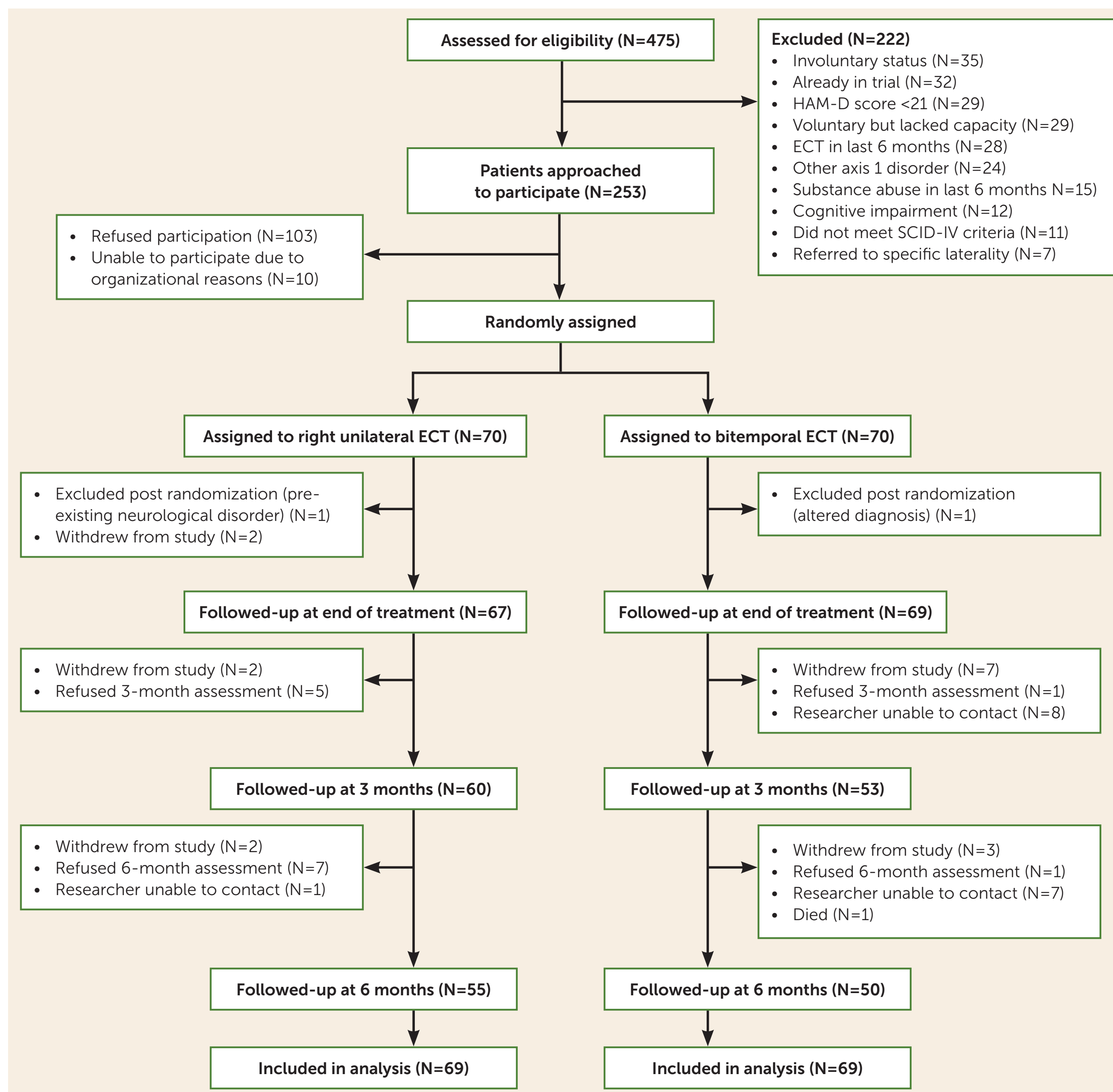
Analyses were on the intention-to-treat principle. ECT measures were summarized by trial arm using relevant descriptive statistics, accompanied by tests of zero group difference where this was not known a priori. We formally compared total numbers of sessions, numbers of sessions to establish seizure threshold (coded "1" or "≥2" sessions), and time to recovery of orientation using regression, logistic regression, and regression of log-transformed times, respectively. In these regression models, randomization stratifiers were included as explanatory variables in addition to trial arm.

The primary statistical analysis was assessment of difference in the 24-item HAM-D scores between arms at the end of treatment. The estimated group difference was supplemented by 95% confidence intervals and this interval compared with the noninferiority threshold (4 points). A regression model was fitted to end-of-treatment HAM-D measures, with prerandomization HAM-D scores, trial arm (unilateral/bitemporal), and randomization stratifiers as covariates. A similar analysis model was assumed for secondary HAM-D outcomes at the 3- and 6-month follow-ups. Among remitters, relapse during the 6-month follow-up was compared between arms using logistic regression as described above.

The main secondary cognitive outcomes of interest (Autobiographical Memory Interview at end of treatment and 3- and 6-month follow-ups) were analyzed using generalized linear models with a binomial distribution and logit-link (31). Posttreatment Autobiographical Memory Interview measures provide the number of baseline items recalled after ECT (27); such "number of items recalled" variables were therefore modeled as arising from binomial distributions, with maximum number of possible recalls set to the number of items obtained at baseline. An overdispersion parameter was introduced to account for recall of individual events being driven by subject characteristics. The covariates of these models were trial arm and randomization stratifiers.

Similar regression models were employed to describe nonprioritized continuous secondary outcomes: other cognitive tasks and subjective side effects (now also including baseline values as a covariate). Time outcomes (i.e., Trail-Making Tests, parts A and B) were log-transformed before analysis to acknowledge positively skewed distributions. The same approach was applied for count outcomes displaying positive skewness (subjective side effects total and cognitive scores). Group effects for these outcomes were therefore quantified by the ratio of outcome in the bitemporal arm to that in the unilateral arm.

FIGURE 1. Trial Profile[a]



[a] HAM-D=24-item Hamilton Depression Rating Scale; SCID-IV=Structured Clinical Interview for DSM-IV.

For physical safety analyses, we assessed proportions of patients who had adverse events by treatment group and compared proportions using logistic regression modeling as for ECT measures.

Handling of missing data is described in the online data supplement. We used Stata (version 13) and SPSS 19.0 (IBM Corp., Armonk, N.Y.) for statistical analyses.

RESULTS

Participant Flow

The trial profile is shown in Figure 1. A total of 475 patients (mean age: 62 years [SD=15.1]; 67.7% female) were referred for ECT during the recruitment period (May 2008–October 2012), accounting for 32.9% of all ECT referrals in Ireland (N=1,480; average age: 57.3 years; 66.5% female). Seventy patients, all white Irish, were assigned per group. One patient per trial arm was excluded postrandomization because they were found not to fulfill eligibility criteria. Comparing the 138 participants to the 113 potentially eligible nonparticipants, participants were younger (mean age: 56.7 years [SD=14.8] versus 63.4 years [SD=14.3]; t=3.64, p=0.0001) but did not differ significantly regarding gender (% female: 63% versus 67%; χ^2=0.48, p=0.52), baseline CGI severity (mean: 5.3 [SD=0.7] versus 5.2 [SD=0.9]; N=101; U test: z=0.93, p=0.35), and MMSE scores (mean: 27.7 [SD=2.1]; N=119 versus 27.8 [SD=2.5]; N=85; t=−0.27, p=0.79).

All patients adhered to allocated treatment, although five (7.2%) unilateral patients had thresholds >200 mC (225 mC,

N=1; 250 mC, N=3; 500 mC, N=1) and thus could not be treated at a fully 6× seizure threshold. Nearly all participants (N=136; 98.6%) were assessed for primary outcome at the end of treatment, while 82% and 76%, respectively, were followed up at 3 and 6 months.

Treatment guesses were made by patients (119/138) and raters (118/138): 12 patients could not guess, and 26/56 in the unilateral group and 36/51 in the bitemporal group correctly guessed (χ^2=3.27, p=0.07; kappa=0.17 [low coefficient of beyond-chance agreement]). For raters, 30/57 of the guesses for the unilateral group and 36/61 for the bitemporal group were correct (χ^2=1.61, p=0.21; kappa=0.12). Thus, masking was successful for patients and raters.

Baseline and Treatment Characteristics

Summaries of baseline characteristics were comparable between trial arms as would be expected under random allocation (Table 1). Age (mean: 56.7 years [SD=14.8]), gender (63% female), psychosis status (21%), bipolarity (23%), baseline 24-item HAM-D scores (mean: 29.9 [6.2]), and depression episode median duration (19.5 weeks) for the total sample were similar to that found in previous relevant trials (8, 9, 11–13) and large observational studies (5, 29).

Anesthesia doses were similar for the two groups (Table 2). In line with previous studies (8–12), we found that threshold was lower with unilateral ECT, and total stimulus charges were higher in the unilateral group, while seizure durations were similar between groups. Ninety-three percent of patients had an adequate seizure in the first session. Although it took fewer sessions to establish threshold in the unilateral group (p=0.002), there was no significant difference between groups for total number of ECT sessions (p=0.26). Median time to recovery of orientation following the initial titration session in the unilateral group was half that of the bitemporal group (p<0.001), and this cognitive advantage was maintained, though to a lesser extent, during the remainder of the course.

Primary and Secondary Mood Outcomes

High-dose unilateral ECT was noninferior to bitemporal ECT at the end of treatment. Changes in the 24-item HAM-D scores are shown in Figure 2A. The prespecified noninferiority margin was no more than a –4-point difference at the end of treatment between the bitemporal and unilateral groups (Figure 2B). The predicted difference at the end of treatment was 1.08 (95% confidence interval [CI]=–1.67 to 3.84; unilateral, N=67; bitemporal, N=69). Noninferiority was evident at both the 3-month (3.48, 95% CI=–0.046 to 7.0; unilateral, N=60; bitemporal, N=53) and 6-month (0.26, 95% CI=–3.33 to 3.85; unilateral N=55, bitemporal N=50) follow-ups.

These results translated into similar proportions of responders (unilateral: 42/69 [60.8%]; bitemporal: 35/69 [50.7%]) and remitters (unilateral: 32/69 [46.4%]; bitemporal: 29/69 [42.0%]) in the two groups at the end of treatment. The median number of ECT sessions for both responders and remitters was 7 (range: 3–12), which was less than the median number of 9 (range: 3–12) ECT sessions for both nonresponders and nonremitters (for both Mann-Whitney U test, p<0.001). During the 6-month follow-up, there was no significant difference between the proportion of remitters who relapsed in the unilateral (8/32; 25.0%) and bitemporal (11/29; 37.9%) groups (odds ratio [unilateral/bitemporal]=0.56, 95% CI=0.17–1.79, z=0.99, p=0.32).

Cognitive Secondary Outcomes

The cognitive outcome of main interest post-ECT was retrograde amnesia as measured by the Autobiographical Memory Interview. Autobiographical memory scores for the unilateral (46.9 [SD=9.7]; N=66) and bitemporal (44.4 [SD=10.3]; N=64) groups were similar at baseline. The percent consistency of recall of baseline memories was lower in the bitemporal group at the end of treatment (odds ratio=0.66, 95% CI=0.513–0.85, p=0.001; unilateral, N=64; bitemporal, N=64), and this was maintained at follow-up after 3 months (odds ratio=0.59, 95% CI=0.45–0.78, p<0.001; unilateral, N=56; bitemporal, N=48) and 6 months (odds ratio=0.59, 95% CI=0.45–0.79, p<0.001; unilateral, N=49; bitemporal, N=42) (Figure 3). Distributions of individual percent recall consistencies are presented in Table S3 in the online data supplement.

Assessment completion levels varied for nonprioritized secondary outcomes. End-of-treatment completion rates ranged from 93.5% (category fluency) to 71.7% (verbal learning). Three-month completion rates varied from 62.3% (category fluency) to 47.8% (Trail-Making Test, part B) and at 6 months from 59.4% (category fluency) to 42.8% (Trail-Making Test, part B).

There were few differences between groups for the other cognitive tasks (see Table S4 in the data supplement). At the end of treatment, the only statistically significant difference was for better performance in the unilateral group on verbal learning for immediate recall (p=0.034), though not delayed recall (p=0.22). There were no differences between groups on these verbal learning and memory tasks at the 3- and 6-month follow-ups. At 3 months, performance was better in the unilateral group for both auditory attention (p=0.021) and verbal working memory (p=0.049), but these cognitive advantages were not evident at the end of treatment or at the 6-month follow-up.

There were no significant differences between groups on the Subjective Side-Effects Schedule for total side effects at any time point (see Table S4 in the data supplement), although the number and severity of side effects declined substantially over time, probably in line with improved mood (16, 28). However, significantly fewer subjective cognitive side effects were reported by the unilateral group at the end of treatment (p=0.02) and after 6 months (p=0.025). Thus, there were both objective and subjective cognitive advantages for unilateral compared with bitemporal ECT.

TABLE 1. Baseline Clinical and Demographic Characteristics[a]

Characteristic	Total Sample (N=138)		Right Unilateral ECT (N=69)		Bitemporal ECT (N=69)	
	Mean	SD	Mean	SD	Mean	SD
Age (years)	56.7	14.8	56.6	15.3	56.8	14.4
Education (years)	13.1	3.4	13.7	3.0	12.6	3.8
Hamilton Depression Rating Scale-24 item	29.9	6.2	30.4	6.1	29.5	6.3
Mini-Mental State Examination[a,b]	27.7	2.1	28.0	1.8	27.4	2.4
National Adult Reading Test[a,c]	108.3	6.8	109.2	5.6	107.4	7.8
Clinical Global Impression-Severity[d]	5.3	0.7	5.4	0.7	5.3	0.7
Number of psychotropic medications	4.2	1.4	4.3	1.3	4.2	1.5
	Median	Range	Median	Range	Median	Range
Episode duration[e]	19.5	2–104	18.0	4–104	21.0	2–104
Number of previous episodes	4.0	0–23	4.0	0–20	3.0	1–23
	N	%	N	%	N	%
Female gender	87	63.0	40	58.0	47	68.1
Socioeconomic group						
Professional	24	17.4	10	14.5	14	20.3
Managerial or technical	23	16.7	15	21.7	8	11.6
Skilled occupations	36	26.1	23	33.3	13	18.8
Partly skilled occupations	22	15.9	8	11.6	14	20.3
Unskilled occupations	31	22.5	11	15.9	20	29.0
Not specified	2	1.4	2	2.9	0	0.0
Marital status						
Married	76	55.1	38	55.1	38	55.1
Single	35	25.4	17	24.6	18	26.1
Widowed/divorced	25	18.1	12	17.4	13	18.8
Not specified	2	1.4	2	2.9	0	0.0
Bipolar depression	32	23.2	16	23.2	16	23.2
Presence of psychosis	29	21.0	16	23.2	13	18.8
Treatment resistance[f]	99	72.8	45	66.2	54	79.4
History of previous ECT	53	38.4	26	37.7	27	39.1
Primary reason for ECT referral[d]						
Refractory to medication	75	54.3	37	53.6	38	55.1
Rapid response required	57	41.3	29	42.0	28	40.1
Acute suicidality	5	3.6	2	2.9	3	4.3
Physical deterioration	1	0.7	1	1.4	0	0.0
Psychotropic medications						
Selective serotonin reuptake inhibitors	29	21.0	15	21.7	14	20.3
Serotonin and noradrenaline reuptake inhibitors	67	48.6	32	46.4	35	50.7
Tricyclic antidepressants	39	28.3	20	29.0	19	27.5
Tetracyclic antidepressants	6	4.3	6	8.7	0	0.0
Mirtazapine	46	33.3	24	34.8	22	31.9
Agomelatine	2	1.4	1	1.4	1	1.4
Lithium	56	40.6	28	40.6	28	40.6
Anticonvulsants[g]	39	28.3	18	26.1	21	30.4
Benzodiazepines	81	58.7	35	50.7	46	66.7
Antipsychotics	97	70.3	48	69.6	49	71.0
Nonbenzodiazepine hypnotics	69	50.0	34	49.3	35	50.7
Tryptophan	2	1.4	1	1.4	1	1.4
Buproprion	4	2.9	2	2.9	2	2.9
Monoamine oxidase inhibitor	1	0.7	0	0.0	1	1.4
Buspirone	1	0.7	0	0.0	1	1.4

[a] Data are not available for all participants.
[b] N=119 (59 right unilateral, 60 bitemporal).
[c] N=112 (54 right-unilateral, 58 bitemporal).
[d] As recorded by referring physician.
[e] Capped at 104 weeks.
[f] Treatment resistance was based on the Antidepressant Treatment History Form (N=136).
[g] Anticonvulsants include lamotrigine, sodium valproate, and pregabalin prescribed as mood stabilizers or anxiolytic.

TABLE 2. ECT Session Measures

Variable	Right Unilateral ECT[a] (N=69)		Bitemporal ECT (N=69)		Formal Test[b]		
	Mean	SD	Mean	SD	t	df	p
ECT treatment characteristics							
Anesthesia[c]							
Methohexitone (mg/kg)	1.1	0.2	1.0	0.2			NA
Suxamethonium (mg/kg)	0.8	0.2	0.8	0.2			NA
Stimulus charge (mC), all sessions	620.3	223.5	368.1	192.0			NA
Stimulus charge (mC), nontitration sessions[d]	741.6	275.6	403.5	207.6			NA
Total number of sessions	7.8	2.5	8.3	2.4	1.13	131	0.26
	Median	Range	Median	Range			
Initial seizure threshold (mC)	75	50–500	150	50–500			NA
Motor seizure duration (seconds)	28	12–55	28	14–63			NA
EEG seizure duration	42	17–87	40	16–116			NA
	N	%	N	%	z		p
Number of sessions to establish the seizure threshold					3.07		0.002
1 session	56	81	39	56			
2 sessions	12	18	28	41			
3 sessions	1	1	2	3			
	Median	Range	Median	Range	t	df	p
Recovery of orientation[e]							
Time to recovery (minutes), initial titration session	10	5–60	20	5–60	6.82	130	0.001[f]
Time to recovery (minutes), nontitration sessions[d]	19.1	10–55	26.4	10–60	3.88	130	0.001[f]

[a] Data are not available for all participants (N=67–68).
[b] All models used to construct inferences were conditioned on stratifiers.
[c] Six patients received propofol during their ECT course at standard doses due to temporary shortage of methohexital, four in the bitemporal group and two in the unilateral group.
[d] A mean time was estimated for all sessions following the definite establishment of the seizure threshold.
[e] Recovery of orientation was defined as correctly answering 4/5 reorientation questions.
[f] Formal inferences carried out after log-transformation.

Adverse Events

There were no differences between the unilateral and bitemporal groups for occurrence of headaches (26.5% versus 27.5%; odds ratio=0.93, 95% CI=0.42–2.04; z=0.20, p=0.84), nausea (16.2% versus 11.6%; odds ratio=1.54, 95% CI=0.56–4.17; z=−0.84, p=0.40), or muscle pain (11.8% versus 8.7%; odds ratio=1.37, 95% CI=0.44–4.17; z=−0.55, p=0.58).

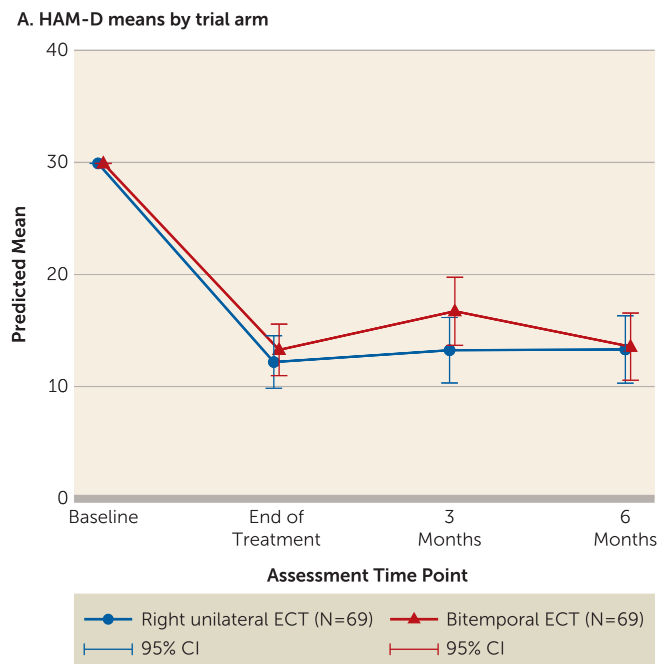
Regarding major adverse events, six patients required β-blockers for ECT-related hypertension (unilateral, N=4; bitemporal, N=2). In the unilateral group, one patient developed laryngospasm with temporary drop in oxygen saturation, one developed tachyarrhythmia necessitating ECT termination, and one attempted suicide during the course. In the bitemporal group, three patients developed interictal confusion resulting in postponement/termination of ECT, one developed bronchospasm, one required β-blocker treatment for sinus tachycardia, one developed bradyarrhythmia, and one developed a pulmonary embolus after the fifth treatment. None of these events led to trial dropout.

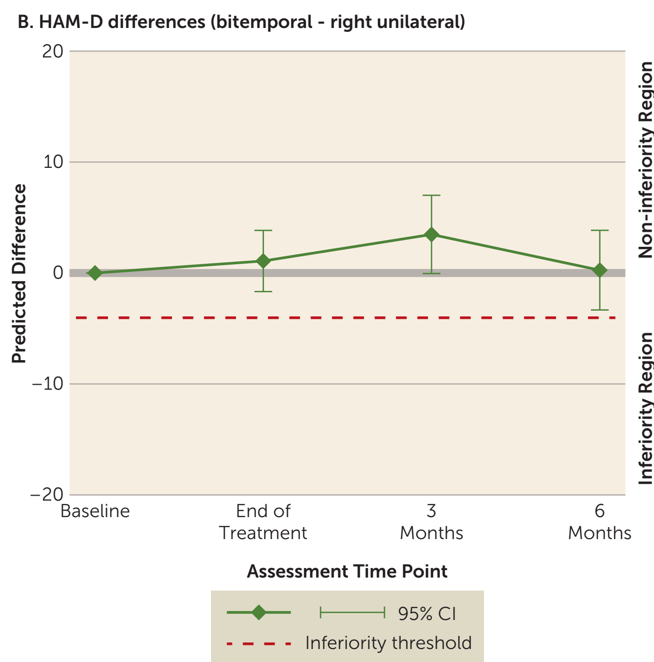
DISCUSSION

Our findings show that twice-weekly high-dose unilateral ECT is noninferior to bitemporal ECT for severe depression in regular clinical practice, which included continued antidepressant pharmacotherapy, and this was maintained over 6 months. The proportions of responders and remitters, as well as relapse rates, are consistent with this. Furthermore, we found high-dose unilateral ECT to be less taxing on autobiographical memory than bitemporal ECT. The unilateral group showed significantly higher autobiographical memory consistency with baseline recall than the bitemporal group at the end of treatment and the 3- and 6-month follow-ups. Other cognitive advantages of unilateral ECT included quicker recovery of orientation following treatments, better verbal learning at the end of treatment, and fewer subjective cognitive side effects. Both forms of ECT were well tolerated. The numbers of common physical side effects and serious adverse events were similar in both groups, in line with previous studies reporting harms (12, 32).

Our findings for the primary outcome, the 24-item HAM-D score, are consistent with results of previous, non-pragmatic, thrice-weekly efficacy trials (8–13). However, the overall remission rate (44.2%) was lower than the rate in some trials (range: 46%−65%) (8, 9, 11–13) but similar to that in a large community study (46.7%) (33), while the overall 6-month relapse rate (31.1%) was at the lower limit reported in a recent meta-analysis of post-ECT relapse (26). These

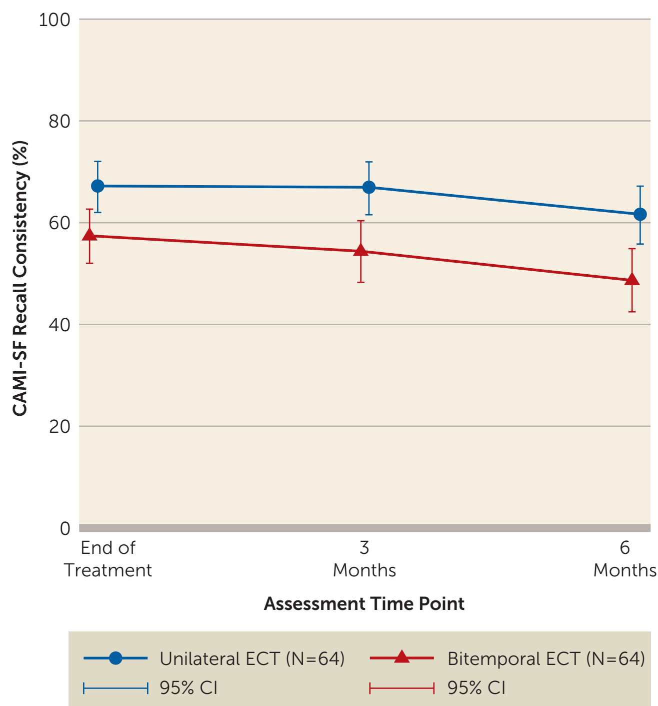
FIGURE 2. Intention-to-Treat Analyses of Primary Outcome[a]





[a] The top graph (A) shows the predicted mean 24-item Hamilton Depression Rating Scale (HAM-D) (95% CI) scores for the unilateral and bitemporal ECT groups at the end of treatment plus the 3- and 6-month follow-ups. Means are predicted for patients with average baseline outcome value who are of younger age (≤65 years), referred from St. Patrick's Mental Health Services, and have no previous experience of ECT. All analyses were carried out using multiple imputation with 200 imputations (see the Statistical Analysis section and the online data supplement). All models used to construct inferences were adjusted for baseline HAM-D scores and conditioned on stratifiers. In the bottom graph (B), the dashed line is the predefined noninferiority margin (−4 points on the HAM-D). The green line shows the predicted mean (95% CI) differences in HAM-D scores at the same time points as shown in graph A.

FIGURE 3. Autobiographical Memory Following ECT: Recall Consistency (%) With Baseline Scores for Unilateral and Bitemporal ECT Groups[a]



[a] CAMI-SF=Columbia Autobiographical Memory Interview-Short Form.

differences most likely reflect the pragmatic nature of our trial, in which the number of treatments was decided by the patients and referring physicians rather than by the protocol, as well as a naturalistic follow-up, and are unlikely to be related to concomitant use of antidepressants, which may improve ECT efficacy (12). Cognitive outcomes at the end of treatment were consistent with previous thrice-weekly ECT trials (8, 9, 11, 12). Regarding autobiographical memory, as measured by the Autobiographical Memory Interview, our findings differed only from two previous trials (9, 13) that found both treatments to have comparable effects. This might be explained by the higher stimulus charge used for the unilateral group (8× seizure threshold) for one trial (9) and/or use of thrice-weekly ECT (9, 13), as both result in larger cognitive deficits.

Strengths and Weaknesses

Trial strengths include noninferiority design, pragmatic attitude, relatively large sample size, and adequate power. We showed excellent adherence and end-of-treatment completion rates. Retention at both follow-ups was satisfactory for the primary and main cognitive outcomes and superior to previous relevant trials. Indeed, existing efficacy trials either lacked follow-up (8, 12, 13), had shorter follow-ups (1–2 months) (9, 10), and/or had smaller follow-up samples (19–22 per group) (9, 11). None was designed to test noninferiority of high-dose unilateral ECT compared with bitemporal ECT. The randomized sample was representative of the general population referred for ECT and similar to

potentially eligible nonparticipants. Our findings, therefore, have good generalizability to countries where twice-weekly ECT is normal practice.

Our study has some limitations. First, we did not include involuntary patients who could not consent due to illness severity (7.4% of referrals), for whom bitemporal ECT may be better (13). Second, other than for autobiographical memory, there are high levels (13%−54%) of missing variables for secondary cognitive outcomes at the follow-ups. Nevertheless, this study presents the best available evidence, to our knowledge, of long-term cognitive correlates of high-dose unilateral and bitemporal ECT. A third limitation concerns the Autobiographical Memory Interview. We selected this instrument to situate our trial within existing research evidence, as most previous trials used a variant of it (8, 11–13). However, it does not allow quantification of retrograde amnesia attributable directly to ECT even though it is sensitive in detecting differences between treatment allocations on autobiographical memory recall (6, 7, 34, 35). Nevertheless, the present trial shows that high-dose unilateral ECT affects autobiographical memory less than bitemporal ECT. Fourth, all trial participants were in-patients, but this reflected routine practice. Fifth, the relatively lower remission rate may be due to the pragmatic design when compared with other trials (8–13) performed under more stringent, but less clinically general, conditions.

CONCLUSIONS

Our study has important clinical implications. In terms of harms/benefits ratio, high-dose unilateral ECT was noninferior to bitemporal ECT but showed a better cognitive profile, especially for preserving retrograde personal memories and fewer subjective cognitive side effects. While there is much interest in other modifications to maintain effectiveness but reduce side effects (e.g., ultrabrief pulse-width ECT), these require further refinement and characterization for optimization (36, 37). Our findings justify considering high-dose unilateral ECT as the preferred ECT option for treating depression and may help improve acceptability and availability of this effective treatment.

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