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Visceral adiposity and oesophageal adenocarcinoma: Exploring the role of the insulin like-growth factor axis

A dissertation submitted to the University of Dublin, Trinity College for the degree of Doctor of Philosophy

by

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Under the supervision of Dr GP Pidgeon & Prof JV Reynolds

July 2012

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This work is supported by an Irish Cancer Society Research Scholarship (CRS09DON).

Declaration

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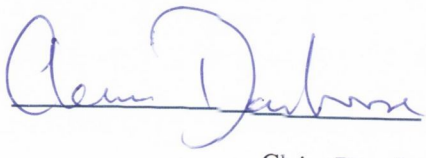

Claire Donohoe

July 2012

Summary

In the modern era there is a consistent reported trend of markedly improved operative and oncologic outcomes from high-volume oesophageal programmes within academic medical centres. The current benchmark survival rate in patients treated with curative intent is between 35 to 50%, but this does not account for the majority of patients who have advanced disease and are unsuitable for treatment with curative intent. Clearly, new treatment strategies are warranted.

Unlike standard chemotherapeutic agents, targeted therapies are chosen for their ability to interact with targets which are integral to the processes used by cancer cells to proliferate and metastasise. It is hoped that using profiling technologies, these agents may be applied to patients with molecularly-defined tumours in whom it is known which targeted oncoproteins are upregulated.

The rising incidence of oesophageal adenocarcinoma is attributed to increases in the prevalence of obesity. The mechanisms which underpin the association between obesity and cancer are poorly understood. It is thought that central visceral obesity rather than subcutaneous obesity is of more relevance to the pro-carcinogenic alterations in systemic metabolism which drive carcinogenesis in the obese. A number of putative alterations have been suggested including the state of chronic inflammation, raised adipokines and increased insulin-like growth factor (IGF) activity. Strong epidemiological evidence demonstrates that the increased risk of oesophageal adenocarcinoma in obese individuals in greater than most other malignancies.

Furthermore, Barrett's oesophagus, a known precursor of oesophageal adenocarcinoma had been associated with obesity. Thus, oesophageal adenocarcinoma is an exemplar model in which to study the mechanisms linking obesity and carcinogenesis.
Given the putative roles of IGF1R in cancer development and progression, many drug discovery efforts are ongoing to identify agents that selectively block the IGF1R pathway in tumour cells. The rationale for this, is the growing *in vitro* and *in vivo* evidence of involvement of the IGF axis in cancer development. The overall aim of this

thesis was to explore the role of the insulin-like growth factor axis in oesophageal adenocarcinoma.

In this study, there was increased expression of circulating free and total IGF-1 as well as increased IGF1R mRNA and protein in viscerally obese patients versus normal weight oesophageal cancer patients. IGF1R protein expression was associated with significantly poorer prognosis in patient with oesophageal adenocarcinoma. Using oesophageal cancer cell lines, the functional effects of IGF1R inhibition using a specific tyrosine kinase inhibitor of IGF1R, picropodophyllin (PPP) was investigated. Treatment led to reduced proliferation, G2/M cell cycle arrest and induction of apoptosis. In addition, treatment with PPP also reduced IGF1R expression on the cells, a potential component of the functional response demonstrated with PPP treatment and which in itself may be a useful indicator of treatment responsiveness in the clinical setting.

An oral analogue of PPP, AXL-1717 is undergoing phase III clinical trials at present. Initial trial data demonstrate that tumour necrosis was visible in treated non-small cell lung cancer tumours – an effect not previously observed in clinical trials of IGF1R inhibitors. In fact, results of recent trials of IGF1R inhibitors have not demonstrated broad clinical efficacy. This may be due to the development of treatment resistance and a potential mechanism involving reciprocal activity in alternate PI3K pathway activators was explored.

Despite inhibition of IGFIR with PPP, there was no decrease in signalling via PI3K pathway. IGF1R inhibition led to a significant increase in VEGF production by treated cells and neutralisation of this VEGF lead to a decrease in proliferation and in phosphorylated Akt activity. In patient tumour samples, the subgroup of patients with negative IGF1R expression and low VEGF expression had fewer advanced T stage (T3/4) tumours, fewer node positive tumours and fewer poorly or undifferentiated tumours than the rest of the cohort. These cell lines were relatively resistant to mTOR inhibition and mTOR inhibition can lead to decreases in VEGF production. Therefore, there may be persistant activation of the mTOR pathway despite IGF1R inhibition. These data may indicate that co-targeting of IGF1R and either VEGF or mTOR activity may be necessary in order to prevent resistance to targeted agents developing.

Whilst IGF1R is a promising target in the treatment of oesophageal adenocarcinoma, a greater understanding of the molecular profile of oesophageal tumours is required in order to fully understand the implications of IGF1R inhibition – both to accurately target patient populations likely to respond to treatment and to rationally combine treatments in order to adequately subjugate resistance mechanisms.

Acknowledgements

I gratefully acknowledge the generous scholarship from the Irish Cancer Society in support of this work, without which this work could not have been undertaken. It is a pleasure to thank the many people who made this thesis possible.

It is difficult to overstate my gratitude to Prof Reynolds. He has many inspirational qualities, including his work ethic, his patient focus and his critical thinking skills. The unit, both clinical and academic, which he has created and allowed me join, has opened my horizons. He has always been there to correct my work, offer encouragement and set goals. I have gained so many skills from this period of research and central to this has been his respect for my opinions, which had given me the confidence to continue on. I can only aspire to his insight, energy and vision. He has given me a deep respect for the academic approach to surgery and shown me the various important facets of being a surgeon – as thinker, teacher and operator.

With his enthusiasm and his motivating talks, Dr Graham Pidgeon helped to make this thesis possible. Thank you for listening to my outlandish great schemes of work, reining me back to reality and giving me the freedom to pursue independent work.

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I dedicate this thesis to the memory of my grandparents (and surrogate parents!), John and Ellen O'Rourke – it has always been my wish to make them proud, despite knowing that they would have been in any case.

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"To those whom much is given, much is expected" – John Fitzgerald Kennedy

List of tables

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Abbreviations

Ab	Antibody
ACM	Adipose conditioned media
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BMI	Body mass index
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CD14	Cluster of differentiation 14
CT	Computerised tomography
CT	Cycle threshold
Cu	Copper
CXCL12	C-X-C chemokine ligand 12
CXCR4	C-X-C chemokine receptor type 4
DFS	Disease-free survival
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
E2	Oestradiol
EDTA	ethylene-diamine tetra-acetic acid
EGFR	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
EMT	epithelial mesenchymal transition
EPIC	European Prospective Investigation into Cancer and Nutrition
ER	oestrogen receptor
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERK	Extracellular signal-regulated kinases
FBS	Foetal bovine serum
FFA	Free fatty acid
FGFR	Fibroblast growth factor receptor
fIGF1-1	Free insulin-like growth factor-1
FITC	Fluorescein isothiocyanate
G phase	Growth phase
GH	Growth hormone
GHRH	GH-releasing hormone
H	Hours
H_20	Water
HER-2	human epidermal growth factor receptor
HIF-1α	Hypoxia inducible factor -1 alpha
HRP	Horseradish peroxidase
HRQL	Health related quality of life

HSP	Heat shock protein
IC_{25}	Inhibitory concentration 25
IC_{50}	Inhibitory concentration 50
IC_{75}	Inhibitory concentration 75
ICGC	International Cancer genome Consortium
IE	Invasive edge
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor receptor 1
IGFBP	insulin-like growth factor binding protein
IL	Interleukin
Inc	Incorporated
IP-10	Interferon inducible protein-10
IR	insulin receptor
IRS-1	insulin receptor substrate-1
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LPS	Lipopolysaccharide
M phase	Mitosis phase
M stage	Metastasis stage
MAB	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemotactic protein
MIP	Macrophage inflammatory protein
MMP9	matrix metalloproteinase-9
mRNA	messenger ribonucleic acid
MSTR1/RON	MET family receptor macrophage stimulating 1 receptor/recepteur d'origine nantais
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N stage	Nodal stage
neo-CRT	Neoadjuvant chemoradiotherapy
NFκB	Nuclear factor-κB
NO	Nitric oxide
NSCLC	Non-small cell lung cancer
OAC	Oesophageal adenocarcinoma
OCCAMS	Oesophageal cancer clinical and molecular stratification
OGJ	Oesophagogastric junction
P	Probability
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFS	Progression free survival
PGE2	Prostaglandin E2
pH	power of Hydogren
PI	Propidium iodide

PI3K	Phosphatidylinositol 3-kinases
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PPP	Picropodophyllin
PTEN	Phosphatase and tensin homolog
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
RBP4	Retinol binding protein 4
RHEB	Ras homologue enriched in brain
RICTOR	RPTOR-independent companion of mTOR, complex 2
ROS	reactive oxygen species
RQ	Relative quantification
RT	room temperature
RTPCR	reverse transcription polymerase chain reaction
S phase	synthesis phase
sACM	Subcutaneous adipose conditioned media
SCC	Sqaumous cell carcinoma
SD	standard deviation
SDS	sodium dodecyl sulphate
SDW	sterile distileed water
SEM	standard error of the mean
SIN1	stress-activated map kinase-interacting protein 1
siRNA	small interfering RNA
SMS	Somatostatin
SNP	single nucleotide polymorphism
SPECT	Single photon emission computed tomography
STAT3	signal transducer and activator of transcription 3
STMN1	stathmin 1
T	Testosterone
T stage	Tumour stage
TAC	tonsil, appendix and carcinoma
TAM	tumour associated macrophage
TBST	Tris-Buffered saline with Tween
TEMED	N,N,N',N'-tetramethylethylenediamine
TGFBR3	transforming growth factor beta receptor 3
TKI	tyrosine kinase receptor inhibitor
TKR	tyrosine kinase receptor
TMA	tissue microarray
TMB	Tetramethylbenzidine
TNF-α	tumour necrosis factor-alpha
TNM	tumour-node-metastasis
TS	tumour stroma
TSC	tuberous sclerosis protein
UK	United Kingdom
USA	United States of America
vACM	visceral adipose conditioned media

VEGF	vascular endothelial growth factor
VFA	visceral fat area
WC	waist circumference
WHO	World Health Organisation

Units

bp	base pairs
°C	degrees Celcius
g	grams
h	hours
µg	microgram
µl	microlitre
µM	micromolar
kDa	kilodaltons
kg	kilogram
hz	hertz
L	litre
M	molar
mA	milliamp
mg	milligram
ml	millilitre
mM	millimolar
n	number size
nM	nanomolar
ng	nanograms
rpm	revolutions per minute
U	units
w/v	weight per volume
v/v	volume per volume

Presentations and publications

Poster presentations

- The association of Insulin-like growth factor 1 and its receptor with obesity status in oesophageal adenocarcinoma.' Donohoe CL, Doyle SL, Lysaght J, Cathcart MC, Pidgeon GP, Reynolds JV. Institute of Molecular Medicine, 13th annual meeting, November 2010

- **Best poster award:** Establishing a role for targeted therapies in oesophageal adenocarcinoma. Donohoe CL, Doyle SL, Lysaght J, Cathcart MC, Pidgeon GP, Reynolds JV. Association of Upper GI Surgery (AUGIS), Belfast 2011.

Oral presentations

National

1. Establishing a role for targeted therapies in oesophageal adenocarcinoma. Donohoe CL, Doyle SL, Lysaght J, Cathcart MC, Pidgeon GP, Reynolds JV. 36th Sir Peter Freyer Surgical Symposium, National University of Ireland (Galway) - September 2011
2. 'The IGF axis, visceral adiposity and picropodophyllin.' Donohoe CL, Doyle SL, Lysaght J, Cathcart MC, Pidgeon GP, Reynolds JV. St Luke's Young Investigator Award, February 2011.
3. The association of Insulin-like growth factor 1 and its receptor with obesity status in oesophageal adenocarcinoma.' Donohoe CL, Doyle SL, Lysaght J, Cathcart MC, Pidgeon GP, Reynolds JV. Sylvester O'Halloran Prize session, March 2011.

International

1) The association of Insulin-like growth factor 1 and its receptor with obesity status in oesophageal adenocarcinoma.' Donohoe CL, Doyle SL, Lysaght J, Cathcart MC, Pidgeon GP, Reynolds JV. Oral presentation for Patey Prize. Society for Academic and Research Surgery conference, Dublin, January 2011
2) Establishing a role for targeted therapies in oesophageal adenocarcinoma. Donohoe CL, Doyle SL, Lysaght J, Cathcart MC, Pidgeon GP, Reynolds JV. Oral presentation for prize; 3rd International Cancer Conference, Trinity College Dubin, September, 2011.
3) VEGF as a co-target for IGF1R inhibition. ***BJS Plenary session, Overall winner***: European Society of Esophagology, Newcastle, November 2011.
4) Oesophageal adenocarcinoma targeted therapy and resistance. ***Patey Prize session***. Society for Academic and Research Surgery conference, Nottingham, January 2012.

Publications

First author relevant publications

1) Obesity and gastrointestinal malignancy. **Donohoe CL**, Lysaght J, Pidgeon GP, Reynolds JV. *British Journal of Surgery*. 2010; 97: 628-42 PMID: 20306531
2) Visceral adiposity, insulin and cancer risk. **Donohoe CL**, Doyle SL, Reynolds JV. *Diabetology & Metabolic Syndrome*, 2011. Jun 22;3(1):12; PMID: 21696633
3) Role of Insulin-like growth factor 1 axis with visceral adiposity in oesophageal adenocarcinoma. **Donohoe CL**, SL Doyle, S McGarrigle, MC Cathcart, T O'Grady, E Daly, J Lysaght, GP Pidgeon, John V Reynolds. BJS 2011. PMID: 22241325

Co-author relevant publications

1) IGF-1 and Its Receptor in Esophageal Cancer: Association with Adenocarcinoma and Visceral Obesity. Doyle SL, ***Donohoe CL***, Finn SP, Howard JM, Lithander FE,

Reynolds JV, Pidgeon GP, Lysaght J. American Journal of Gastroenterology, 2011. PMID: 22146489

2) Pro-inflammatory and tumour proliferative properties of excess visceral adipose tissue. Lysaght J, van der Stok EP, Allott EH, Casey R, ***Donohoe CL***, Howard JM, McGarrigle SA, Ravi N, Reynolds JV, Pidgeon GP. Cancer Letters, 2011. PMID: 21890265.

3) Visceral obesity, the metabolic syndrome, insulin resistance and cancer. Doyle SL, ***Donohoe CL***, Lysaght J, Reynolds JV. Proceedings of the Nutrition Society, 2011. Awaiting PMID:

4) T-lymphocyte activation in visceral adipose tissue of patients with oesophageal adenocarcinoma. Lysaght J, Allot EH, ***Donohoe CL***, Howard JM, Pidgeon GP, Reynolds JV. British Journal of Surgery, 2011. PMID: 21520028

5) MMP9 expression in oesophageal adenocarcinoma is up regulated with visceral obesity and correlates with poor tumour differentiation. Allott EH, Lysaght J, Cathcart MC, ***Donohoe CL***, Cummins R, McGarrigle S, Ravi N, Reynolds JV, Pidgeon GP. Molecular carcinogenesis, 2011. PMID: 22121096.

6) Diet, Obesity and Cancer. Reynolds JV, **Donohoe CL**, Doyle SL. *Irish Journal of Medical Science.* 2010 – PMID: 21174166

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Chapter 1 General introduction

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General introduction

Published in part:

Donohoe CL, Pidgeon GP, Lysaght J, Reynolds JV. *Obesity and gastrointestinal cancer.* Br J Surg. 2010 May;97(5):628-42. PMID: 20306531

and

Donohoe CL, Doyle SL, Reynolds JV. *Visceral adiposity, insulin resistance and cancer risk.* Diabetol Metab Syndr. 2011 Jun 22;3:12. PMID: 21696633

1.1 Targeted therapies, IGF-1 axis and oesophageal adenocarcinoma

1.1.1 Personalised medicine: the potential role of Targeted therapies

Greater understanding of the heterogeneity in the molecular evolution of cancers had uncovered new therapeutic avenues (McDermott et al., 2011). The aim of personalised medicine is to link established clinicopathological indices with molecular profiling to create more detailed and effective diagnostic, prognostic and therapeutic strategies. In principle, targeted therapies are directed against cancer cell-specific attributes essential for survival. We are currently re-considering our understanding of oncogenic transformation and are at the start of an era of precise treatment with predictable responses.

There are a number of associated challenges with the translation of knowledge of molecular processes into clinical practice. The first of these is complexity – molecular subtyping has demonstrated that cancers at one site with similar histological appearances can have very different genetic backgrounds (Sorlie et al., 2001). Analysis of genomic signatures to re-classify molecular subtypes requires complex analytic tools (Chung et al., 2002). Furthermore, the interplay of different factors which combine together to determine clinical outcomes, both genetic and environmental factors, will require a similarly complex way of analysing and interpreting data.

The second challenge is to understand which patients may benefit from targeted agents and, in what context. Mirroring the heterogenous composition of tumours, there are heterogenous treatment responses to targeted agents and predictive clinical biomarkers need to be identified in order to accurately target the use of these agents. Patients with solid cancers treated with targeted agents ultimately develop disease recurrences. Therefore, a combination approach based on understanding the interplay between complementary pathways will be necessary, in order to subdivide diagnostic categories and refine therapeutic choices.

1.1.2 The requirement for targeted therapies in oesophageal adenocarcinoma

Oesophageal cancer is the eight most common disease site for cancer worldwide but the sixth most common cause of cancer related mortality, indicating the lethal nature of the disease (Ferlay et al., 2010). Oesophageal cancer overall has a dismal prognosis, with fewer than 17% of patients diagnosed with the condition alive five years after the diagnosis (Society, 2010). Surgery, alone or preceded by chemotherapy or combination chemoradiation, is the mainstay of treatment approach for localised disease (Reynolds et al., 2012). Despite radical treatment, the disease recurs in the majority of patients and the median disease free interval in these patients is only between 12 and 24 months (Reynolds et al., 2012). The 5 year survival rate in England from 1998 to 2007 ranged from 3.7-15.6% (Coupland et al., 2012). These poor outcomes highlight the need for effective and well-tolerated treatment regimes to supplement the existing standard of care.

Oesophageal cancer research currently receives only 2.1% of the National Cancer Institute's annual budget (2010). Recent advances in treatment have led to improved treatment outcomes, yet despite radical treatment the 5 year survival rate of patients with localised disease undergoing treatment is only 40% (Reynolds et al., 2011). Clearly, there is a requirement for research into the mechanisms of disease development and progression in order to develop new diagnostic and treatment modalities for patients with this cancer subtype. Oesophageal cancer research may also have implications for other cancer types. Oesophageal adenocarcinoma (OAC) research, in particular, is of interest as it has a pre-malignant precursor lesion, Barrett's oesophagus which allows the development of models of early disease. Additionally, the strong epidemiologic association between obesity and OAC means that this disease may potentially allow the testing of obesity and tumourigenesis related hypothesis, which can have implications for other obesity-associated cancers.

1.1.2.1 Oesophageal cancer: Pathology and staging

Oesophageal cancer comprises two main histological subtypes: oesophageal adenocarcinoma (OAC) and squamous cell carcinoma (SCC). Staging modalities may include computerised tomography, endoscopic ultrasound, positron emission tomography (PET) and laparoscopy. OAC is classified according to the American Joint Committee on Cancer (AJCC) tumour-node-metastasis (TNM) classification system, 7th edition. Tumours are classified according to depth of invasion (T stage), lymph node involvement (N stage) and presence or absence of distant metastases (M stage) (Table 1.1 and 1.2)

Table 1.1: American Joint Commission on Cancer (AJCC) staging system for oesophageal cancer: TNM definitions

Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ/ High grade dysplasia
T1	Tumour invades: T1a: invades lamina propria or muscularis mucosae T1b invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades adventitia
T4	Tumour invades adjacent structures 4a: invades resectable structures including pericardium, pleura or diaphragm 4b: Unresectable tumour invading other adjacent structures e.g. aorta, trachea, vertebrae etc
Regional lymph nodes (N)	
NX	regional lymph node metastasis cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis: 1-2 nodes involved
N2	Regional lymph node metastasis: 3-6 nodes involved
N3	Regional lymph node metastasis: >7 nodes involved
Distant metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 1.2 AJCC 7th edition staging system for oesophageal adenocarcinoma, Adapted from (Rice et al., 2010)

Grade	T1		T2		T3	T4 a	T4 b
	1/2	3	1/2	3			
N0	IA	IB	IB	IIA	IIB	IIIA	IIIC
N1	IIB		IIB		IIIA	IIIC	
N2	IIIA		IIIA		IIIB	IIIC	
N3	IIIC		IIIC		IIIC	IIIC	

The age standardised incidence rate, and correspondingly the mortality rates, for Ireland are amongst the highest in Europe (Figure 1.1).

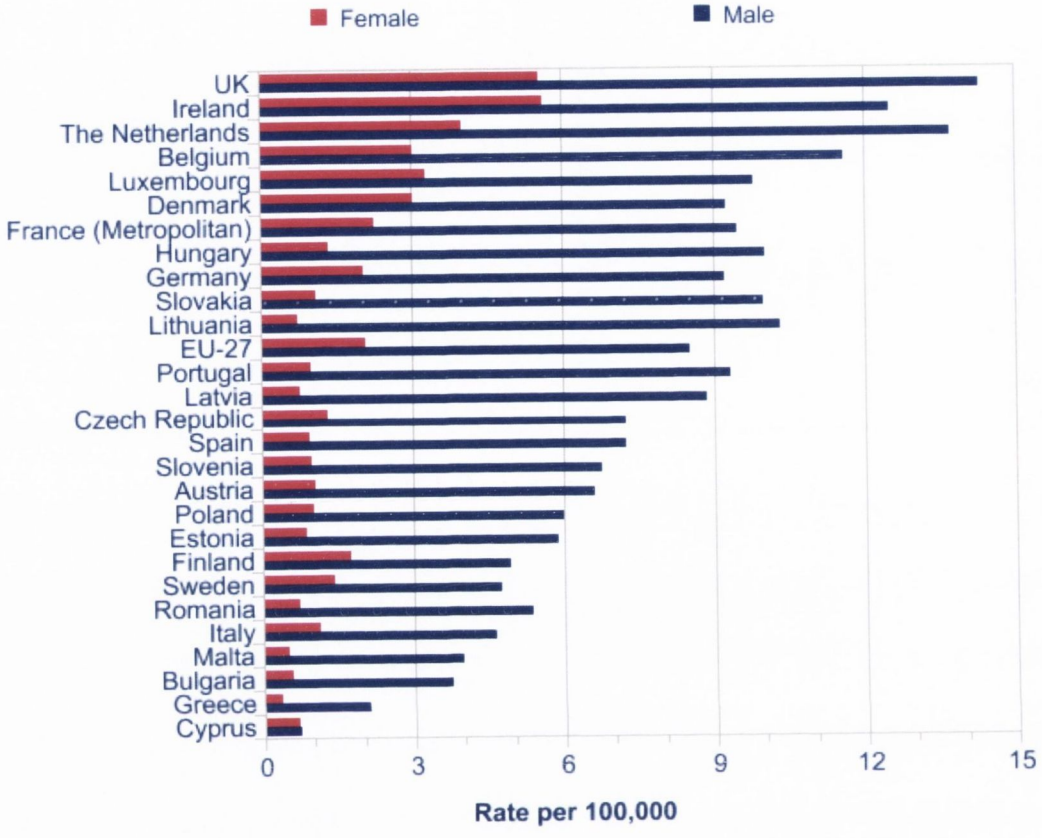


Figure 1.1 Oesophageal cancer European age-standardised incidence rates, EU-27 countries, 2008 estimates Ireland has the second highest age standardised incidence rate for women and third highest incidence rate for men in European Union. (downloaded from: http://info.cancerresearchuk.org/cancerstats/types/oesophagus/incidence/ on 18/01/12).

1.1.2.2 Oesophageal cancer treatment

Surgery alone or preceded by chemotherapy or combination chemoradiation is the mainstay of treatment for localised disease, and these approaches are associated with a marked impairment in health-related quality of life (HRQL) in the months following treatment. This has been reported in several series, the effect is most marked in the first

six months with a trend for recovery in disease-free patients beyond that time (Reynolds et al., 2006, Djarv et al., 2010, Blazeby et al., 2000, Blazeby et al., 2005, Donohoe et al., 2011c). Therefore, radical treatment attempts are reserved only for patients where there is a potential for long-term disease-free survival. Where patients have localised disease (Stage II or less) and have an adequate performance status, options for treatment with curative intent include oesophagectomy using a transthoracic or transhiatal approach. Patients with more advanced, locally invasive disease may be treated with neoadjuvant chemo- or chemoradiation therapy followed by surgery or by radical chemoradiotherapy. The current standard of care in this unit at St. James's Hospital, comprises neoadjuvant chemoradiotherapy (neo-CRT) for patients with node positive or locally advanced T3/4 disease. While neo-CRT has been shown to increase survival compared to surgery alone in some randomised controlled trials these findings are not unanimous (Wijnhoven et al., 2009). Most meta-analyses find in favour of the addition of neo-CRT and the most recent meta-analysis included 12 trials of chemoradiation therapy versus surgery alone and found a hazard ratio (HR) of 0.78 (95% CI 0.70-0.88; $p<0.0001$) with neo-CRT (Sjoquist et al., 2011). However, the largest trial of neo-CRT using modern chemotherapeutic agents demonstrates only an absolute overall survival benefit of approximately 7% at 5 years in the group receiving neo-CRT versus surgery alone (van Hagen et al., 2012). Hence, there is a need to uncover new therapeutic targets to supplement standard neoadjuvant treatment protocols.

Although, there have been recent improvements in the diagnosis and management of oesophageal carcinoma (Reynolds et al., 2012), the overall five-year survival rate remains approximately 8% (Figure 1.2)

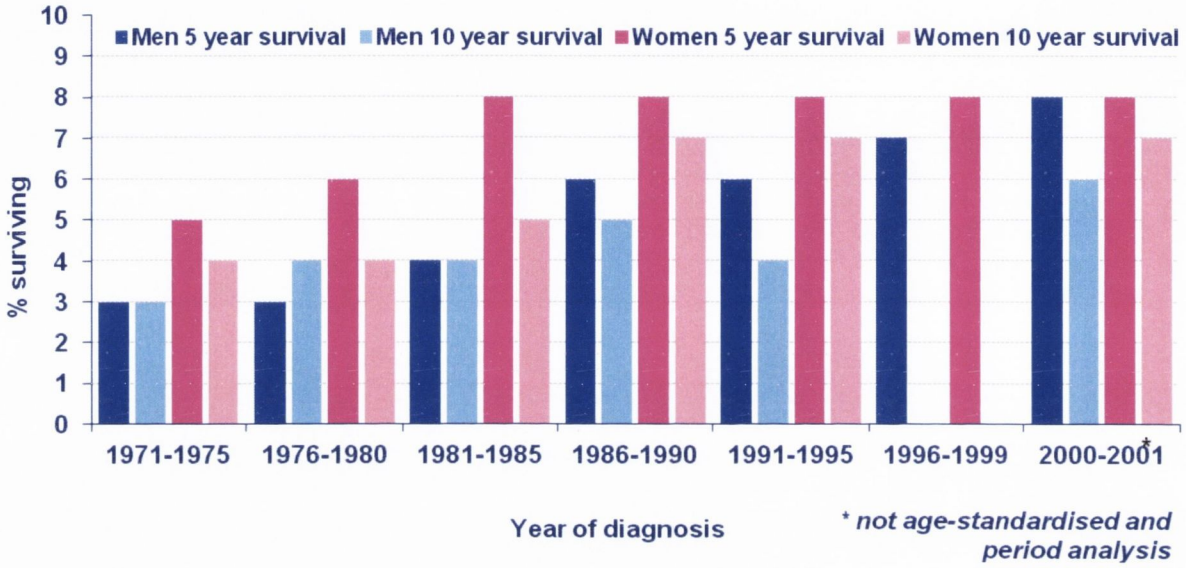
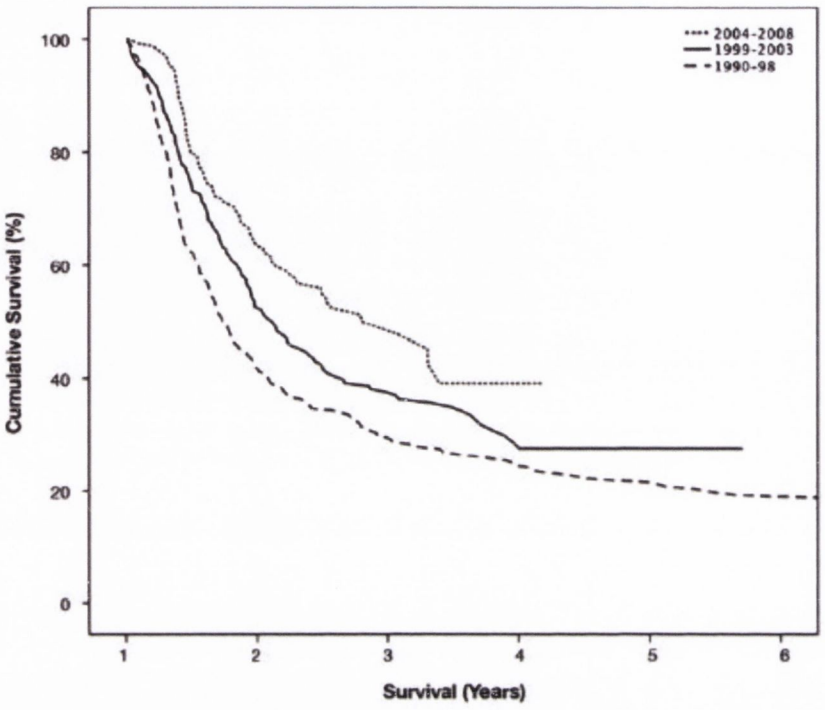


Figure 1.2: Five- and ten-year relative age-standardised survival for oesophageal cancer patients aged 15-99, England and Wales, 1971-2001. While age-standardised survival rates have increased, the overall survival rate for all patients diagnosed is only approximately 8%. (downloaded from: http://info.cancerresearchuk.org/cancerstats/types/oesophagus/incidence/ on 18/01/12).

Many patients are elderly (median age at diagnosis is 72 years (Gilbert FJ, 2002)) and survival decreases with increasing age (Figure 1.4) – this observation is likely multi-factorial. It may be due to treatment related morbidity and selection of younger patients for treatment with curative intent. It may also reflect differing disease processes or differing rates of non-oesophageal cancer related mortality in older patients. Data previously published from our unit has demonstrated that young age (<50 years at the time of diagnosis) is an

independent predictor of survival (Donohoe et al., 2011b). Disease-specific survival rather than overall survival may be a more relevant end-point for the assessment of treatment efficacy in oesophageal cancer patients, since there are a significant proportion of non-cancer related deaths amongst this largely elderly population.

Published data from analysis of the prospectively maintained database in this department demonstrate a significant improvement in disease-specific survival in patients treated with curative intent during the period 2004-08 compared to outcomes from the preceding 15 years (Reynolds et al., 2012). These improvements in outcome have also been reported in other centres (Gertler et al., 2011, Law et al., 2003). Improvements are related to increased numbers of early stage cancer diagnoses, improved clinical and pathological staging and integration of the multidisciplinary team approach. Despite this, the majority of patients are unsuitable for treatment with curative intent and the cancer-specific mortality rate even for patients treated with curative intent remains poor. Clearly, new treatment strategies are warranted.



	1990-98		1999-2003		2004-08		p-value (log-rank)
	N	95% CI	n	95% CI	n	95% CI	
Median survival (months)	17.38	14.89-19.87	22.93	18.29-27.58	32.85	24.34-38.15	0.001

Survival	1990-98			1999-2003			2004-2008		
	No at risk	Deaths	% survival	No at risk	Deaths	% survival	No at risk	Deaths	% survival
0 years	471	0	100	254	0	100	342	0	100
1 year	462	151	65	208	51	75	253	65	80
3 years	241	103	36	144	60	43	144	60	55
5 years	151	27	27	99	13	35	84	13	44
10 years	102	15	19	63	8	28	n/a	n/a	n/a

Figure 1.3 Improved disease-specific survival in patients treated with curative intent. Data from this department demonstrates improved disease-specific survival in patients treated during the latest time period (2004-08) versus the preceding 15 years (Reynolds et al., 2012).

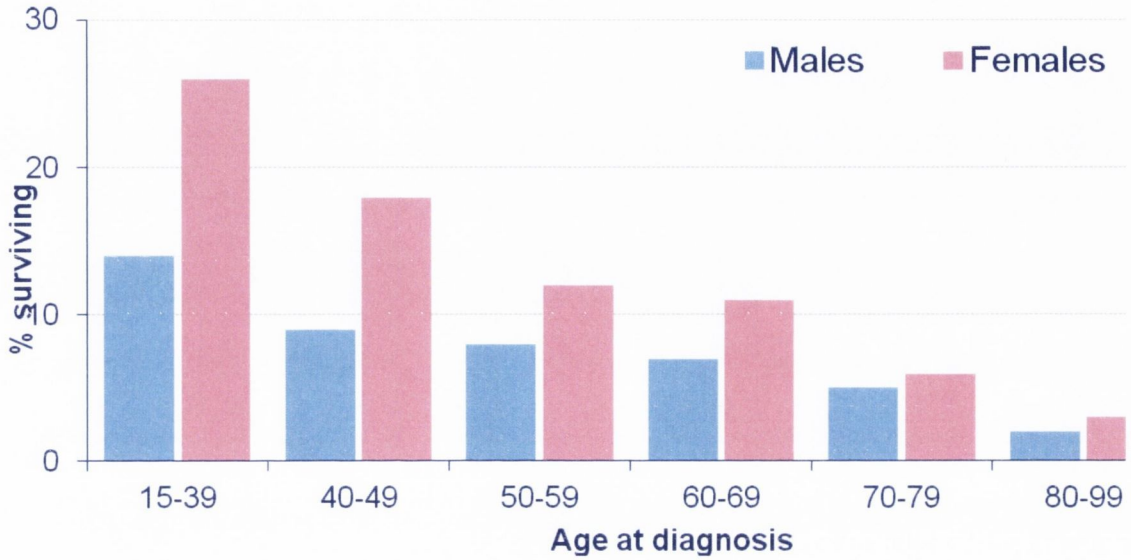


Figure 1.4: Five-year relative survival for patients diagnosed with oesophageal cancer in England and Wales during 1986-1990 by age at diagnosis. The proportion of patients surviving to five years is greater in younger patients. This is likely multi-factorial. (downloaded from: http://info.cancerresearchuk.org/cancerstats/types/oesophagus/incidence/ on 18/01/12).

1.1.2.3 Potential targeted therapies in oesophageal adenocarcinoma

Cancers arise as a result of acquired changes in the DNA of cancer cells. Numerous anomalies develop with time, however, not all contribute to cancer formation and therefore, mutations may be thought of as either driver or passenger mutations. Whereas passenger mutations lead to no direct consequences on cellular function, a driver mutation is one that is causally implicated in the development of a cancer by inferring a growth advantage to the cancer cell (Stratton et al., 2009). Driver mutations are positively selected for in the microenvironment of the tissue. The mutation may not be a

requirement for the maintenance of the tumour, although it often is. It may lead to the expression of or facilitate the activity of an oncoprotein. This oncoprotein's activity is thus a therapeutic target – silencing of which may lead to induction of apoptosis. The concept of cancer cells preferentially utilising a small number of oncoproteins to lend a survival advantage is termed oncogene addiction (Weinstein and Joe, 2006).

There are two alternate hypotheses for how oncogene addiction develops– the first is that cancer cells are dependent primarily on one oncoprotein - thought of as the Achilles' heel of each cancer type, which if silenced leads to the induction of apoptosis.

The other hypothesis is that cancer cells utilise a number of different oncoproteins in order to maintain a pro-survival advantage over pro-apoptotic stimuli. Thus, when one of the pro-survival signals due to activation of an oncoprotein is downregulated, the balance between pro-survival and pro-apoptotic signals is disrupted and apoptosis ensues during a period denoted "oncogenic shock" (Sharma et al., 2006). (Figure 1.5)

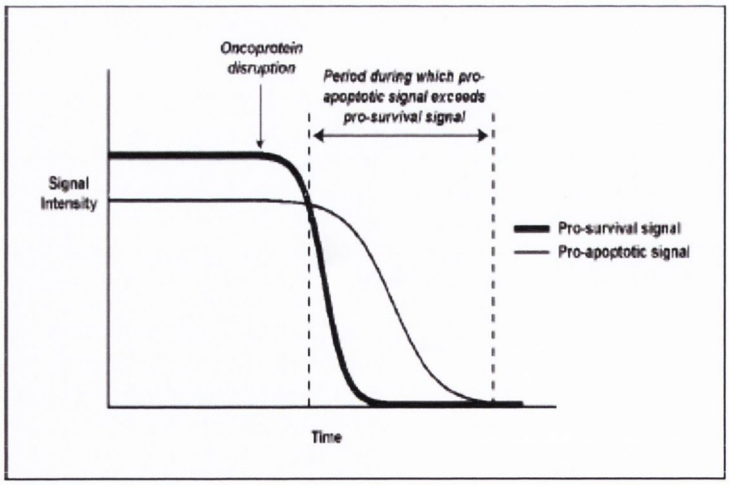


Figure 1.5: Oncogenic shock in the tumour cell following targeted treatment. A schematic illustration of the role of differential signal attenuation in oncogenic

addiction. The model proposes that pro-survival and pro-apoptotic signals emanating from an active oncoprotein in a tumour cell are normally balanced such that the survival output predominates. However, on acute disruption of oncogene function, pro-survival signals dissipate very rapidly whereas pro-apoptotic signals linger sufficiently long such that the cell becomes committed to an apoptotic death. "Signal intensity" is an arbitrary value that describes the relative strength of pro-survival and pro-apoptotic signals emanating from the active oncoprotein. Reproduced from Sharma et al., 2006.

This second hypothesis may explain a number of phenomena observed in the clinical use of targeted agents – namely that they often enhance sensitivity to traditional chemotherapeutic agents and radiotherapy; that secondary resistance often arises – when pro-survival signals in the armamentarium of cancer cells overcome pro-apoptotic signals in response to knockdown of one oncoprotein.

At present, an understanding of the molecular constitution of oesophageal adenocarcinoma is lacking. To date, studies have concentrated on single mutations or proteins in the hope of uncovering relevant driver mutations or oncoproteins as therapeutic targets in this cancer type, but with no real translational success in oesophageal adenocarcinoma.

The target nearest to reaching use in the clinical setting is human epidermal growth factor receptor 2 (HER2; also known as ERB2). The recent TOGA II trial included a group of patients with gastro-oesophageal junctional (OGJ) cancers amongst patients with metastatic gastric cancer and demonstrated an improvement in overall survival from 11.1 months to 13.8 months in patients treated with transtuzumab – the

monoclonal antibody against HER2 (Bang et al., 2010). This trial treated only the 22.1% of patients from 3807 patients tested who were positive for overexpression of HER2. HER2 positivity rates were higher in OGJ tumours than gastric tumours: 33.2% vs 20.9%, $p<0.001$. In independent patient samples, HER2 overexpression has been demonstrated in oesophageal adenocarcinomas – usually in the order of 15% of patients tested (Reichelt et al., 2006, Schoppmann et al., 2010), however it has not been demonstrated to be an independent prognostic factor in the disease in these small cohorts nor has there been any extensive characterisation of its role in tumourigenesis in oesophageal adenocarcinoma. A phase I/II trial of adjuvant transtuzumab with paclitaxel, cisplatin and radiation for locally advanced oesophageal adenocarcinoma only included 19 patients but there was no increase in toxicity (Safran et al., 2007). Further studies are underway.

Other promising targets may include any of those listed in Table 1.3 which are currently undergoing investigation in other cancer subtypes. EGFR mutations may be rare in OAC (Sudo et al., 2007, Miller et al., 2003) but have been reported to be prognostic factors in OAC (Gibson et al., 2003, Wilkinson et al., 2004, Okines et al., 2011, Wang et al., 2007a).

A more rational approach to the identification and selection of targets is the Oesophageal Cancer Clinical and Molecular Stratification (OCCAMS) incorporating International Cancer Genome Consortium (ICGC) multicentre study (Stratton et al., 2009). It is proposed to determine novel biomarkers for prognosis and targets for

therapy from whole genome sequencing and transcriptomics performed on primary tumour material (before and after neo-adjuvant treatment) and selected lymph node metastases in over 500 patients with oesophageal cancer. Gene expression arrays to date have compared Barrett's oesophagus and oesophageal adenocarcinoma samples from small numbers of patients (Hao et al., 2006, Wang et al., 2006). OCCAMS, however, should have enough detailed data to lead to an ability to molecularly subtype OAC and to uncover new therapeutic targets.

Table 1.3 Targeted therapeutics in cancer. A large repertoire of potential agents are currently under investigation. (McDermott et al., 2011)

Table 1. **Targeted Therapeutics in Cancer.***

Gene	Genetic Alteration	Tumor Type	Therapeutic Agent
Receptor tyrosine kinase			
EGFR	Mutation, amplification	Lung cancer, glioblastoma	Gefitinib, erlotinib
ERBB2	Amplification	Breast cancer	Lapatinib
FGFR1	Translocation	Chronic myeloid leukemia	PKC412, BIBF-1120
FGFR2	Amplification, mutation	Gastric, breast, endometrial cancer	PKC412, BIBF-1120
FGFR3	Translocation, mutation	Multiple myeloma	PKC412, BIBF-1120
PDGFRA	Mutation	Glioblastoma, gastrointestinal stromal tumor	Sunitinib, sorafenib, imatinib
PDGFRB	Translocation	Chronic myelomonocytic leukemia	Sunitinib, sorafenib, imatinib
ALK	Mutation or amplification	Lung cancer, neuroblastoma, anaplastic large-cell lymphoma	Crizotinib
c-MET	Amplification	Gefitinib-resistant non–small-cell lung cancer, gastric cancer	Crizotinib, XL184, SU11274
IGF1R	Activation by insulin-like growth factor II ligand	Colorectal, pancreatic cancer	CP-751,871, AMG479
c-KIT	Mutation	Gastrointestinal stromal tumor	Sunitinib, imatinib
FLT3	Internal tandem duplication	Acute myeloid leukemia	Lestaurtinib, XL999
RET	Mutation, translocation	Thyroid medullary carcinoma	XL184
Non–receptor tyrosine kinase			
ABL	Translocation (BCR-ABL)	Chronic myeloid leukemia	Imatinib
JAK2	Mutation (V617F), translocation	Chronic myeloid leukemia, myeloproliferative disorders	Lestaurtinib, INCB018424
SRC	Overexpression	Non–small-cell lung cancer; ovarian, breast cancer; sarcoma	KX2–391, dasatinib, AZD0530
Serine–threonine–lipid kinase			
BRAF	Mutation (V600E)	Melanoma; colon, thyroid cancer	SB-590885, PLX-4032, RAF265, XL281
Aurora A and B kinases	Overexpression	Breast, colon cancer; leukemia	MK-5108 (VX-689)
Polo-like kinases	Overexpression	Breast, lung, colon cancer; lymphoma	BI2536, GSK461364
MTOR	Increased activation	Renal-cell carcinoma	Temsirolimus (CCI-779), BEZ235
PI3K	PIK3CA mutations	Colorectal, breast, gastric cancer; glioblastoma	BEZ235
DNA damage or repair			
BRCA1 and *BRCA2*	Mutation (synthetic lethal effect)	Breast, ovarian cancer	Olaparib, MK-4827 (PARP inhibitors)

* PARP denotes poly(adenosine diphosphate–ribose) polymerase.

1.1.2.4 Oesophageal adenocarcinoma epidemiology

The incidence of oesophageal cancer is rapidly increasing and the pattern of histological distribution is changing (Figure 1.5). Formerly regarded as an uncommon form of cancer, OAC is now the fastest growing form of cancer in the last 25 years in the United States (Blot et al., 1991). The incidence of oesophageal cancer has increased by 27% in Ireland over the last 15 years (National Cancer Registry, 2011). This is accounted for by a 50% increase in the rate of oesophageal adenocarcinoma (OAC) whilst the rate of oesophageal squamous cell carcinoma had fallen by 4% over the same period (National Cancer Registry, 2011). Rates of adenocarcinoma are set to or already have exceeded those of squamous cell carcinoma in Western societies (Brown et al., 1995, Horner MJ, Wayman et al., 2001). The rising rate of OAC was initially observed in Western societies and the rate of OAC is now beginning to increase in Asia – which may reflect increasing Westernisation of dietary, lifestyle and body habitus patterns in this region.

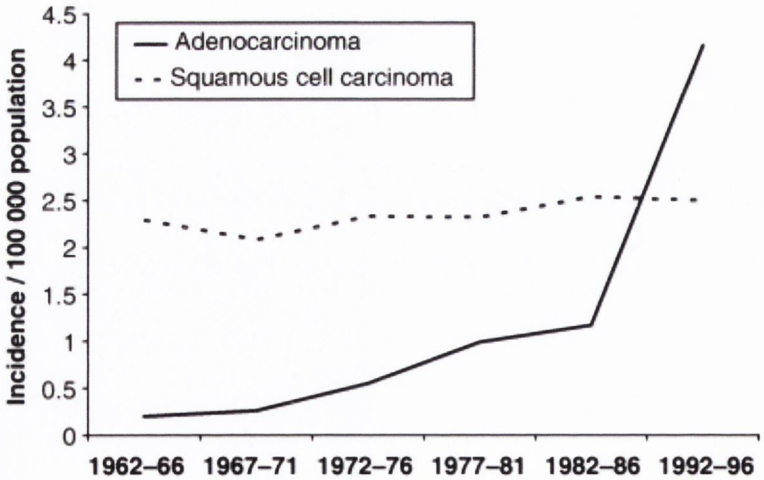


Figure 1.6 Increase in incidence of oesopahgeal adenocarcinoma in UK males 1962-1996. The increasing rate of oesophageal carcinoma is primarily driven by the increasing incidence of oesophageal adenocarcinoma. A similar increase in oesophageal

cancer rates has been observed in Ireland. (Reproduced from (Peters and Fitzgerald, 2007)).

Epidemiological studies have shown that oesophageal carcinoma is largely attributable to lifestyle factors (Engel et al., 2003). Other factors implicated in the development of oesophageal carcinoma include: male sex with a male preponderance of 6:1; smoking and alcohol, particularly for the SCC variant; increasing age and ethnicity (Caucasians have a fivefold higher risk than African Americans).

1.2 Obesity and cancer

The relationship between obesity and development of cardiovascular disease has long been recognised (Coakley et al, 2001). In recent years, there has been an increased acknowledgement of the fundamental impact of obesity on the development of a large number of cancer types. More recently the impact of obesity on cancer-specific mortality rates has been described (Calle et al. 2003), implying a role in tumour progression. Rates of obesity have become a growing problem, particularly in Westernised societies.

Obesity is characterised by increased storage of fatty acids in an expanded adipose tissue mass (Galic et al., 2010). Increased adipose tissue mass, especially visceral adipose tissue, is associated with insulin resistance, hyperglycaemia, dyslipidemia, hypertension, and other components of the metabolic syndrome (Schroeder et al., 2005).

Adipocytes from obese subjects exhibit an altered endocrine function and secretory profile, leading to an increased release of pro-inflammatory molecules resulting in a chronic low-grade inflammatory state that has been linked to the development of chronic diseases, including cancer (Schroeder et al., 2005). Despite an expanding body of epidemiological evidence in support of the link between obesity and cancer, the underlying molecular mechanisms responsible are poorly characterised.

1.2.1 Obesity epidemiology

In the United States 71% of men and 62% of females are overweight or obese (Ogden et al., 2006). Rates are increasing in a similar fashion in Western Europe, with 65% of men and 56% of women being overweight or obese in the United Kingdom (Zaninotto P, 2006). Obesity prevalence shows no signs of decreasing. Over the past decade in the United States mean BMI and waist circumference measurements have increased, particularly amongst men with the mean BMI in 2008 of 28.5kg/m^2 and mean waist circumference 100.8cm (Ford et al., 2011). The prevalence of abdominal obesity (WC>102 cm in men and >88cm in women) was 43.7% in men and 61.8% in women (Ford et al., 2011) in the US.

The prevalence of obesity in Irish adults is currently 18%, with men at 20% and women at 16%. A further 47% of men and 33% of women are overweight (BMI 25.0-29.9 kg/m^2) (McCarthy et al., 2002). It has been estimated that ingestion of 5% more

calories than expended may result in an accumulation of 5kg of adipose tissue in a single year (Klein et al., 2002).

Obesity rates are increasing amongst children (Troiano and Flegal, 1999) and overweight children tend to become overweight adults. (Serdula et al., 1993) Obesity rates are increasing exponentially: rates have doubled in Australia over the last 20 years (Dunstan et al. 2002) and in the United States over the last 30 years (National Health and Nutrition Examination Survey (1971-74 to 2003-06) (http://www.cdc.gov/nchs/fastats/overwt.htm)). Over the same time period European obesity rates have tripled (WHO report 2007).

1.2.2 Visceral adiposity

Adipose tissue is principally deposited in two compartments – subcutaneously and centrally (Figure 1.7). It is thought that centrally deposited, or visceral, fat is more metabolically active than peripheral subcutaneous fat (Kershaw and Flier, 2004, Vohl et al., 2004, Galic et al., 2010). Visceral adipose tissue is largely comprised of omental adipose tissue but also includes other intra-abdominal fat sources such as mesenteric fat. Visceral adipose tissue secretes a number of adipokines and cytokines leading to a proinflammatory, procoagulant and insulin resistant state collectively known as the metabolic syndrome (Despres and Lemieux, 2006). The importance of adipose tissue location in terms of dysmetabolism risk is evident as central obesity is more strongly associated with increased risk of insulin resistance, the metabolic syndrome and

cardiovascular diseases than BMI alone (Nedungadi and Clegg, 2009). For any given amount of total body fat, the subgroup of individuals with excess visceral fat (versus subcutaneous fat) is at higher risk of developing insulin resistance (Kissebah et al., 1982) and the features of the metabolic syndrome (Despres et al., 1990). Visceral fat remains more strongly associated with an adverse metabolic risk profile even after accounting for the contribution of other standard anthropometric indices (Snijder et al., 2006). These systemic effects exerted by visceral adiposity are putatively involved in cancer biology (van Kruijsdijk et al., 2009) and are the focus of current research.

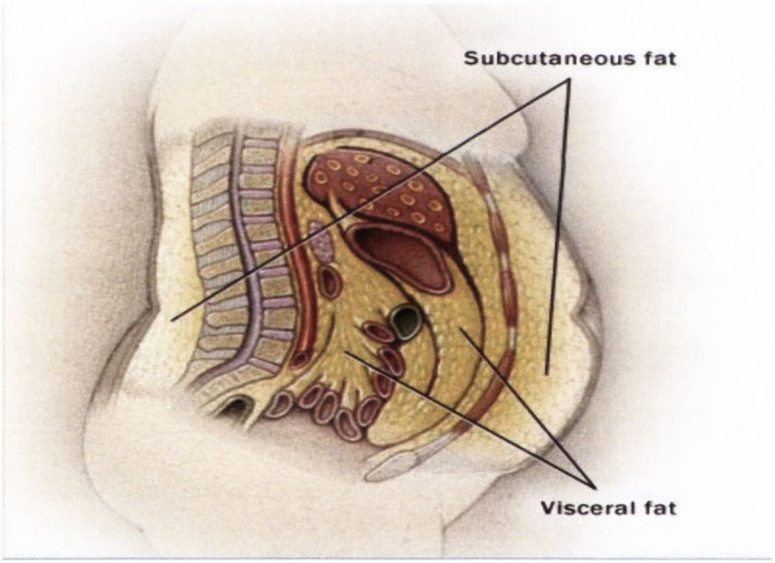


Figure 1.7: Distribution of adipose tissue: Adipose tissue is deposited in two compartments, centrally (visceral adipose tissue: including peri-organ, omental and mesenteric adipose tissue) and peripherally (subcutaneous adipose tissue). Image downloaded from http://www.mayoclinic.com/images/image_popup/w7_belly_fat.jpg; accessed on 18/01/12).

1.2.3 Measuring obesity

The World Health Organisation defines obesity as an abnormal or excessive fat accumulation in adipose tissue, to the extent that health is impaired. The classification of obesity for epidemiological purposes defines overweight as body mass index (BMI) greater than 25kg/m^2 and obesity as BMI greater than 30kg/m^2. (WHO, 2000) (Table 1.4).

Table 1.4: Methods of measuring obesity: Body Mass Index (BMI)

	BMI (kg/m^2)
Underweight	<18.5
Normal weight	18.5-25
Overweight	25-30
Obese class I	30-35
Obese class II	35-40
Obese class III	>40

It is important to note that within each category of BMI there can be substantial individual variation in total and visceral adiposity. This is also true within what is at present considered the 'normal' or 'healthy' range of BMI (18.5–24.9) (Lean et al., 1995). Commonly used BMI cut-off values to diagnose obesity have high specificity, but low sensitivity to identify adiposity, as they fail to identify those with excess body fat (Okorodudu et al., 2010). BMI does not distinguish lean tissue mass from adipose tissue and does not reflect adipose tissue distribution. (Hu, 2007, Wajchenberg, 2000a) Since the biologic activity of these fat compartments is distinct, discriminating between visceral and subcutaneous fat has important implications for disease (Balentine et al., 2010).

Measurement of abdominal fat area by computed tomography (CT) of the abdomen is an accurate technique for evaluation of the abdominal fat distribution in visceral and superficial fat areas (Beddy et al., 2010). Cross-sectional abdominal visceral and subcutaneous fat areas are measured using an established protocol; whereby a cross-sectional scan at the disc space between the L3 and L4 vertebral body is obtained using a radiograph of the skeleton as a reference (Beddy et al., 2010). Total abdominal adipose tissue area is calculated by delineating the surface with a graph pen and then computing the adipose tissue surface using an attenuation range of −50 to −150 Hounsfield units (Beddy et al., 2010). VFA is measured by drawing a line within the muscle wall surrounding the abdominal cavity. VFA corresponds to the surface inside the circumscription fulfilling the attenuation criteria of adipose tissue (Lemieux et al., 1996). SFA is calculated by subtracting the VFA from the total abdominal adipose

tissue area (Lemieux et al., 1996, Bray, 1988, Beddy et al., 2010). In a study published by Balentine *et al* in 2010, VFA and SFA were quantified by CT at the levels of L2-L3, L4-L5, and mid-waist; the results obtained were shown to be independent predictors for medical conditions known to be associated with obesity. The location used for imaging did not affect predictive power (Balentine et al., 2010). One of the limitations of this technique, however, is that it is not applicable in extremely obese subjects because of the weight limits for the table attached to the CT scanner (Yoshizumi et al., 1999).

Given its accuracy in determining the distribution of body fat, Computerised Tomography (CT) measurements of visceral adiposity are considered the gold standard, (Rossner et al., 1990) with waist circumference the next best proxy (Rankinen et al., 1999).

WC is the anthropometric index most commonly used for the prediction of visceral adipose tissue (Lemieux et al., 1996). An increased WC has been demonstrated to be a good predictor of cardiovascular disease, and is an important marker for the metabolic syndrome (Hans et al., 1995). On the basis of prior studies WC has been shown to directly reflect abdominal fat mass (Kashihara et al., 2009). Although WC is an inexpensive and practical screening tool for diagnosing central obesity and serves as a surrogate indicator for visceral fat, its inclusion of subcutaneous fat or skeletal muscle mass is problematic (Misra et al., 1997)

Table 1.5: Waist measurements as a predictor of risk to health, WHO (1998)

	Increased risk	Substantial risk
Caucasian men	> 94 cm (37 in)	> 102 cm (40 in)
Caucasian women	> 80 cm (32 in)	> 88 cm (35 in)
Asian men	No data	> 90 cm (38 in)
Asian women	No data	> 80 cm (32 in)

Several studies have indicated that assessment of abdominal adiposity by waist circumference or waist-hip ratio measurements are better indictors of obesity related morbidity and mortality than BMI (Pouliot et al 1994; Onat et al 2004; Price et al. 2006; Simpson et al 2007), while others have found no significant difference between these alternative measurements (Dalton et al. 2003; Flegal & Graubard, 2009). Waist circumference measurements are highly correlated with CT-measured visceral fat area in numerous studies (Pouliot et al., 1994, Lemieux et al., 1996, Han et al., 1997). (Figure 1.8)

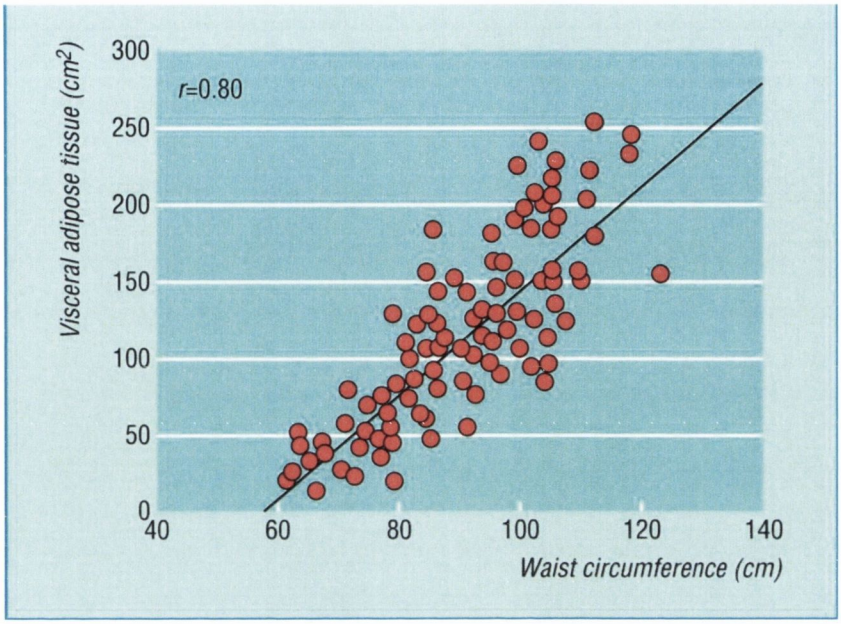


Figure 1.8 Waist circumference measurements are highly correlated with CT-measured visceral fat area. Figure from (Després et al., 2001); adapted from data in (Pouliot et al., 1994)

1.2.4 Metabolic alterations in visceral adipose tissue

The vascular anatomy and the metabolic activity of visceral fat may be the key factors predisposing to complications of obesity as only visceral adipose tissue is drained by the portal venous system and has a direct connection with the liver (Wajchenberg, 2000b). Mobilisation of free fatty acids (FFAs) is more rapid from visceral than from subcutaneous fat cells because of the higher lipolytic activity in visceral adipocytes, in both non obese but particularly in obese individuals, which probably contributes significantly to the FFA levels in the systemic circulation of obese individuals (Snijder

et al., 2006). The higher lipolytic activity in VFA in comparison with SFA tissue can be attributed to regional variation in the action of the major lipolysis-regulating hormones, catecholamines and insulin; the lipolytic effect of catecholamines being more pronounced and the anti-lipolytic effect of insulin being weaker in visceral than in subcutaneous adipose tissue (Wajchenberg, 2000b). In addition, specific proteins and hormones produced by omental and mesenteric adipose tissue, such as inflammatory molecules, angiotensinogen and cortisol can also contribute the systemic inflammatory condition associated with obesity.

1.2.5 Obesity and gastrointestinal cancer incidence

Epidemiological studies have provided convincing evidence for the association of obesity with cancer (Renehan et al., 2008b, Calle et al., 2003). The World Cancer Research Fund used a standardised approach to the analysis of the evidence and concluded that there is convincing evidence of association between obesity and oesophageal adenocarcinoma, pancreas, colorectum, breast (postmenopausal), endometrium and kidney. (Fund, 2007) Meta-analyses pertaining to obesity and the gastro-intestinal malignancies are summarised in Table 1.6. The International Agency for Research on Cancer working group estimated elevated body weight accounted for 11% of colon cancers and 39% of oesophageal adenocarcinoma. (Vainio H, 2002)

The largest meta-analysis to date includes 282,000 patients from prospective observational studies with over 133 million person-years of follow-up. (Renehan et al.,

2008b) This comprehensive analysis shows that high body mass index is associated with an increased incidence of many types of cancer. The association is modest with risk estimates of 1.1 to 1.6 per 5kg/m^2 incremental increase in BMI. This 5kg/m^2 increase in BMI corresponds to 15kg weight gain in men and 13kg in women with an average BMI of 23kg/m^2. Associations are sex specific and while there is an increased risk of colon cancer in men (1.24, $p<0.0001$ with increase of 5kg/m^2 BMI), a similar association between colon cancer was not found in women (relative risk <1.2). The risk of oesophageal adenocarcinoma was raised in both sexes (RR 1.52 and 1.51, $p<0.0001$ in men and women respectively) but there was a non-significant weak association between obesity and pancreatic or gastric cancer (Renehan et al., 2008b).

1.2.6 Obesity and cancer outcomes

It has been estimated that current patterns of overweight and obesity in the United States could account for 14% of all deaths from cancer in men and 20% in women (Calle et al., 2003). This is based on findings of a prospective study of 900,000 adults which found increased death rates from all cancer types in those of excess BMI, in both sexes. Those with a BMI greater than 40 had a death rate 52% higher for men and 62% higher in women when compared to those of normal weight. Table 1.7 summarises the relative risk of mortality according to BMI for both sexes.

Table 1.6: Meta-analyses of obesity and incidence of gastrointestinal cancers

Cancer type	Authors	Year	Incident cancers	BMI designated as obese	Design	Relative Risk (RR)	95% CI	P value
Oesophageal adeno-carcinoma	Renehan (Renehan et al., 2008b)	2008	2050	RR defined per 5kg/m2 increase in BMI	10 datasets	Men: 1.52 Women: 1.51	1.33-1.74 1.31-1.74	<0.0001 <0.0001
	Kubo & Corley (Kubo and Corley, 2006)	2006	2488	≥28	12 case control; 2 cohort	Overall: 2.4 Men: 2.4 Women: 2.1	2.0-2.8 1.9-3.2 1.4-3.2	<0.01 0.35 0.94
Gastric	Yang et al.(Yang et al., 2009)	2009	9492	≥30	10 cohorts	Non-cardia:1.26 Cardia: 2.06	0.89-1.78 1.63-2.61	0.2 <0.00001
	Renehan(Renehan et al., 2008b)	2008	1142	RR defined per 5kg/m2 increase in BMI	10 datasets	Men:0.97 Women: 1.04	0.88-1.06 0.9-1.20	0.49 0.56

Pancreatic	Larsson et al.(Larsson et al., 2007)	2007	8062	RR defined per 5kg/m2 increase in BMI	20 cohort, 1 nested case control	Men: 1.16 Women: 1.10	1.05-1.28 1.02-1.19	0.12 0.58
	Renehan(Renehan et al., 2008b)	2008	4443	RR defined per 5kg/m2 increase in BMI	16 cohort	Men: 1.07 Women: 1.12	0.93-1.23 1.02-1.22	0.33 0.01
	Berrington de Gonzalez et al.(Berrington de Gonzalez et al., 2003)	2003	6391	≥30	6 case control, 8 cohort	1.19	1.1-1.29	0.1
Liver (Hepato-cellular)	Larsson& Wolk(Larsson and Wolk, 2007c)	2007	5039	≥30	11 cohort	Men: 2.42 Women: 1.67	1.83-3.20 1.37-2.03	<0.001

	Renehan(Renehan et al., 2008b)	2008	2070	RR defined per 5kg/m2 increase in BMI	4 cohort	Men: 1.24 Women: 1.07	0.95-1.62 055-1.08	0.12 Only 1 study
Gallbladder	Larsson& Wolk(Larsson and Wolk, 2007b)	2007	3288	≥30	8 cohort, 3 case control	1.66	1.47-1.88	0.31
	Renehan(Renehan et al., 2008b)	2008	2039	RR defined per 5kg/m2 increase in BMI	4 cohort	Men: 1.09 Women: 1.59	0.99-1.23 1.02-2.47	0.12 0.04
	Bergström et al.(Bergstrom et al., 2001)	2001	273	Per unit increase in BMI	2 case control studies	1.06	1.0-1.12	
Colorectal	Harriss et al (C-CLEAR	2009	Colon: 43415	RR defined per 5kg/m2 increase in	26 cohort and 3 nested case	Men: colon: 1.24 Rectum: 1.09	1.2-1.28 1.05-1.14	0.121 0.497

	group)(Harriss et al., 2009)		Rectal: 23946	BMI	control	Women: colon: 1.09 Rectum: 1.02	1.04-1.12 0.99-1.43	0.007 0.054
	Guh et al.(Guh et al., 2009)	2009	Not reported	≥30	12 cohort	Men: 1.95 Women: 1.66	1.59-2.29 1.52-1.81	0.17 0.01
	Moghaddam (Moghaddam et al., 2007)	2007	69619	≥30	23 cohort, 8 case control	1.19 Men: colon:1.53 Rectum: 1.27 Women:colon: 1.09 Rectum: 1.02	1.08-1.30 1.33-1.75 1.17-.137 0.93-1.28 0.85-1.22	
	Renehan(Renehan et al., 2008b)	2008	67361	RR defined per 5kg/m2 increase in BMI	29 datasets	Men: colon: 1.24 Rectum:1.09 Women: colon:1.09 Rectum:1.02	1.20-1.28 1.06-1.12 1.05-1.13	<0.0001 <0.0001 <0.0001

							1.00-1.05	0.26
	Dai et al.(Dai et al., 2007)	2007	6458	Highest to lowest quintiles of BMI	15 cohort studies	Men: colon: 1.59	1.35-1.86	0.82
						Rectum:1.16		
						Women: colon:1.22	0.93-1.46	0.03
						Rectum:1.23	1.22	0.15
							0.98-1.77	0.93
	Bergström et al.(Bergstrom et al., 2001)	2001	3115	Per unit increase in BMI	4 cohort and 2 case control	Both sexes, colon only: 1.03	1.02-1.04	
	Larsson& Wolk(Larsson and Wolk, 2007a)	2007	Colon: 45116 Rectal: 22232	RR defined per 5kg/m2 increase in BMI	30 cohort and 1 case control	Men: colon: 1.3	1.25-1.35	0.13
						Rectum: 1.12	1.09-1.16	0.8
						Women: colon: 1.12	1.07-1.18	<0.001
						Rectum: 1.03	0.99-1.08	0.37

Table 1.7: Relative risk of cancer-related mortality according to BMI (adapted from Calle *et al.* 2003; published in Donohoe et al, 2010.)

	Body mass index (kg/m^2)				
	18·5–24·9	25·0–29·9	30·0–34·9	35·0–39·9	*P* for trend
Oesophageal cancer					
Men	1·0	1·15 (0·99, 1·32)	1·28 (1·00, 1·63)	1·63 (0·95, 2·8)	0·008
Women	1·0	1·2 (0·86, 1·66)	1·39 (0·86, 2·25)		0·13
Gastric cancer					
Men	1·0	1·01 (0·88, 1·16)	1·2 (0·94, 1·52)	1·94 (1·21, 3·13)	0·03
Women	1·0	0·89 (0·72, 1·09)	1·3 (0·97, 1·74)	1·08 (0·61, 1·89)	0·46
Pancreatic cancer					
Men	1·0	1·13 (0·97, 1·84)	1·41 (1·19, 1·66)	1·49 (0·99, 2·22)	<0·001
Women	1·0	1·11 (1·0, 1·24)	1·28 (1·07, 1·52)	1·41 (1·01, 1·99)	<0·001
Colorectal cancer					
Men	1·0	1·2 (1·12, 1·3)	1·47 (1·3, 1·66)	1·84 (1·39, 2·41)	<0·001
Women	1·0	1·1 (0·8, 1·31)	1·33 (1·17, 1·51)	1·36 (1·06, 1·74)	<0·001

There are some non-statistically significant differences amongst overweight patients for the common GI malignancies but these effects achieve significance and the magnitude of the relative risk increase is raised with increased BMI. This study by Calle *et al.*, 2003, is limited in that it only compares overall mortality rates after diagnosis with cancer regardless of stage. Survival rates may be influenced by differences in diagnosis, operative outcomes and treatment of cancer in the obese, as well as the true biological effect of adiposity on cancer progression. For example, data from our unit has shown that obesity is associated with increased respiratory complications and anastomotic leak rates in a cohort of patients who underwent oesophagectomy for cancer treatment (Healy et al., 2007). Such complications may impact on the oncologic outcome of surgery, as well as directly influence the mortality rate. However, other studies have also implicated obesity as an indicator of poorer prognosis from cancer, (Li et al., 2009, Meyerhardt et al., 2003, Haydon et al., 2006, Dignam et al., 2006) and even accounting for increased peri-operative mortality rates, there appears to be an additional effect by which obesity impacts on survival from cancer. Whether this is merely due to

differences in stage at diagnosis or whether adipose tissue plays a role in promoting cancer growth and metastasis has yet to be elucidated. Further study into the influence of obesity on cancer progression and response to treatment is warranted.

1.2.7 Visceral adiposity and cancer risk

There appears to a sex differential with respect to risk of cancer development with men having a higher risk of developing cancer at increased BMI than women (Moore et al., 2004, Renehan et al., 2008b). This may be due to the differing hormonal milieu in females or it may reflect the fact that BMI poorly reflects central adiposity in females. Since females generally only deposit central adipose tissue once total fat volumes are raised, overweight BMIs do not correspond with visceral fat volume in females as they do in males, which may account for the differences in cancer risk seen when BMI is used to determine obesity status.

In studies which use measures of visceral adiposity such as WC or VFA, visceral adiposity is associated with increased risk of cancer development (Moore et al., 2004, Wang et al., 2008, Steffen et al., 2009); is a stronger predictor of cancer risk than BMI (Moore et al., 2004) and the cancer risk is similar in males and females (Moore et al., 2004, Wang et al., 2008). Further, larger studies using measures of visceral adiposity across cancer sites are awaited in order to clarify whether there is a clear differential effect of visceral versus subcutaneous obesity.

Visceral adiposity (and not subcutaneous adiposity) is associated with development of features of the metabolic syndrome (taken as a proxy measure of a dysmetabolic profile in viscerally obese patients) (Donohoe et al., 2010). Most of the components of the syndrome, alone (Cowey and Hardy, 2006, Giovannucci, 2007) or in combination

(Colangelo et al., 2002, Bowers et al., 2006, Trevisan et al., 2001), have been individually link with cancer development at various subsites. A prospective international population-based study of 580,000 people (Me-Can Study) is underway to identify whether the metabolic syndrome is independently associated with cancer development (Stocks et al., 2010a). Initial findings suggest that a combination of components of the metabolic syndrome is associated with risk of colorectal cancer development (Men: RR: 1.25 (95% CI; 1.18-1.32; Women: RR 1.14 (95% CI; 1.02-1.18)(Stocks et al., 2010b), endometrial cancer (RR 1.37, 1.28-1.46) (Bjørge et al., 2010), bladder cancer in men (RR: 1.1, 1.01-1.18) (Häggström et al., 2010) and pancreatic cancer in women (RR,1.58; 1.34-1.87) (Johansen et al., 2010).

1.2.8 Obesity and oesophageal adenocarcinoma

Obesity has been strongly implicated in the pathogenesis of oesophageal adenocarcinoma. (Calle et al., 2003, Renehan et al., 2008b, Ryan et al., 2006, Lagergren et al., 1999b, Chow et al., 1998, Vaughan et al., 1995, Brown et al., 1995, Engel et al., 2003) The association is stronger than for most other solid tumours (Renehan et al., 2008b, Calle et al., 2003) (see Figure 1.9).

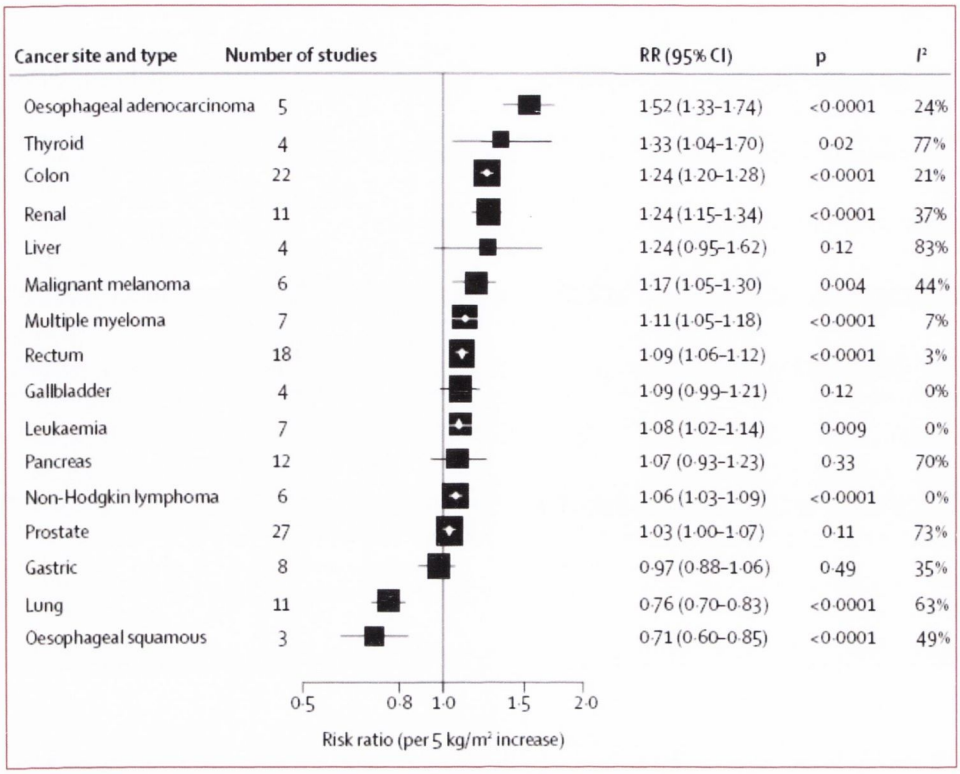


Figure 1.9.1: Association between body mass index and cancer incidence: Summary risk estimates by cancer sites for men (Renehan et al., 2008b).

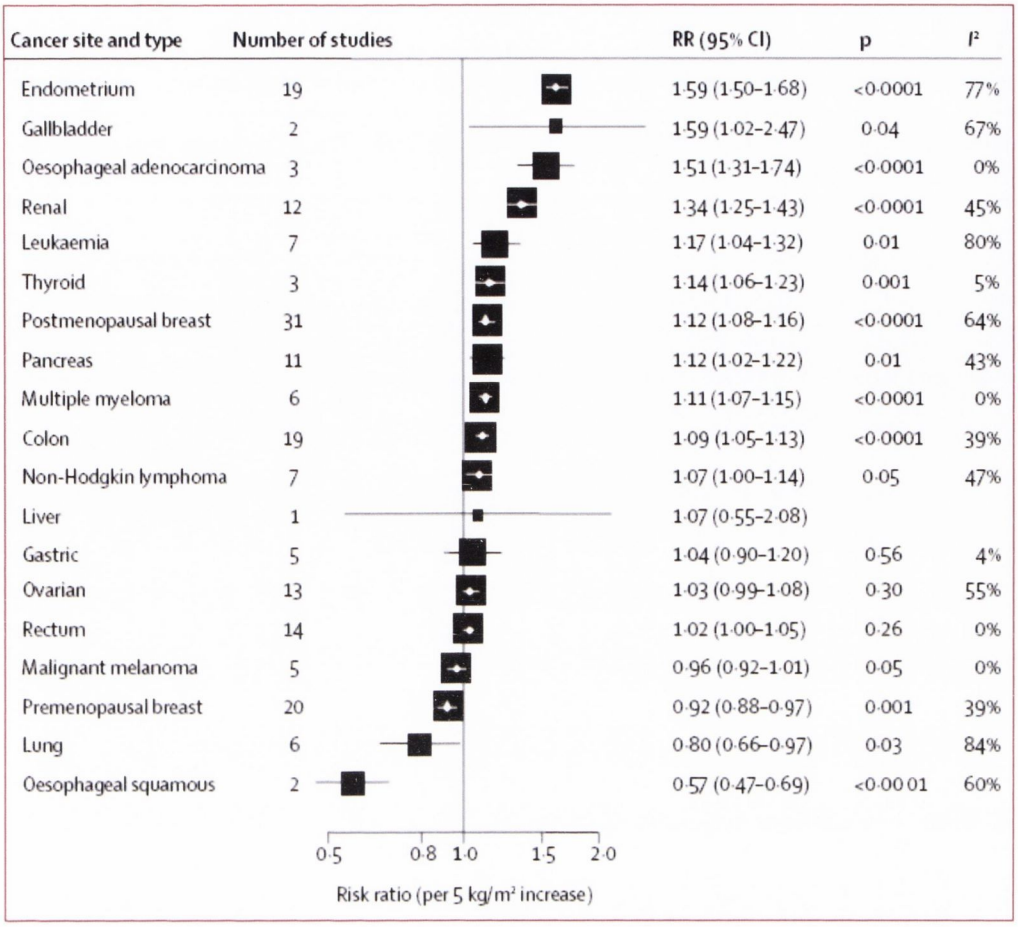


Figure 1.9.2: Association between body mass index and cancer incidence: Summary risk estimates by cancer sites for women (Renehan et al., 2008b).

The rising incidence of oesophageal adenocarcinoma parallels that of the obesity epidemic (Enzinger and Mayer, 2003). Centrally located fat, independent of overall BMI is associated with increased risk of developing gastro-oesophageal reflux, Barrett's oesophagus and OAC (Lagergren et al., 1999a, Corley et al., 2007a). Intuitively, the raised intra-abdominal pressure due to visceral adiposity places mechanical pressure on the stomach to increase reflux. However, after adjustment for gastro-oesopahgeal reflux , rates of OAC remain increased amongst those with obesity. Certainly, Barrett's

oesophagus seems to be more closely correlated with increased waist circumference (Corley et al., 2007b, Edelstein et al., 2007, Cook et al., 2008, Akiyama et al., 2009) than raised body mass index (Cook et al., 2008).

There have been three prospective studies into the association between abdominal obesity and OAC. A prospective cohort study (Steffen et al., 2009) using the European Prospective Investigation into Cancer and Nutrition (EPIC) population revealed positive associations between OAC risk and BMI and waist circumference (highest versus lowest quintile; relative risk (RR), 2.60; 95%CI 1.23-5.51; p trend<0.01; RR, 3.07; 1.35-6.98; p trend <0.01 respectively). On multivariate analysis, neither factor was significant although there were only 88 incidence cases of OAC in the cohort and thus the sample size is probably too small to comment on whether abdominal obesity is an independent predictor of risk above BMI alone.

In an Australian cohort study there was a significant positive association with waist circumference (MacInnis et al., 2006). In a nested case-control study, increased abdominal diameter was associated with higher OAC risk even after adjustment for BMI (Corley et al., 2008).

A recognised precursor to adenocarcinoma is specialised intestinal metaplasia or Barrett's Oesophagus. Patients with Barrett's changes have a 30-to 40 fold increase risk of developing adenocarcinoma of the oesophagus. Patients develop a transformation of the normal stratified squamous epithelium of the oesophagus into columnar intestinal epithelium characteristic of the stomach lining (Reid et al., 2010). The existing consensus is that long-standing acid and bile reflux leads to chronic inflammation, triggering a metaplasia-dysplasia sequence. Not all patients with chronic gastro-oesophageal reflux disease develop Barrett's.

It is known that Barrett's patients are more likely to be obese and one proposed mechanism for this association is that of increased acid reflux in patients with high levels of visceral adiposity (Lagergren et al., 1999a),(Corley et al., 2007a). However, a study from our Unit found that patients with Barrett's oesophagus were more likely to have features of the metabolic syndrome and central adiposity, particularly if they had long segments of specialised intestinal metaplasia (Ryan et al., 2008). This raises the possibility that visceral fat plays an additional role in the pathogenesis of Barrett's beyond simple reflux. A prospective study of Nurses' Health Study cohort revealed an OR for the development of Barrett's oesophagus of 1.52 (1.02-2.28) in women of BMI>30 after controlling for symptoms of reflux (Jacobson et al., 2009). There was no increase for women who had overweight category BMI and women only tend to have significant visceral fat deposits once they are within the obese category of BMI as opposed to men, who are at an increased risk of Barrett's when both overweight and obese, but who also deposit visceral fat in preference to subcutaneous fat.

Amongst symptomatic refluxers, men are twice as likely to have Barrett's oesophagus (Cook et al., 2005) and 5-8 times more likely to develop OAC than women (El-Serag et al., 2002).

1.2.9 Obesity as a causative factor: reversibility of cancer risk with weight loss

According to Hill's postulates (Hill, 1965), for a correlation to assume a causal role in the pathogenesis of a disease, there needs to be strength of association, coherence, biological plausibility, consistency across populations, specificity, temporality and biologic gradient. The aspect of biologic plausibility may be partially demonstrated experimentally by observing the effect of weight loss on cancer incidence.

Although cancer incidence is clearly increased in obese individuals, it is not clear whether weight loss decreases cancer incidence. There is limited evidence that intentional weight loss may reduce morbidity and mortality from cancer, particularly in women (Williamson et al., 1995, Williamson et al., 1999, Parker and Folsom, 2003). Since few obese patients manage to lose weight (Levy et al., 2007) and maintain significant amounts of weight loss in the long-term (Kassirer and Angell, 1998), it is difficult to design adequately powered interventional studies. Observational studies are hampered by the fact that the disease process may affect weight and thus, intentional weight loss, as a means of reducing cancer incidence, may be masked by increased incidence of cancer in those who have lost weight.

Since direct evidence for the efficacy of weight loss in reducing the risk of cancer development is lacking, other models have been used to assess the impact of weight loss on cancer incidence. One such model is the difference in cancer incidence in bariatric surgery patients versus matched obese controls (Sjostrom et al., 2009, Christou et al., 2008, Adams et al., 2009). The largest of these studies compared 6596 patients who had gastric bypass with 9,422 matched obese controls.(Adams et al., 2009) Over a follow-up period of 24 years, total cancer incidence was lower in the surgery group (HR=0.76;CI 95%: 0.65-0.89, p=0.0006). Bariatric surgery had a significant effect on cancer incidence in women (HR 0.73; p=0.0004) but not in men (HR 1.02; p=0.91). Interestingly, this sex difference has been replicated in other studies (Sjostrom et al., 2009). Cancer mortality was 46% lower in surgery patients although it was not clear whether this was due to differences in diagnosis, treatment or responses to treatment in surgery patients versus the obese controls (Sjostrom et al., 2009). Since reduction in cancer incidence following bariatric surgery is different for men and women and the

reduction is independent of the amount of weight lost, it appears that reduction of cancer risk associated with bariatric surgery is mediated by mechanisms other than weight loss alone.

1.3 Obesity and carcinogenic mechanisms

Despite convincing epidemiological data implicating obesity as an aetiological factor in cancer, the underlying mechanisms are not yet clear. Long thought of as inert, adipose tissue, particularly visceral fat, (Wajchenberg, 2000a) is an important metabolic tissue which secretes factors which alter the immunological, metabolic and endocrine milieu and promote insulin resistance (Kershaw and Flier, 2004).

The obese state may be thought of as a pro-tumourigenic environment which can act to facilitate tumour development by promotion of the acquisition of some of the hallmarks properties that characterise cancerous lesions (Figure 1.10) (Hanahan and Weinberg, 2000, Mantovani, 2009).

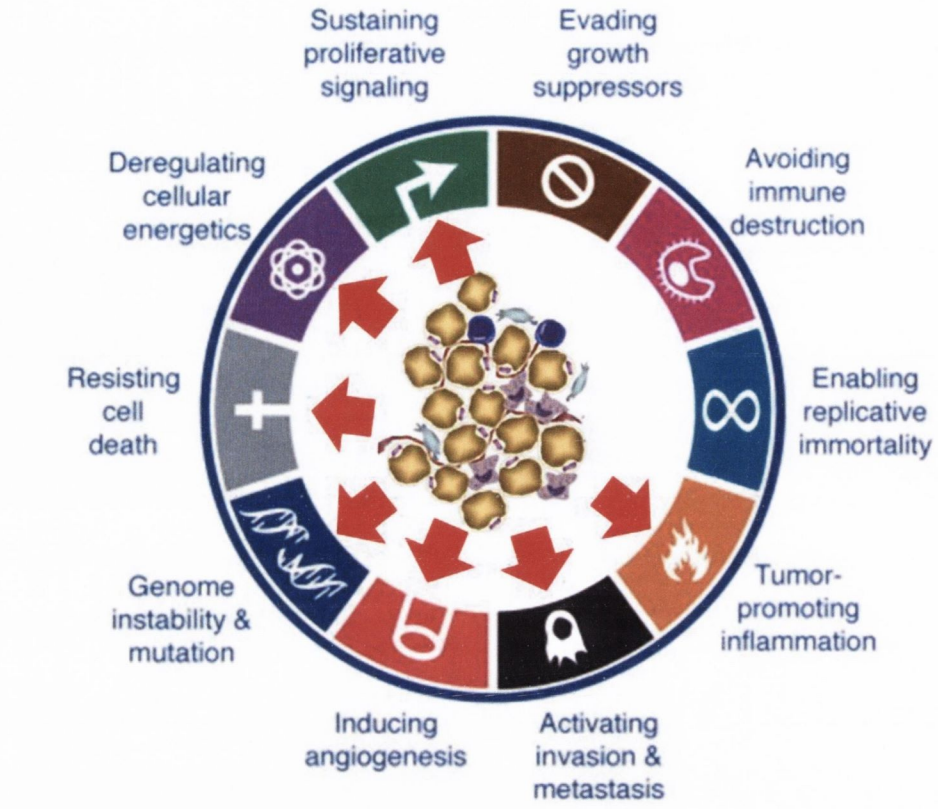


Figure 1.10 Hallmarks of cancer and potential role of visceral obesity in tumourigenesis. Hanahan and Weinberg, 2000 proposed six hallmarks that define properties that tumours acquire in order to maintain a malignant phenotype. Mantovani (Mantovani, 2009) proposed that addition of a seventh property: an inflammatory microenvironment. In 2011, a further two hallmarks were added including genomic instability and mutation, and deregulating cellular energetics (Hanahan and Weinberg, 2011). Adipose tissue through the systemic alterations associated with obesity may support the development of malignant potential in susceptible cells through supporting invasion and metastases, evasion of apoptosis, promotion of angiogenesis, genomic instability and systemic inflammation. Adapted from Hanahan and Weinberg,

1.3.1 Paracrine mechanisms of obesity and tumourigenesis

Adipose tissue may act in both a paracrine and systemic manner. At a local level, adipose tissue is involved in a number of mechanisms which may promote tumour development (Figure 1.11). Obese mice have reduced levels of oxygen within their epididymal adipose tissue (Ye et al., 2007). The tumour microenvironment in solid tumours is often characterised by low oxygen tensions and hypoxia within the peri-tumoural fat may promote tumour-site hypoxia. Hypoxia upregulates the hypoxic-inducible factor (HIF-1α) which can lead to altered expression in over 60 target genes involved in angiogenesis, glycolysis, cell proliferation and apoptosis, leading to cellular adaptation to low oxygen conditions (Vaupel, 2004). Hypoxia has been associated with metastasis and poor prognosis (Zhong et al., 1999) and also induces pro-angiogeneic and inflammatory cytokine secretion (Trayhurn and Wood, 2004, Feldser et al., 1999, Fukuda et al., 2002).

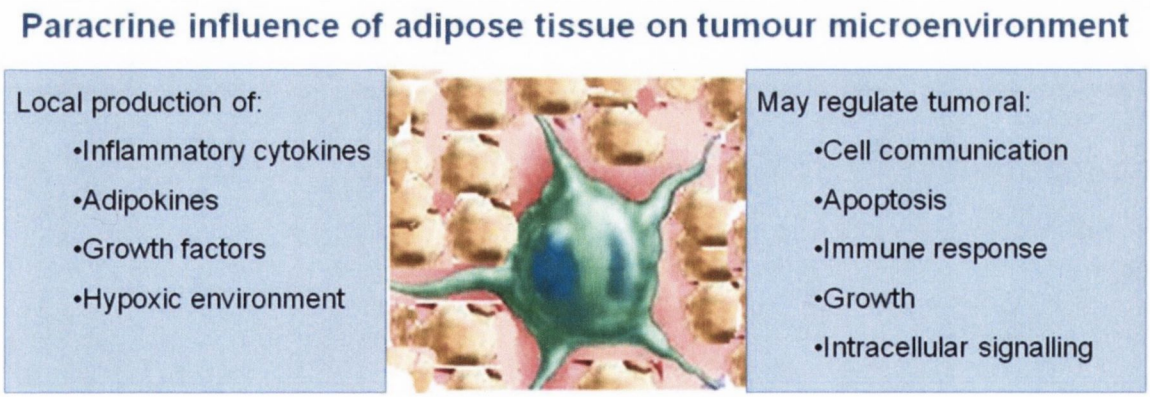


Figure 1.11: Paracrine mechanisms linking adipose tissue and cancer development

Local production of inflammatory cytokines, adipokines, growth factors and a hypoxic environment by peri-tumoral adipose tissue may be implicated in the development of a pro-tumorigenic microenvironment by altering tumour cell communication, apoptosis, immune response, growth and intracellular signalling. Reproduced from Donohoe et al., (2011a).

Inflammatory cytokines produced in adipose tissue can upregulate nuclear factor-κB (NFκB) which leads to an increase in nitric oxide (NO), a substrate for reactive oxygen species (ROS). Cytokines and ROS can contribute to insulin resistance and the resultant excess circulating glucose, free fatty acids and insulin can further induce inflammation (Sonnenberg et al., 2004). The key inflammatory pathways NFκB and STAT3 are activated by adipose tissue products leading to transcription of genes which mediate proliferation, invasion, angiogenesis, survival and metastasis (Aggarwal et al., 2009b).

The observation that epithelial tumour cell growth is enhanced by injection into fat pads rather than subcutaneously (Elliott et al., 1992) supports the hypothesis that chemokine production within the adipose tissue provides conditions which enhance tumour cell growth, although the potential mechanism(s) underlying the observation were not studied in detail in this paper. A proteomic study of mammary fat revealed the production of a wide variety of proteins involved in diverse processes such as cell communication, growth, immune response, apoptosis and numerous signalling molecules including hormones, cytokines and growth factors (Celis et al., 2005).

While the fat which surrounds individual organs may act in a paracrine manner to influence tumour development or progression, this mechanism has been less fully investigated and most research efforts have concentrated on systemic alterations in obesity and how these may influence cancer development and progression. Adipokine production and inflammatory alterations in the obese state described in detail hereafter may act to influence tumourigenesis in either a systemic or paracrine manner or a combination of both.

1.3.2 Obesity and Systemic mechanisms of tumourigenesis

Systemic alterations in obesity include chronic systemic inflammation, increased adipokine production and an altered immunological status (Figure 1.12). Additionally, there are associated changes in the sex hormone profile. Insulin resistance develops as a consequence of visceral adiposity and there is a rise in insulin production, which may be associated with activation of the insulin-like growth factor (IGF) system. All of these changes which occur in tandem with the development of obesity have the ability to interact with each other. It is this altered systemic milieu which is thought to fuel cancer development and progression.

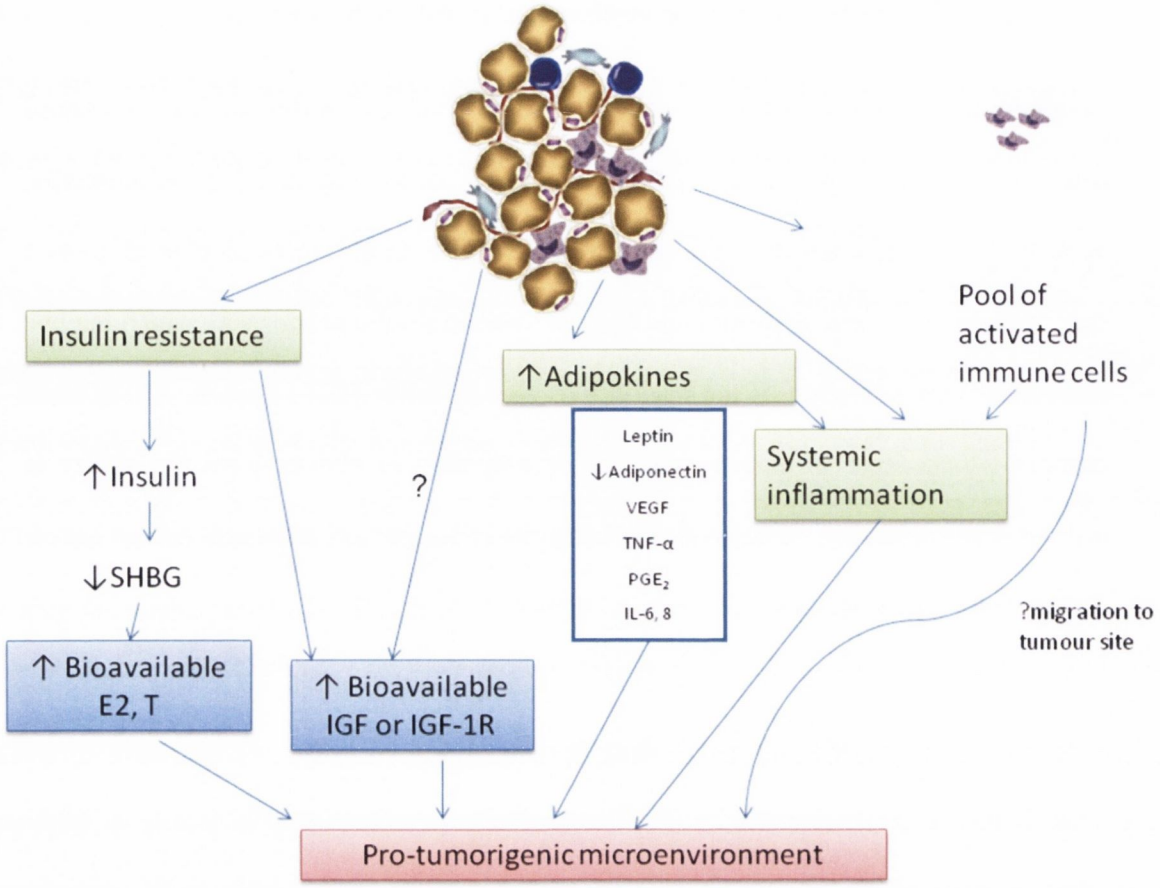


Figure 1.12: Systemic alterations associated with visceral obesity. These potential mechanisms include insulin resistance, increased adipokine production and systemic inflammation. There is also an alteration in bioavailable sex hormones (Estradiol and testosterone) and the Insulin-like growth factor axis. These systemic factors associated with visceral obesity may create a pro-tumourigenic microenvironment. Reproduced from Donohoe et al., (2011a).

?= Area of controversy (see main text)

Abbreviations: E2: estradiol, T: testosterone; VEGF: Vascular Endothelial Growth Factor; TNF-α: Tumour Necrosis Factor-alpha; PGE2: Prostaglandin E2; IL-6, -8: Interleukin -6, -8; IGF: insulin-like growth factor; IGF1R: insulin-like growth factor receptor.

1.3.2.1 Visceral adiposity and insulin resistance

Excess adipose tissue is associated with raised free fatty acids and insulin. Nutritionally induced insulin resistance develops as a metabolic adaptation to increased circulating levels of free fatty acids (FFAs), which are constantly released from adipose tissue, especially from visceral fat stores (Calle and Kaaks, 2004). Increased FFA levels force liver muscle and other tissues to shift towards increased storage and oxidation of fats for their energy production (Bergman and Ader, 2000). The compensatory effect is a reduced capacity of these tissues to absorb, store and metabolise glucose. In addition to increased FFA levels, high concentrations of cytokines produced by adipose tissue, such as TNF-α, interleukin (IL) 6 and IL-1β, and low concentrations of adiponectin have deleterious effects on glucose homeostasis leading to chronic hyperinsulinaemia and insulin resistance (Greenberg and McDaniel, 2002b). The cellular and molecular mechanisms leading to insulin resistance includes a reduction in cellular insulin-receptor levels and reduced responsiveness of some intracellular transduction pathways mediating the effects of insulin binding to its receptor (Moller and Flier, 1991). Insulin resistance leads to increased insulin production and insulin can act as a mitogen and has been associated with several cancers. The tumourigenic effects of insulin could be directly mediated by insulin receptors in the pre-neoplastic target cells, or might be due to related changes in endogenous hormone metabolism, secondary to hyperinsulinaemia (Calle and Kaaks, 2004).

Epidemiological evidence has implicated insulin resistance in the development of colorectal cancer. Colon cancer risk is increased in those who consume a diet low in fruit, vegetables and fibre and high in refined carbohydrates. It was thus proposed that

this effect was mediated by insulinaemia. Specifically, it was proposed that high levels of insulin, such as in those with insulin resistance and type 2 diabetes mellitus, was related to the development of cancer (Giovannucci, 1995). Cohort studies have demonstrated increase risk of colorectal cancer in those with insulin resistance (Trevisan et al., 2001, Colangelo et al., 2002, Schoen et al., 1999), the metabolic syndrome (Ahmed et al., 2006) and type 2 diabetes (Larsson et al., 2005). Interestingly, the risk of colorectal cancer in type 2 diabetic patients treated with insulin appears to be even higher (Yang et al., 2004).

Levels of adipose tissue affect the body's handling of glucose (Brochu et al., 2000) and adipokines are thought to be involved in the pathogenesis of insulin resistance (Fasshauer and Paschke, 2003, Rajala and Scherer, 2003). High concentration of cytokines produced by adipose tissue, such as TNF-α, IL-6, IL-1β, and low concentrations of adiponectin have deleterious effects on glucose homeostasis leading to chronic hyperinsulinaemia and insulin resistance in Type 2 Diabetes Mellitus (Greenberg and McDaniel, 2002a, LeRoith, 2002).

1.3.2.2 Chronic inflammation and adipokines

Adipocyte-conditioned media can promote tumourigenesis in cancer cells, by increasing cell proliferation, invasive potential, angiogenesis and induction of cross-talk between cancer cells and the surrounding extracellular matrix (Iyengar et al., 2003). This is thought to be mediated by production of biologically active proteins such as adiponectin, leptin and numerous cytokines including TNF-α, IL-6, IL-8, IL-10 and IL-1 receptor agonist (IL-1Ra) (Tilg and Moschen, 2006). These secreted proteins can be collectively termed adipokines.

Cytokines secreted by adipose tissue include: TNF-α, IL-6, IL-8, IL-10, IL-1 receptor agonist (IL-1Ra), macrophage inflammatory protein-1 (MIP-1) and monocyte chemoattractant protein-1 (MCP-1) (Table 1.8). It has been suggested that hypoxia in adipose tissue induces the secretion of these factors in order to promote angiogenesis (Trayhurn and Wood, 2004, Feldser et al., 1999, Fukuda et al., 2002). Inflammatory cytokines can further upregulate NFκB which leads to an increase in nitric oxide (NO), a substrate for reactive oxygen species (ROS). Cytokines and ROS can contribute to insulin resistance and the resultant excess circulating glucose, free fatty acids and insulin can further induce inflammation (Sonnenberg et al., 2004). The key inflammatory pathways NFκB and STAT3 are activated by adipose tissue products leading to transcription of genes which mediate proliferation, invasion, angiogenesis, survival and metastasis (Aggarwal et al., 2009b). Vascular endothelial growth factor (VEGF) has become a novel therapeutic target, particularly in the treatment of colorectal cancer (Kerbel and Folkman, 2002). This pro-angiogenic factor is produced by adipose tissue and circulating levels have been correlated with visceral fat versus subcutaneous fat (Miyazawa-Hoshimoto et al., 2003, Hausman and Richardson, 2004, Silha et al., 2005).

Excess adipose tissue results in elevated levels of pro-inflammatory adipokines, resulting in an imbalance between increased inflammatory stimuli and decreased anti-inflammatory mechanism leading to persistent low-grade inflammation (Esposito and Giugliano, 2004, Wajchenberg, 2000a, Das, 2001). The level of adipokine production from adipose tissue is strongly influenced by immune cell populations present in adipose tissue (Kershaw and Flier, 2004, Schaffler et al., 2006, Xu et al., 2003, Weisberg et al., 2003). Adipose tissue in obese people is infiltrated with macrophages

and the number of macrophages correlates with the degree of adiposity (Neels and Olefsky, 2006) (Figure 1.13).

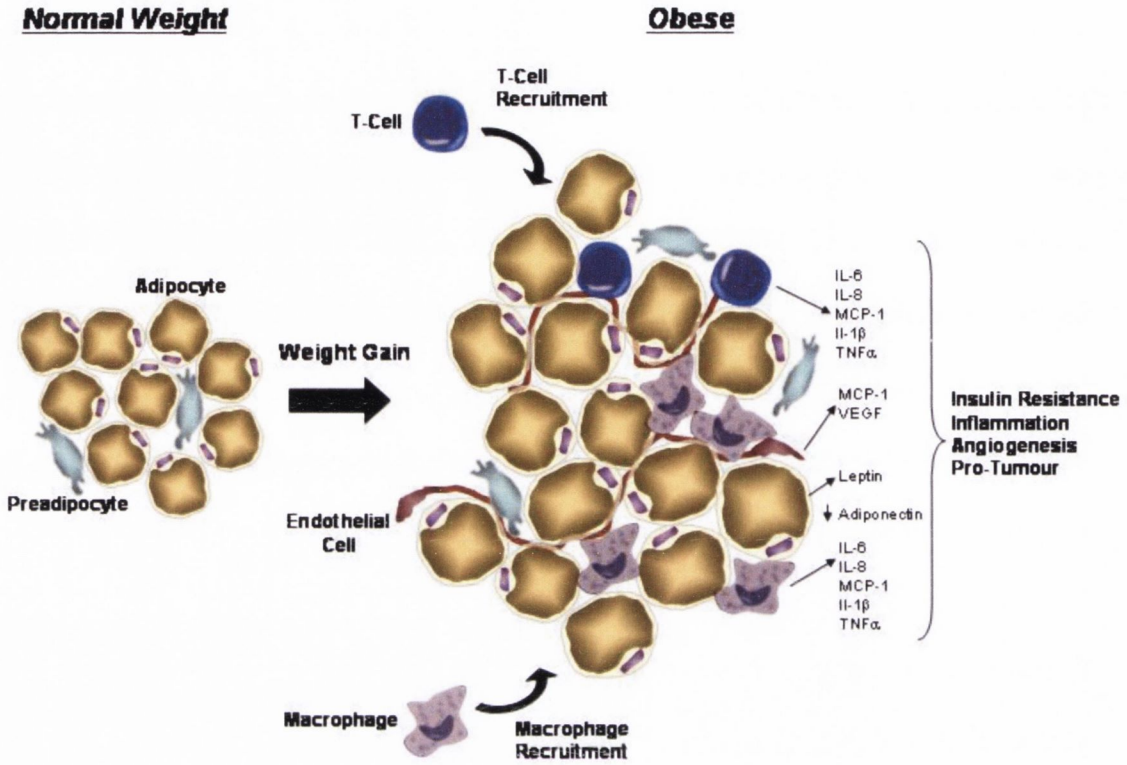


Figure 1.13 Altered immunological profile of visceral adipose tissue in obesity

In obese patients, adipose tissue becomes inflamed. Adipocytes become hypertrophied and the tissue becomes infiltrated with macrophages and T cells. Together these cells act to produce adipocytokines which are involved in the development of inflammation, insulin resistance and conditions which favour angiogenesis and tumour proliferation. Reproduced from Donohoe et al., (2010).

Peripheral monocytes are recruited by monocyte chemoattractant protein (MCP)-1 and TNF-α, and can differentiate into activated macrophages (Curat et al., 2004). Pre-adipocytes also have the ability to differentiate into macrophages (Schaffler et al., 2006). The products of activated macrophages can impact on adipocyte function and are postulated to be involved in altering adipose tissue glucose handling and thus contribute

to insulin resistance (Sartipy and Loskutoff, 2003, Wellen and Hotamisligil, 2003). Research has shown that co-culture of adipocytes with macrophage-conditioned media causes increased adipokine and inflammatory cytokine production by adipocytes (Bassols et al., 2009), further supporting this hypothesis.

Insulin can modulate adipokine production and interacts with two of the most abundant adipokines: leptin and adiponectin. Insulin is a positive regulator of leptin and increases its gene expression to suppress appetite (Yamauchi et al., 2001). Adiponectin acts as an insulin sensitising agent (Yamauchi et al., 2001). In addition to modulation of insulin sensitivity, these adipokines can directly affect tumour cells (Schaffler et al., 2005). Adiponectin is anti-tumour: it increases apoptosis (Dieudonne et al., 2006), inhibits proliferation, inflammation and angiogenesis (Brakenhielm et al., 2004) and can prevent the interaction of growth factors with their receptors (Wang et al., 2007b). There is a consistent inverse relationship with cancer incidence and circulating adiponectin levels (Roberts et al., 2010). The pro-tumour effects of leptin are the direct opposite of those of adiponectin (Roberts et al., 2010), although the epidemiological association between circulating levels and cancer risk is less consistent (Renehan et al., 2008a). Circulating levels of leptin positively correlate and adiponectin levels negatively correlate with all measures of obesity (BMI, WC and visceral fat area) (Kadowaki and Yamauchi, 2005, Cummings and Foster, 2003, Howard et al.).

Thus altered adipokine production by adipose tissue, and in particular inflamed visceral adipose tissue, may influence the tumour microenvironment. Obese rat models have increased inflammatory transcription factor expression (TNF-α and NFκB) in their tumours (Jain and Bird, 2010). Furthermore, there is emerging evidence that adipose stromal cells may be a source of stromal cells in tumour microenvironments. In animal

models of obesity, adipose stromal cells and adipose endothelial cells from inflamed visceral adipose tissue migrate to tumour sites (Zhang et al., 2009). Stromal cells in the tumour microenvironment promote angiogenesis and support tumour progression (Coussens and Werb, 2002). There is also evidence that visceral adiposity can influence a patient's treatment outcome, with a study demonstrating increased visceral fat area to be an independent predictor of outcome after first-line bevacizumab treatment in colorectal cancer (Guiu et al., 2010). This finding indicates that angiogenic factors produced by visceral fat may influence tumour progression and response to chemotherapy and is mirrored in animal models as adiponectin, which is reduced in visceral obesity, inhibits tumour growth by reduced neovascularisation (Brakenhielm et al., 2004) . The mechanism(s) for this resistance may uncover important information on how obesity influences the tumour microenvironment.

1.3.2.3 Immunomodulation

While the immune system plays a fundamental role in anti-tumour immunity, under certain circumstances, it can also aid tumour development and progression. The most abundant subsets of infiltrating immune cells within tumours are lymphocytes and macrophages. The density of tumour associated macrophages (TAMs) is correlated in most studies with increased angiogenesis, tumour invasion and poor prognosis (Balkwill, 2004). TAMs produces a large number of tumour promoting factors – including growth factors, matrix metalloproteases and angiogenic factors, such as VEGF. Interestingly, they also produce factors which can suppress adaptive immunity (Sica et al., 2008). That is, they attract T cell subsets, however, these T cells do not have cytotoxic functions. It is thought that TAMs are derived from circulating monocytes, which are attracted to the tumour site by local production of chemokines (Pollard,

2004). TAMs often accumulate in necrotic areas of tumours, which are characterised by low oxygen tension and it is thought that hypoxia-induced factors, such as HIF-1α, VEGF, CXCL12 and CXCR4, result in TAM differentiation at the tumour site (Lewis and Murdoch, 2005, Talks et al., 2000, Knowles and Harris, 2007).

Broadly-speaking, macrophages can be classified as M1 or classically activated macrophages which produce toxic intermediates capable of killing intracellular microbes and tumour cells (Mantovani et al., 2006). M2 or alternatively activated macrophages have anti-inflammatory properties and hence are considered pro-tumour (Figure 1.14). M1 macrophages upregulate inflammation through production of pro-inflammatory cytokines and promote T helper 1 cell differentiation, important in the anti-tumour response. M2 macrophages are activated by T helper 2 cytokines and act to down-regulated inflammation by production of IL-10 and suppression of Type 1 adaptive immunity (Mantovani et al., 2004). They have poor antigen presenting capacity and instead promote wound healing via angiogenesis and tissue remodelling (Mantovani et al., 2002).

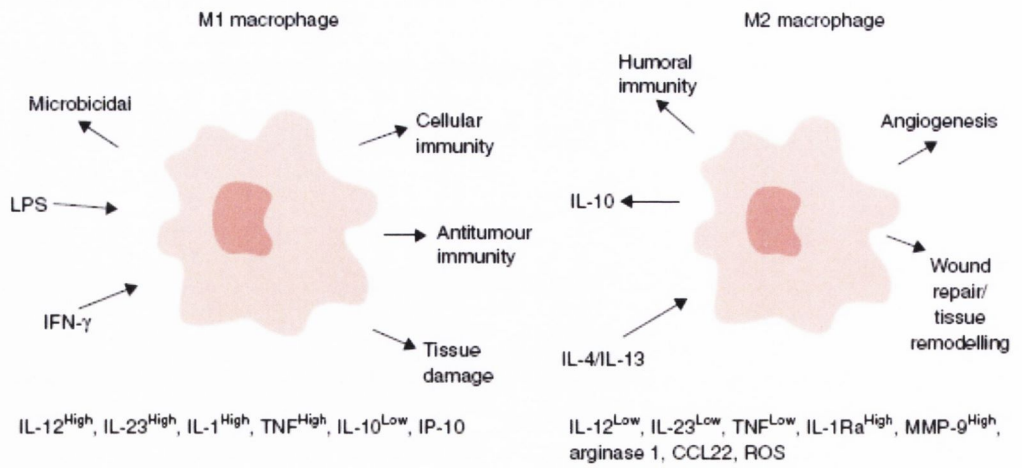


Figure 1.14: Characterisation of M1 and M2 macrophages. "Classically activated" or M1 macrophages are activated upon stimulation with IFN-γ followed by a microbial danger signal such as lipopolysaccharide (LPS). Classically activated macrophages secrete inflammatory cytokines such as IL-12, important for the generation of Th1 immune response and are important in cellular mediated immunity and the anti-tumour immune response. "Alternatively activated" or M2 macrophages are activated by IL-4 or IL-13 and are characterised by poor antigen presenting properties, high IL-10 and arginase-1 production. M2 macrophages play an important role in angiogenesis, tissue remodelling and wound repair and are also thought to play a role in tumour promotion and progression. Abbrev. MMP-9; matrix metalloproteinase-9, TNF; tumour necrosis factor, IP-10; Interferon inducible protein-10, IL-1Ra; Interleukin-1 Receptor antagonist. Reproduced from (Donohoe et al., 2010).

Polarisation of macrophages into M1 and M2 subtypes is directly influenced by cytokines produced by tumour cells. Thus tumours can recruit macrophages and induce them to differentiate into the M2 subtype in order to help them evade immune destruction and to promote angiogenesis. An intriguing hypothesis, which would link obesity to immune cells involved in tumour immunity, is that visceral adipose tissue represents a pool of activated macrophages which can be released from adipose tissue as circulating monocytes and can then infiltrate tumours, promoting their growth. Multiple mouse models of obesity have demonstrated that mesenchymal stromal cells from adipose tissue can be recruited to tumours and may develop into tumour stromal cells

(Zhang et al., 2009). If a similar recruitment process could be demonstrated for adipose tissue macrophages, an alternative means by which adipose tissue influences tumour development and progression could be developed. It is known that adipose tissue macrophages are of the M2 (alternatively activated) phenotype (Mayi et al., 2012, Orr et al., 2012).

Obesity is also associated with an alteration in the function of circulating immune cells. Studies have found decreased T- and B-cell function, increased monocyte and granulocyte phagocytosis and oxidative burst, and raised total leukocyte counts (Nieman et al., 1999, Mendall et al., 1997). Circulating mononuclear cells from obese subjects have been shown to exhibit increased nuclear factor b (NFB) nuclear binding with decreased levels of NFκB inhibitor and increased mRNA expression of IL-6, TNF-α and migration inhibition factor. Markers of macrophage activation correlate with plasma levels of FFAs (Ghanim et al., 2004). Hyperlipidaemia in mice mediates an inflammatory response by signalling through a receptor complex comprising Toll like receptor-4, CD14 and MD-2 to activate the innate immune system (Bjorkbacka et al., 2004).

Members of the IGF-1 family are expressed in immune cell populations (Oberlin et al., 2009) and tumour associated macrophages are a source of IGF-1 (Sunderkotter et al., 1994, Kodelja et al., 1997, Kopfstein and Christofori, 2006). A study previously carried out in this unit demonstrated an increase in $CD68^{+}$ cells, a cell surface macrophage marker, in the stromal tissue surrounding the invasive edge of oesophageal tumours (Figure 1.15) (Doyle et al., 2011). Furthermore, there was increased expression of the insulin-like growth factor receptor in oesophageal adenocarcinoma tumour cells at the

invasive edge (Figure 1.16). These data may indicate that there is a paracrine mechanism involving interplay between tumour-associated macrophage-derived IGF-1 and tumour cells which express IGF1R. In this preliminary study, there were no associations between IGF1R expression and survival – possibly related to the small number of tumours analysed.

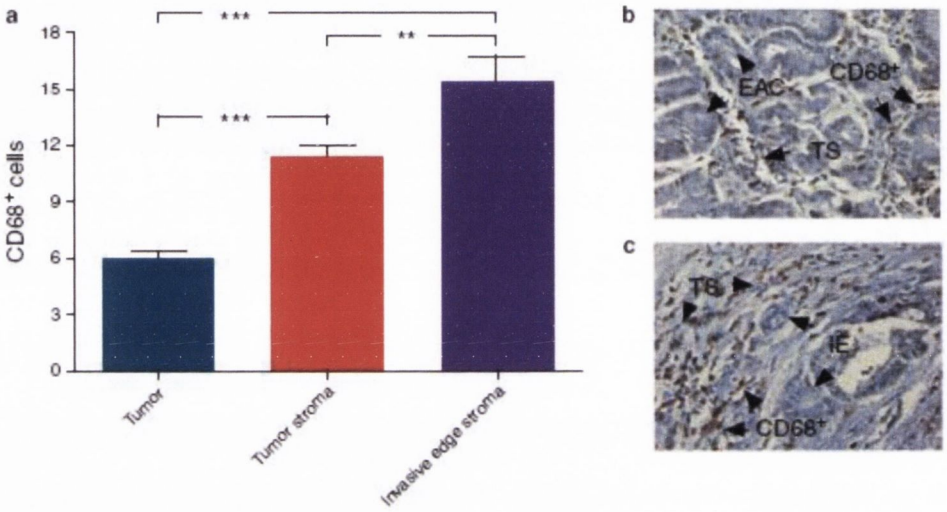


Figure 1.15: Increased CD68$^+$ cell numbers are present in the tumour-associated stroma compared with the tumour. There was a significant variation in CD68$^+$ cell numbers based on location within tumour tissue with increased numbers in tumour stroma (TS) and invasive edge (IE) tumour stroma.

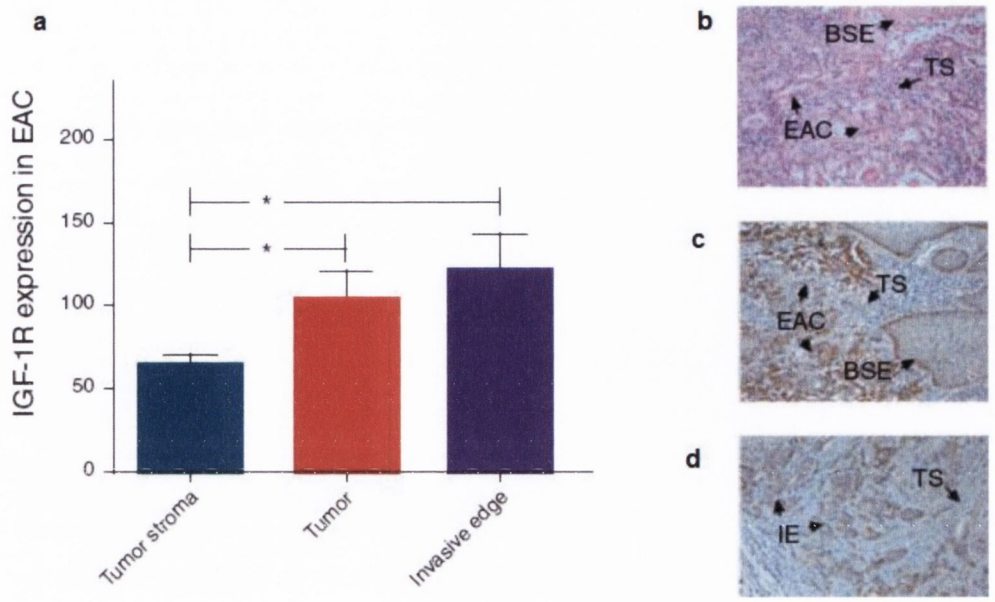


Figure 1.16: IGF1R expression is highest at the invasive edge in oesophageal adenocarcinoma (labelled EAC); Representative images of (b) haematoxylin and eosin section, (c) IGF1R staining of tumour and (d) invasive tumour edge, are shown. Data represent mean overall scores ± SEM; * $p<0.05$.

1.3.2.4 Sex hormones

Epidemiological studies have suggested a difference in the influence of obesity on cancer development between men and women (Calle et al., 2003). While some studies are inconsistent, the risk of colorectal cancer in post-menopausal women does not seem to be related or only weakly associated with obesity (Reeves et al., 2007, Moghaddam et al., 2007). It has been hypothesised that different distributions of adipose tissue between men and women accounts for the difference (Calle, 2007). Whether this is mediated by affecting circulating sex hormone levels is not fully understood. Adiposity is inversely related to testosterone concentration in men but positively related in women (Derby et al., 2006). Excess adipose tissue leads to increased conversion of androgenic precursors to oestradiol by increased aromatase activity (Hemsell et al., 1974). In endometrial

cancer, oestradiol increases cell proliferation via inducing a local increase in IGF-1 (Kleinman et al., 1996).

Different influences of circulating oestrogen levels may influence cancer development (Slattery et al., 2003). Chronic hyperinsulinaemia may promote tumourigenesis in oestrogen-sensitive tissues as it reduces circulating sex-hormone binding globulin and thus increases bioavailable oestrogen (Calle et al., 2003, Pan et al., 2004, Wolk et al., 2001, Calle and Kaaks, 2004). The association between obesity and postmenopausal breast cancer risk is accounted for by the increased serum oestradiol levels as obesity increases (Key et al., 2003) and since in females, most fat deposition occurs peripherally, this fat area may be of more relevance to endometrial and post-menopausal breast cancer risk.

1.3.2.5 Insulin and cancer

Insulin can act as a mitogen and has been associated with several cancers (Argiles and Lopez-Soriano, 2001). The tumourigenic effects of insulin could be directly mediated by insulin receptors in the pre-neoplastic target cells, or might be due to related changes in endogenous hormone metabolism, secondary to hyperinsulinaemia (Calle and Kaaks, 2004). Epidemiological studies are hampered by the heterogeneity of diabetic patients with respect to their degree of glycaemic control which will influence their circulating insulin levels and hence making correlations with cancer development difficult.

Epidemiological studies have shown that serum C-peptide, as a proxy measure of insulin release, is associated with increased risk of cancer of the colorectum, post-menopausal breast, pancreas and endometrium (Pisani, 2008) and that type 2 diabetes is associated, independent of obesity, with breast, pancreas, kidney, endometrial,

colorectal and bladder cancer (Nicolucci, 2010). Cohort studies have demonstrated increase risk of colorectal cancer in those with insulin resistance (Trevisan et al., 2001, Colangelo et al., 2002, Schoen et al., 1999), the metabolic syndrome (Ahmed et al., 2006) and type 2 diabetics (Larsson et al., 2005). The risk of cancer-related mortality is increased in those with high insulin levels or insulin resistance and cancers of the breast (Goodwin et al., 2002), prostate (Amling et al., 2004) and colorectum (Wolpin et al., 2009). Colorectal cancer incidence is higher in type 2 diabetics treated with insulin (Yang et al., 2004).

In vitro studies support epidemiological data, in that insulin increases the neoplastic proliferation of cell lines at both physiological and pharmacological doses (Osborne et al., 1976) and the insulin receptor is commonly expressed in human neoplasms. Under investigation at present is whether there are differential effects downstream signalling effects in normal or transformed epithelial cells compared to insulin-responsive tissues (such as fat, liver and muscle) with receptor activation resulting in cell survival and proliferation rather than altered energy metabolism (Pollak, 2008)

1.4 The insulin-like growth factor axis

The interaction between insulin, body fat and the IGF axis is not well understood. It has been proposed that the IGF system mediates the effect of hyperinsulinaemia. The insulin-like growth factor axis of proteins is involved in the enhancement of cell proliferation, differentiation and apoptosis and has been implicated in tumourigenesis (Samani et al., 2007, Frasca et al., 2008, LeRoith and Roberts, 2003).

The IGF axis is a multifunctional system with a variety of molecular and biological effects. To date, 15 molecular functions and 29 biological processes have been linked to the IGFIR in the gene ontology database (http://www.geneontology.org). Proteins from the axis are ubiquitously expressed but at different levels in different tissues and with varying roles in each tissue. The IGF axis can have auto-, para- and endocrine effects. The main biological processes that the IGF axis is involved with can be summarised as: control of normal growth (Ohlsson et al., 2009, Pollak, 2008) (and perhaps lifespan) (Holzenberger et al., 2003)); maintenance of tissue homeostasis (Sutherland et al., 2008) and a differentiated phenotype (Belfiore et al., 2009); alteration in the balance of proliferation and apoptosis (LeRoith and Roberts, 2003); angiogenesis, cell adhesion, migration and wound healing (LeRoith and Roberts, 2003).

Mounting evidence suggests that both IGF-1 and 2 and their receptors IGF1Rs are involved in the development of cancer. IGF1R has been extensively studied for its role in the proliferation and differentiation of cancer cells. IGF-1 is produced mainly under the control of growth hormone by the liver and is released into the systemic circulation (referred to as circulating IGF-1). It is also produced locally within tissues and the level of local IGF-1 exerting paracrine effects may not correlate with systemic circulating IGF-1 levels (Figure 1.17).

IGF1R is a trans-membrane heterotetrameric protein encoded by the IGF1R gene located on chromosome 15q25-q26. IGF1R is composed of two α and two β subunits. Due to structural homology with the insulin receptor it can heterodimerise with it. It appears that where both receptors are expressed, heterodimers are common (Belfiore et al., 2009), thereby making an association between obesity, insulin resistance and IGF1R expression seem intuitive, although studies in this regard are lacking.

There are also reports of IGF1R and EGFR heterodimers but their distribution and ligand affinity are not clear (Riedemann et al., 2007). The IGFIR receptor binds (ranking from high to low affinity): IGF-1, IGF-2 and Insulin.

IGF-1 and IGF-2 are bound by 6 high affinity binding proteins (IGFBP1-6) and other low affinity binding proteins (IGFBP-related proteins). IGFBP1-5 have higher affinities for IGF-1, whereas IGFBP6 has a higher affinity for IGF-2 (Firth and Baxter, 2002). IGFBPs stabilise and prolong the half-life of IGFs and by binding IGFs prevent their binding to receptors. IGFs are released from IGFBPs by dissociation or protease-mediated IGFBP cleavage (Firth and Baxter, 2002). Thus IGFBPs alter the bioactivity of IGF and in some circumstances act to increase the bioactivity of IGF. This is thought to occur by IGFBPs binding IGFs in proximity to their receptor and acting to concentrate IGFs at receptors and through the slow release of the growth factors can influence the duration of signalling via the receptor (Firth and Baxter, 2002). Another hypothesis is that IGFBP2 binds an integrin-linked kinase to increase IGF bioactivity (Pollak, 2008).

Thus the IGF system is highly regulated via a dynamic system of binding proteins which influence growth factor stability, receptor binding and duration of receptor activation.

IGFBPs may also have independent effects on proliferation, adhesion and motility. Certain IGFBPs modulate Wnt signalling and this modulation is influenced by local concentrations of IGF ligands (Zhu et al., 2008). The tumour suppressor p53, vitamin D, anti-oestrogens, retinoids and TGF-β reduce the bioactivity of IGFs by increasing secretion of IGFBPs (Firth and Baxter, 2002).

There are two principle ligands of the IGF1R – IGF-1 and IGF-2. The binding affinity for IGF-1 far exceeds that of IGF-2 and IGF-2 is thought to act as a negative activity regulator – i.e. binding of IGF-2 to IGF1R reduces the activity of the IGF-1 axis by preventing binding of the more active IGF-1. Most of the total IGF protein in the circulation is bound to IGFBPs and only a small fraction remains unbound, termed free IGF-1 (fIGF-1). The most clinically relevant determination of IGF-1 concentrations – whether it be the total or free fraction- or a ratio of free or total to one of the bioactive IGFBPs, is not known. Epidemiological evidence concerning the bioactivity of circulating IGF levels is summarised in section 1.4.1.

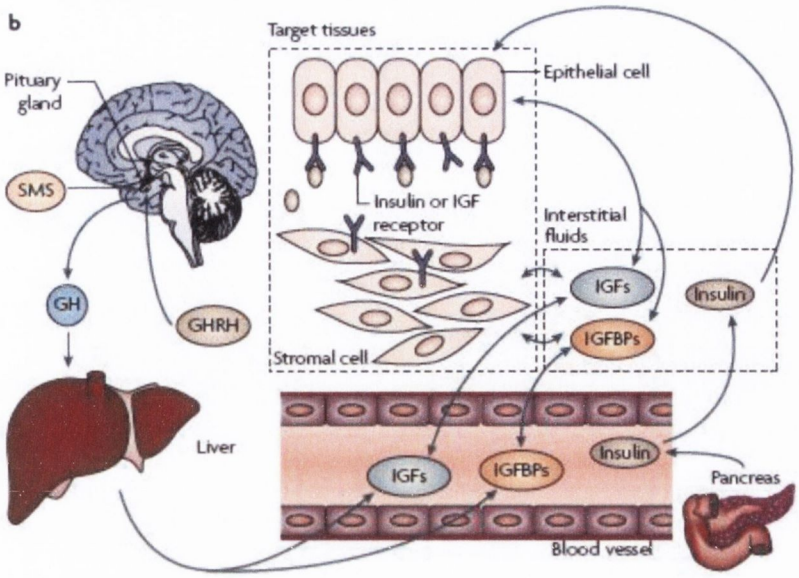


Figure 1.17: IGF-1-Growth hormone axis. At the whole organism level, circulating IGF-1 is produced mainly in the liver, mainly under the control of growth hormone. IGF-1 can also be produced in a paracrine and autocrine manner (Pollak, 2008). Abbr: GH, growth hormone; GHRH, GH-releasing hormone; SMS, somatostatin.

IGF1R is a tyrosine kinase receptor which initiates intracellular signalling upon receptor activation by autophosphorylation and stimulation of tyrosine kinase activity, leading to recruitment and phosphorylation of IRS-1 and Shc. These receptor substrates activate two main signalling pathways: Phosphatidylinositol 3-kinase – Akt (PI3K-Akt) and Ras -Raf- Mitogen Activated Protein Kinase (Ras-Raf-MAPK) which have multiple effects on gene regulation and protein expression, activation and translocation (LeRoith and Roberts, 2003) (Figure 1.18). The availability, location and ratios of receptor substrates influence cellular responses to receptor activation and may also alter the balance of IGF signalling and insulin signalling when heterodimers of IGF/insulinR are bound. Several mechanisms of crosstalk which influence IGFIR receptor function have been described including heterodimers with EGFR (Morgillo et al., 2006) and SOCS interactions

influencing the pro-inflammatory Jak-Stat pathway (Dey et al., 2000). Heterodimeric receptors are both activated upon binding (Belfiore et al., 2009).

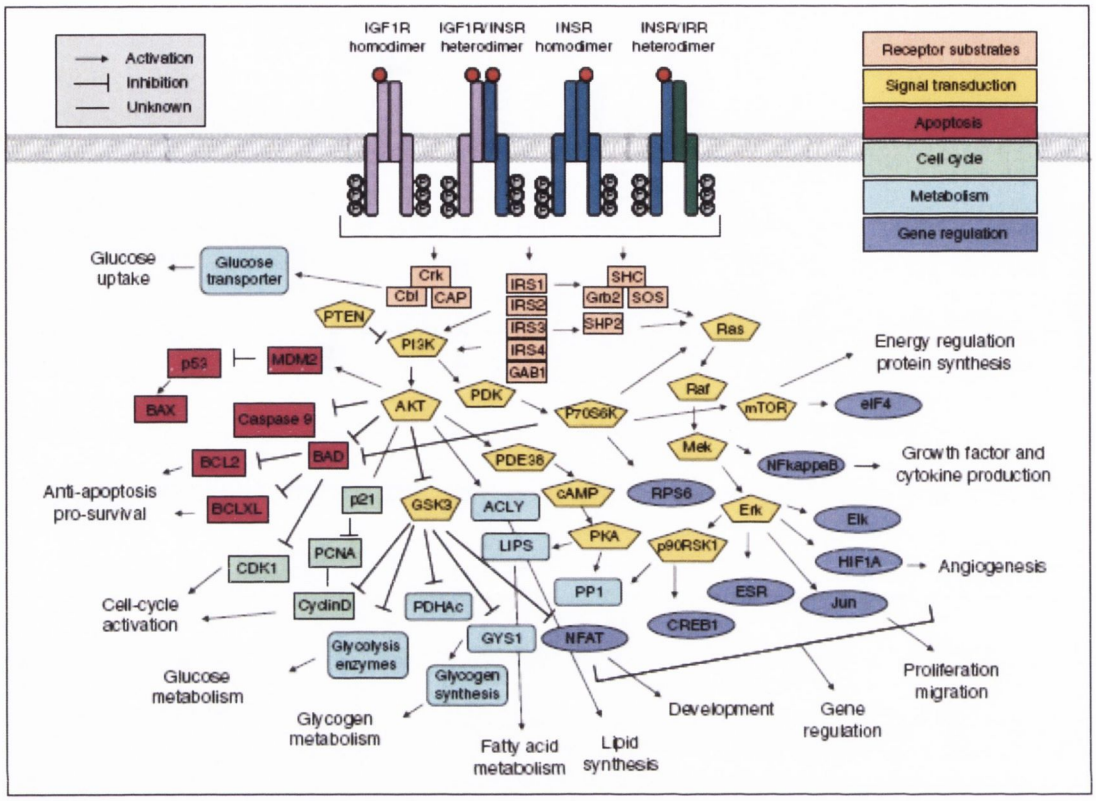


Figure 1.18 Signalling downstream from IGF1R

Abridged PI3K-Akt and Ras-Raf-MAPK signalling pathways are shown for clarity. Signalling activity is influenced by different binding velocities, reaction times, activities, expression levels and sub-cellular locations of signalling substrates. Reproduced from Massoner et al.,(2010).

1.4.1 IGF-1 axis and cancer

Two of the hallmarks of cancer (Hanahan and Weinberg, 2000) are limitless replication and evasion from apoptosis (Figure 1.10). The IGF axis is a central regulator of growth and survival. It has been found that IGF1R plays a role in the establishment and maintenance of cellular transformation (Sell et al., 1993). IGF1R or its ligands are often

over-expressed in human tumours (Hellawell et al., 2002, Law et al., 2008) and its action protects against apoptosis and favours invasion and metastasis (Samani et al., 2007, Frasca et al., 2008, LeRoith and Roberts, 2003). Activation of IGF1R can promote cell migration and the redistribution of E-cadherin and α- and β- catenins from adherens junctions into the cytoplasm (Chan and Lee, 2008). IGF-1 also modulates the activities of integrin-coupled proteins (FAK, p130, Cas and paxillin) through dephosphorylation (Guvakova, 2007) .

The IGF1R is commonly expressed by neoplastic cell lines and human cancers and on circulating tumour cells (de Bono et al., 2007, Hellawell et al., 2002, Law et al., 2008). However, gene amplification is not commonly associated with protein overexpression or ligand-independent activation (Pollak, 2008). The IGF1R has been found to be essential for oncogenic transformation in some cellular systems. Mouse fibroblasts cannot be transformed by the oncogenes: SV40 T antigen, papillomavirus E5 and Ras overexpression if they lack the IGF1R (Sell et al., 1993). Stable (constitutive) activation of IGF1R is insufficient to cause mammary epithelial cell transformation in mouse models (Jones et al., 2006).

Many cell lines are mitogenically responsive to physiological concentrations of IGFs (Pollak et al., 2004b). Increased proliferation in response to raised IGF levels may fuel the development of early cancers. Using prostate cancer as a model, there appears to be an increased likelihood of progression to clinically detectable malignancy in patients with higher IGF-1 levels such that baseline IGF-1 level predicts progression to prostate cancer more accurately than prostate-specific antigen in screened populations (Chan et al., 1998, Chan et al., 2002).

In vivo animal models using natural occurring mutations associated with low IGF levels (Majeed et al., 2005, Yang et al., 1996) or genetic manipulations to influence ligand levels (Pollak et al., 2001, Wu et al., 2003), result in variability of neoplastic growth related to IGF activity. Animal models have shown decreased tumour growth after IGF1R inactivation and with decreased circulating or tissue levels of IGF-1 (Khandwala et al., 2000, Jones and Clemmons, 1995).

Studies of patients with acromegaly (Jenkins, 2006) or Laron dwarfisim (Shevah and Laron, 2007) have been used as proxies to identify cancer risk in relation to IGF-1 excess or deficiency. They provide circumstantial evidence that increased IGF-1 levels are associated with cancer. Other forms of circumstantial evidence include the observation that height and birth weight, which is related to the concentration of IGF-1 in the umbilical cord, are related to the risk of some cancers (Tibblin et al., 1995, Sandhu et al., 2002, McCormack et al., 2003). Mammographic breast density, which is a strong risk factor for breast cancer is related to the level of circulating IGF-1 genes (Diorio et al., 2008, Byrne et al., 2000) and to polymorphisms in IGF-related genes (Diorio et al., 2008, Tamimi et al., 2007).

Population based studies have provided evidence that relate circulating ligand levels as well as polymorphic variation of relevant genes to cancer risk and prognosis. Prospective epidemiologic studies provide evidence of a relationship between circulating IGF-1 and the risk of developing prostate, breast, colorectal and other cancers (Ma et al., 1999, Giovannucci et al., 2000, Palmqvist et al., 2002, Chan et al., 1998, Chan et al., 2002, Harman et al., 2000, Stattin et al., 2000). Individuals at the high end of the normal range of serum IGF-1 have more than double the risk of a subsequent cancer (defined as any incidental cancer, site not specified). Variability between studies,

particularly regarding the potential reciprocal relationship between IGFBP3 and IGF-1 levels may be accounted for by technical challenges in measuring IGF and uncharacterised factors that modify IGF-1 levels including age and diurnal variation. Studies of single nucleotide polymorphisms in the IGF-1 axis genes indicate a potential relationship between SNPs and circulating levels (Diorio et al., 2008, de Graaff et al., 2010) but increased risk of cancer development with IGF SNPs is unclear (Gu et al., 2010). Studies report polymorphic variants associated with colorectal (Wong et al., 2005), oesophageal (McElholm et al., 2010), ovarian (Terry et al., 2009) and non-small cell lung cancer (Zhang et al., 2010) but not with breast or prostate cancer (Schumacher et al., 2010). These studies require confirmation in other populations. Circulating IGF levels seem to better predict cancer risk within the general population than cancer prognosis within cancer populations (Pollak, 2012b).

1.4.2 IGF-1 axis and obesity

Alterations in the IGF axis have been implicated in malignancies that are also associated with obesity, suggesting the IGF axis may play a mechanistic role in linking obesity and cancer. There is a paucity of studies examining the influence of obesity on IGF1R in tumour tissue. An obesity-associated transcriptional signature of 662 genes was indentified from the whole genome analysis of 104 breast tumours. This obesity signature correlated strongly with a gene signature for IGF signalling in independent cohorts of patient tumours and may be associated with shorter time to metastases (Creighton et al., 2011). A study in breast cancer also demonstrated that increasing BMI was positively associated with increased IGF1R expression in both normal mammary gland tissue and breast cancer tissue (Suga et al., 2001). Furthermore research has found that IGF1R expression is significantly higher in the colorectal neoplasms of individuals

with metabolic syndrome than in the lesions of individuals without the syndrome (Papaxoinis et al., 2007). The findings of these studies suggest that the molecular consequence of obesity is the increased expression of IGF1R in both normal and malignant tissue.

As mentioned previously, elevated levels of free IGF-1 have been reported in obese individuals and are believed to be a consequence of hyperinsulinaemia inhibiting production of IGFBP-1 and -2 (Lukanova et al., 2002, Frystyk, 2004) . The chronic systemic inflammation associated with obesity is believed to fuel tumour development and progression, and IGF-1 may mediate obesity-associated inflammation via its effects on immune cells, including macrophages. Studies have shown IGF-1 can lead to macrophage migration and invasion, and also increased macrophage production of proinflammatory cytokines (Heemskerk et al., 1999, Renier et al., 1996). In addition, recent research has supported a functional role for IGF-1 in obesity associated inflammation and tumourigenesis.

In a murine model of obesity and chronic IGF-1 gene deficiency, diet-induced obese mice demonstrated increased local tumour growth and metastases compared to lean controls, but this was not seen in IGF-1 gene deficient mice. In addition, the expression of inflammatory cytokines and cell adhesion molecules was upregulated in obese compared to lean mice, but chronic IGF-1 deficiency was associated with a reduction in these indicators (Wu et al., 2010). Hence it is possible that in obesity IGF-1 can affect tumour development both directly by stimulating tumour growth and indirectly, by creating a microenvironment that is permissive for tumour growth.

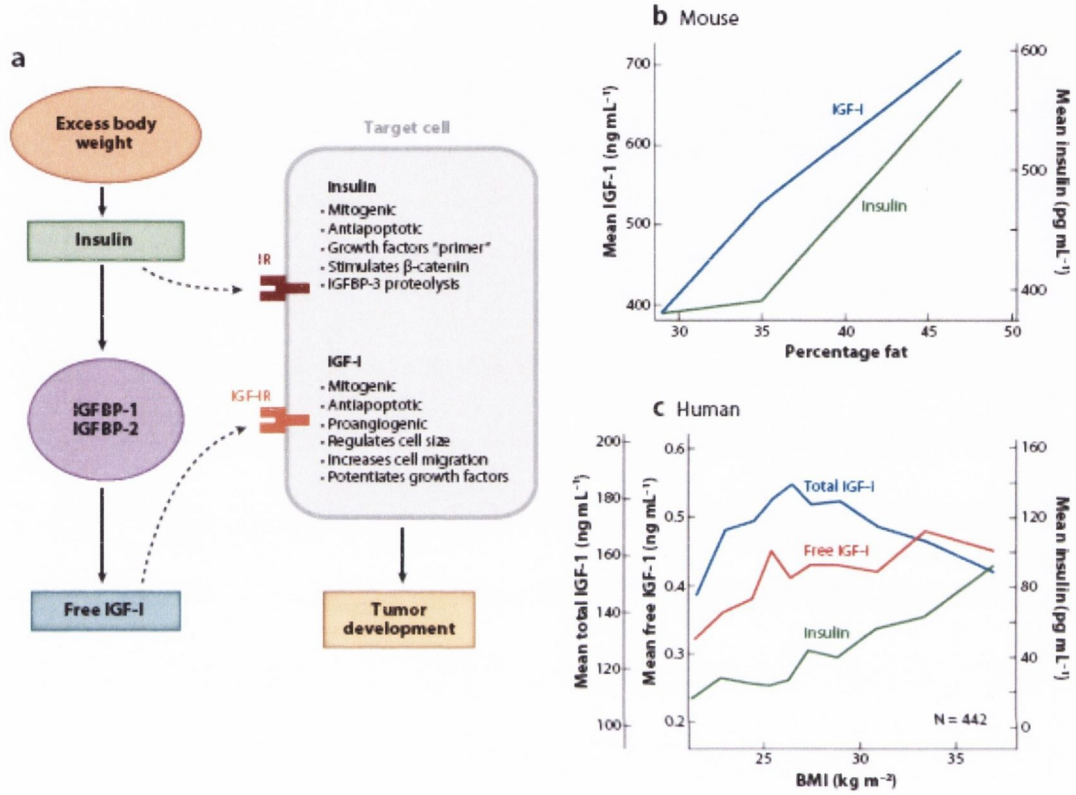


Figure 1.19 The insulin-IGF hypothesis of obesity-related cancer.

(*a*) Obesity is associated with a state of prolonged hyperinsulinemia, which reduces IGFBP-1 and IGFBP-2 production. The decrease in these proteins results in increased levels of free IGF-I. Free IGF-I is believed to be the "bioactive" form of IGF-I. Actions of IGF-I (*listed in grey target cell*) may favour tumour development. Abbreviations: IR, insulin receptor; IGF-IR, insulin-like growth factor-I receptor. (*b*) Linear relationship between circulating total IGF-I and insulin and percentage fat in mice. Original data kindly shared by Prof. Stephen Hursting, Division of Nutritional Sciences, University of Texas at Austin, USA. (*c*) Nonlinear relationship between circulating total IGF-I, free IGF-I, and BMI in humans. The linear relationship with serum insulin is shown for comparison. Reproduced from Roberts et al.,(2010)

1.4.3 IGF-1 axis, Barrett's oesophagus and progression to oesophageal adenocarcinoma

There is some emerging evidence that the insulin-like growth factor may be implicated in the progression from Barrett's oesophagus to oesophageal adenocarcinoma. Increased levels of serum insulin (OR 2.02, 95% CI 1.15 to 3.54) and IGF-1 (OR 4.05, 95%CI 2.01-8.17) were associated with an increased risk of Barrett's oesophagus when compared to screening colonoscopy control (Greer et al., 2012). An increasing proportion of specimens (n=75) have increased phsophorylated insulin receptor susbstrate-1 (p-IRS1) across the spectrum of progression from Barrett's oesophagus (43.2%), through low grade dysplasia (55%) through high grade dysplasia (66.7%) and oesophageal adenocarcinoma (70%) (Greer et al., 2013). Presence of p-IRS1 correlated with p-AKT, p-m-TOR and Ki-67 staining (a marker of proliferation). Elevated IGF1R expression has previously been demonstrated in oesophageal adenocarcinoma tumour tissue (Iravani et al., 2003, Kalinina et al., 2010). Nonetheless, there have been no previous investigations of the association of visceral adiposity, the IGF-1 axis and oesophageal adenocarcinoma nor investigation of the potential implications of inhibition of IGF1R at a cellular level in oesophageal adenocarcinoma.

1.4.4 IGF1R as a therapeutic target

Given the putative roles of IGFIR in cancer development and progression, many drug discovery efforts are ongoing to identify agents that selectively block the IGFIR pathway in tumour cells. The rationale for this is the growing *in vitro* and *in vivo* evidence of involvement of the IGF axis in cancer development. One such strategy is to identify low molecular weight molecules that inhibit the catalytic activity of the IGFIR by binding its active site. The potential difficulty associated with this strategy is the

high degree of structural homology with the insulin receptor (IR). Highly selective inhibition of the IGFIR is necessary to avoid unacceptable toxicity as a result of inhibition of the insulin receptor and the effect on homesostasis.

Different approaches to targeting the IGF axis involve reduction of ligand levels or bioactivity or inhibition of receptor function which may include anti-IGFIr antibodies, Small-molecule IGFIR inhibitors and IGFIR tyrosine kinase inhibitors. Somastostatin analogues were found to cause only a modest reduction of serum insulin and IGF-1 which correlated with lack of anti-neoplastic activity (Pollak et al., 2010). Other strategies include ligand-specific antibodies (Goya et al., 2004) or growth hormone antagonists (Divisova et al., 2006).

An entirely different way of targeting the IGF1R may be to downregulate its expression or downstream signalling activity. Activators of AMP-activated protein kinase (AMPK), such as metformin, result in lower amounts of circulating insulin by inhibiting liver gluconeogenesis and also reduce downstream signalling from insulin and IGFIRs (Dowling et al., 2007, Zakikhani et al., 2006). The biosynthesis and trafficking of the IGFIR involves the chaperone protein heat shock protein 90 (HSP90) and HSP90 targeting agents are under current evaluation (Eccles et al., 2008, Lang et al., 2007).

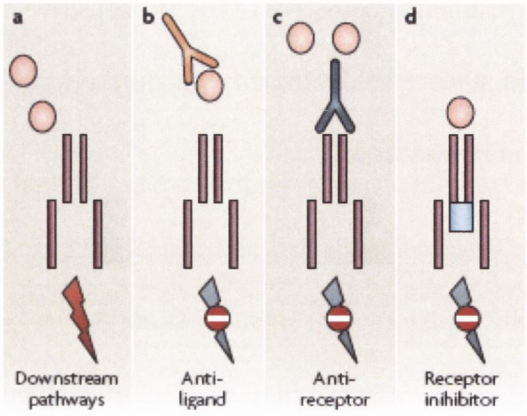


Figure 1.20 Approaches to targeting tyrosine kinase receptors a) Ligand binding to tyrosine kinase receptors induces a variety of downstream signalling pathways. Prevention of downstream signalling may involve any of a number of approaches: b) Ligand targeting strategies involve pharmacological measures that attempt to lower ligand concentration or the use of ligand-specific antibodies to decrease their binding to the tyrosine kinase receptor. c) Receptor specific monoclonal antibodies bind to and prevent downstream activation of the receptor. d) tyrosine kinase inhibitors (TKIs) act to prevent activation of the receptor and thus, downstream signalling (usually by inhibition of phosphorylation of the receptor or prevention of interaction with downstream signalling molecules). Figure from Pollak (2008)

To date, there have been 9 large and 6 small molecules inhibiting the IGF-1 axis trialled in over 150 phase I to III trials in 16 solid tumour types (Gao et al., 2012).

Phase I findings confirmed a compensatory increase in the circulating concentrations of growth hormone and IGF-1 occurs on administration of IGFIR-specific antibodies (Pollak, 2012b). There is no evidence that this can overcome the antibody receptor inhibition. There is an increase in insulin levels as a result of high levels of GH which responds to metformin (Haluska et al., 2007).

Findings from early phase I/II trials demonstrated some disease activity across a range of tumours. For example, using the monoclonal anti-IGF1R antibody Figitumumab (CP-751971) (Pfizer Inc, NY, USA) in early trials demonstrated acceptable toxicity levels without dose-limiting toxicity and achievement of pharmacological end points (de Bono

et al., 2007, Gualberto et al., 2008b, Karp et al., 2008) with an increased partial response rate to chemotherapy in NSCLC from 41% to 72% (Gualberto and Pollak, 2009). However, in phase II trials, it was noted that not all patients respond in a similar manner to IGF1R inhibition (Pollak, 2008).

Trials of IGF1R inhibitors are also beginning in the treatment of gastrointestinal cancers (summarised in Table 1.8)

Table 1.8: Summary of IGF1R inhibitor trials in gastrointestinal cancer

From (Golan and Javle, 2011)

Agent	Cancer	Combination Therapy	Study Phase	Study Status
Cixitumumab	Colorectal (treated with prior EGFR inhibitor, *K-ras* wt)	Cetuximab (Erbitux)	Phase II randomized	Completed
AMG 479 or AMG 102	Colorectal (*K-ras* wt)	Panitumumab (Vectibix)	Phase II randomized	Ongoing, not recruiting
Sch717454	Colorectal	Approved chemotherapy	Phase II randomized	Completed
Cixitumumab	Colorectal (*K-ras* wt)	Irinotecan (Camptosar) and cetuximab	Phase II randomized	Not recruiting
AMG 479 or AMG 655	Colorectal (*K-ras* mutant)	FOLFIRI	Phase II randomized	Recruiting
AMG 479 or AMG 655	Pancreatic	Gemcitabine (Gemzar)	Phase II randomized	Completed
AMG 479	Pancreatic	Gemcitabine	Phase III	Not recruiting
Dalotuzumab	Pancreatic	Gemcitabine ± erlotinib (Tarceva)	Phase II randomized	Recruiting
Cixitumumab	Pancreatic	Gemcitabine, erlotinib	Phase II randomized	Not Recruiting
Dalotuzumab	Colorectal	Irinotecan and cetuximab	Phase II/III	Not recruiting
BIIB022	Hepatocellular	Sorafenib (Nexavar)	Phase II	Not recruiting
Cixitumumab	Hepatocellular	Sorafenib	Phase I	Recruiting
Cixitumumab	Gastoesophageal	Paclitaxel	Phase II randomized	Recruiting

EFGR = epidermal growth factor receptor; wt = wild type.

In the completed trial of patients who had previously failed EGFR directed therapies with metastatic colorectal carcinoma, there was no response detected and this combination is not further studied in this setting (Reidy et al., 2010). Patients included in this setting had only moderate to strong staining of IGF1R in a minority of patients treated, which may account for the lack of efficacy. Results from the other studies cited

are not presently available. The trial of cixutumumab in gastro-oesophageal cancer is designed to evaluate the progression-free survival of patients with metastatic oesophageal or gastro-oesophageal junction cancer treated with paclitaxel with versus without cixutumumab as a second-line therapy and is ongoing (NCT01142388).

Where there were initial positive results from phase II trials, phase III trials have been carried out using various IGF1R inhibitors. Two of the largest trials in NSCLC, named the ADVancing IGF1R in Oncology (ADVIGO) trials of fitigumumab were terminated early due to a combination of adverse events and a low likelihood of meeting the primary endpoint of improved overall survival (Jassem J, 2010). Efforts are now currently focused on identifying which patients developed toxicity and responses to the treatment in an effort to better select patients for future trials.

Most trials to date have investigated anti-IGF1R MABs. TKIs may be anticipated to have more toxicity due to interactions with insulin signalling via the insulin receptor and their ability to cross the blood-brain barrier. However, they may also be more efficacious than antibody therapies by also inhibiting insulin-receptor mediated effects and thereby preventing an insulin-receptor mediated resistance to anti- IGFIR antibodies. Only linsitinib (OSI-906) of TKIs is in advanced clinical studies – as a phase III single agent in patients with locally advanced or metastatic adrenocortical carcinoma (NCT00924989). Some early phase studies have shown that hyperglycaemia may be overcome with some TKIs which inhibit insulin signalling if they do concentrate and thus inhibit muscle insulin receptors (Dool et al., 2011).

Clinical trials in patients with sarcoma have shown anti-tumour activity in cases where the IGF-1 pathway is activated, such as in Ewing Sarcoma, where about one quarter of

patients benefit from treatment (Olmos et al., 2011). However, secondary resistance has been common.

1.4.4.1 Treatment resistance to targeted therapies: relevance to IGF1R targeted therapies

Challenges in identifying disease-activity and resistance have not only been experienced with IGF1R inhibition. A common theme arising from the use of targeted agents in malignancies is that with time, resistance inevitably develops (Ellis and Hicklin, 2009). In cancers with a driver mutation or a dependence on a dominant oncogene, inhibition of this oncogene can lead to marked improvements in response and survival. For example, treatment of Philadelphia chromosome positive chronic myelogenous leukaemia (CML) with imatinib, a tyrosine kinase inhibitor of the aberrantly expressed ABL protein, leads to dramatic clinical responses in most patients (Gambacorti-Passerini et al., 2003). Mechanisms of resistance described to date in the literature are summarised in Figure 1.21.

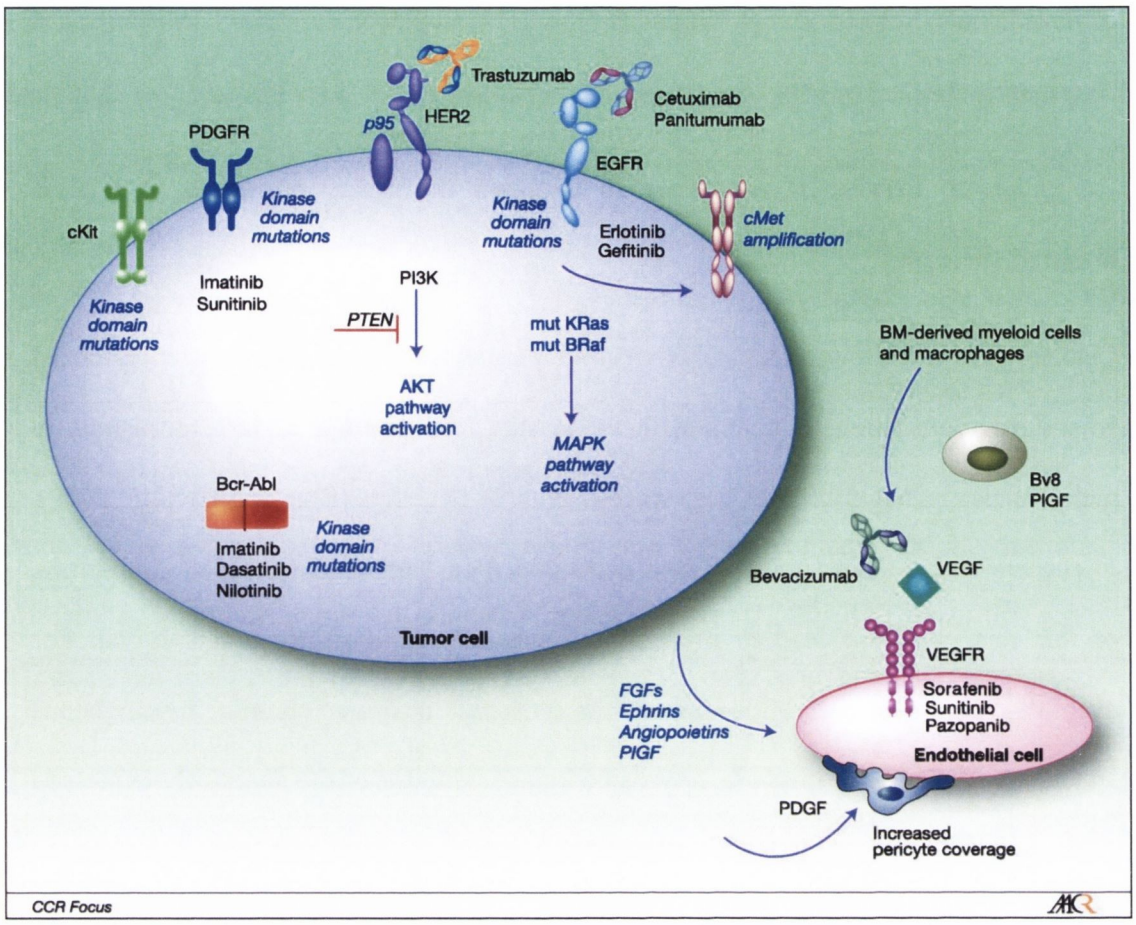


Figure 1.21: Overview of resistance pathways to targeted therapies. Current targeted therapies are directed at inhibiting the kinase activity of dominant oncogenes and/or growth factor receptors with either TKIs or mAbs. Resistance in tumour cells may be inherent or acquired. Resistance is likely to be multifactorial. Common acquired resistance mechanisms include the selection of tumour cells with specific kinase mutations rendering drugs incapable of binding to the ATP pocket, and activation of redundant signalling pathways. Inherent resistance to targeted therapies may be due to activation and/or mutation of downstream signalling pathways or mutations in kinase domains, which do not allow access of the drugs to the ATP pocket. Resistance to angiogenic-VEGF-targeted therapies may be due to redundant angiogenic pathways, survival signals from perivascular cells, or myeloid-derived infiltrating cells that secrete pro-angiogenic factors. Reproduced from Ellis and Haskins, 2009.

However, many solid cancers are more complex than these cancer types and are not addicted to a single oncogene or quickly develop secondary resistance to targeted agents. In addition, intrinsic (primary) resistance seems to be an issue with most targeted agents, whereby only subgroups of patients benefit from drugs targeting different oncoproteins. In a study of NSCLC tumours, driver mutations were found in 54% (CI: 50-59%) of the 516 tumours investigated. The most common mutation was KRAS at 22%. The second most common mutation was EGFR at 17%, and the third most common was EML4-ALK at 7%. Some of the other rarer mutations include BRAF at 2%, PI3K, HER2-neu, MET amplification, MEK, KRAS, and AKT1. Therefore, it seems that driver mutations only occur in half of tumours and 97% of the driver mutations were mutually exclusive (Kris et al., 2011). A similarly detailed understanding of common mutations within oesophageal cancer tumours is lacking.

1.4.4.2 Mechanisms of resistance to anti- IGF1R treatment

Individualised tailored therapy with anti-IGF1R therapies will be aided by increased understanding of the interactions of the IGF axis and other growth factors as well as developments in the ability to characterise receptor expression and activation and alterations in signalling molecules such as PTEN, p53 and k-Ras. For example, patients with PTEN activation would be expected to have constitutive activity downstream from the IGF1R and thus be unresponsive to anti-IGF therapy (Pollak et al., 2004a).

Investigation of primary resistance to IGF1R inhibition in the *in vitro* setting has proposed resistance mediated via compensatory increased insulin-receptor signalling (Buck et al., 2010, Ulanet et al., 2010). IGF1R inhibitors with some anti-insulin receptor activity may thus be an attractive option although with potential for hyperglycaemic mediated toxicity. Understanding the interplay between networks of tyrosine kinases

will be critical to uncovering resistance mechanisms. In a sarcoma model, using siRNAs to look for synthetic lethality alongside an IGF1R inhibitor demonstrated that one such intrinsic resistance mechanism may be via MSTR1/RON (MET family receptor macrophage stimulating 1 receptor/recepteur d'origine nantais) (Potratz et al., 2010).

Another potential mechanism is via cross-talk with EGFR family of receptors which has been well-documented in a variety of different tumour types *in vitro*. The IGF1R is thought to heterodimerise with EGFR (Morgillo et al., 2006) and this may be involved in resistance to anti-EGFR therapies. EGFR protein expression is correlated with IGF1R protein expression in operable non-small cell lung cancer (Dziadziuszko et al., 2010). IGF1R/EGFR heterodimers are also thought to be responsible for transtuzumab resistance in breast cancer cell lines (Nahta et al., 2005, Lu et al., 2001). Inhibition of EGFR-directed MAPK pathway (using erlotinib) activity leads to enhanced IGF1R-driven PI3K pathway activity in epithelial like tumours (Buck et al., 2008, Chakravarti et al., 2002, Guix et al., 2008). This may be mediated via reductions in IGFBP3 expression (Guix et al., 2008). EGFR-resistant breast and prostate cancer cell lines have increased IGF1R mediated signalling (Jones et al., 2004). This pre-clinical evidence has led to phase II/III trials combining EGFR and IGF1R inhibition, without any reported successes (although many trials are still recruiting) (Ramalingam et al., 2011).

Similarly crosstalk between IGF1R and HER2 has been described in ovarian and breast cancer cell lines with activation of HER2 conferring resistance to IGF1R inhibition (Haluska et al., 2008). There appears to be a high degree of co-expression of IGF1R and HER2 in oesophageal adenocarcinoma tumour specimens (Kalinina et al., 2010).

1.4.4.3 IGF1R as a resistance mechanism for other chemotherapeutic agents

The other side of the coin is that IGF1R signalling may lead to resistance to other anti-neoplastic agents, particularly to other tyrosine kinase growth factor receptors. Synthetic lethality describes the concept of the combination of inhibition of two genes leading to cell death whereas inhibition of one gene leaves the cell viable. IGF1R inhibition may be synergistic with traditional chemotherapies (Benini et al., 2001) (Cohen et al., 2005) or radiation (Allen et al., 2007). Combined anti-IGFIR therapy may important in the prevention or treatment of acquired resistance to rapamycin and its analogues (O'Reilly et al., 2006, Wan et al., 2006), transtuzumab (Lu et al., 2001) (Best et al., 2006) and anti-EGFR therapies (Huynh et al., 1996) (Barr et al., 2007). In BRAF resistant melanoma cells a combination of IGF1R/PI3K and MEK inhibition is required to overcome resistance (Villanueva et al., 2010).

Given the initial clinical success in adjuvant transtuzumab treatment in metastatic OGJ tumours in the TOGA II trial (described in section 1.1.2.4; (Bang et al., 2010)) , transtuzumab may be an agent with therapeutic efficacy in oesophageal adenocarcinoma. As seen in breast cancers, resistance to this treatment is inevitable (Nahta et al., 2006). Based on analysis of HER2-amplified tumours, Staaf *et al* (Staaf *et al.*, 2010) derived a gene expression signature that predicts outcome in HER2-positive breast cancer with a mixed stage, histologic grade and ER status. One subtype (cluster 2) had a significantly worse outcome that the others. Increased PI3K signalling (as measured by STMN1 expression (Saal et al., 2007)), IGF1R levels and observations of lower lymphocytic infiltration have been reported in poor responders to transtuzumab in breast cancer. These observations correlated with the HDPP cluster 2 classification – those with poor prognosis i.e. amongst HER2 amplified cancer a subgroup have

increased invasiveness (decreased CXCR4, PLAU, TGFBR3 and STAT5A) and lower lymphocytic infiltration. Resistance may be mediated via an IGF1R/HER-2 heterodimer (Nahta et al., 2005) or by decreased expression of PTEN (Nagata et al., 2004).

The mTOR pathway function has been intimately linked with that of IGF1R. Following activation of IGF1R and subsequent increased PI3K activity, mTORC1 complex acts to downregulate IRS and thus by negative feedback decreases PI3K pathway activity (Figure 1.22). Thus, in order to prevent resistance to mTOR inhibition developing, the IGF1R pathway may need to be co-inhibited to prevent compensatory IGF1R-PI3K activation (O'Reilly et al., 2006).

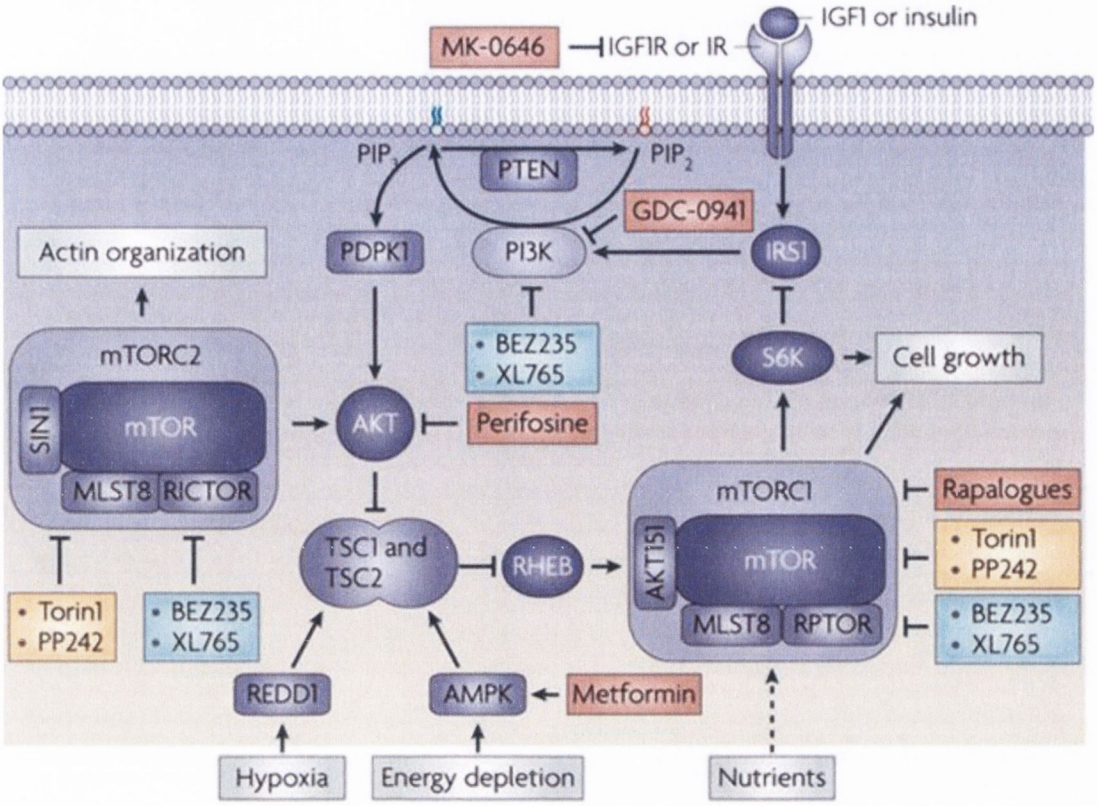


Figure 1.22 IGF1R and mTOR negative feedback. mTORC1 leads to downregulation of PI3K activity following IGF1R activation. Abbreviations: AKT1S1, AKT1 substrate 1; AMPK, AMP-activated protein kinase; IGF, insulin-like growth factor; IGF1R, IGF-1 receptor; IR, insulin receptor; IRS1, IR substrate 1; MLST8, target of rapamycin complex subunit LST8; PDPK1, 3-phosphoinositide-dependent protein kinase 1; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; RPTOR, regulatory-associated protein of mTOR; RHEB, Ras homologue enriched in brain; RICTOR, RPTOR-independent companion of mTOR, complex 2; SIN1, stress-activated map kinase-interacting protein 1 (also known as MAPKAP1); TSC, tuberous sclerosis protein. Inhibitors and activators are in red. Inhibitors for both mTORC1 and mTORC2 are highlighted in yellow. Dual inhibitors for PI3K and mTORC1 and mTORC2 are highlighted in blue. Reproduced from Tennant et al., (2010).

However, how do we investigate mechanisms of resistance? A rational way to design combination therapies may be to develop a library of candidate mechanisms of resistance based on known interacting intracellular pathways to investigate whether inhibition of any one of these genes leads to synergistic synthetic lethality. For example,

in one study centred on EGFR resistance, co-treatment with inhibition of protein kinase C, aurora kinase A and signal transducer and activator of transcription 3 (STAT3) synergised with EGFR antagonists to reduce cell viability and tumour size (Astsaturov et al., 2010).

1.4.4.4 Predictive biomarkers

Initial investigations of therapeutic targeted agents have been disappointing when used to treat undifferentiated cancer populations. For example, the EGFR tyrosine kinase inhibitor gefitinib does not show a clear benefit when given to unselected NSCLC patients but patients with mutations in the TK domain of EGFR are sensitive to this agent (Mok et al., 2009). Predictive biomarkers for response allow targeted therapies to use selectively in patients who are likely to derive benefit. Table 1.9 lists some examples of predictive biomarkers used in clinical practice.

Table 1.9 Examples of predictive biomarkers for drug response

Biomarker	Cancer type	Drug therapy	Drug target
HER2 (gene amplification)	Breast	Trastuzumab	HER2
Estrogen receptor (protein expression)	Breast	Tamoxifen	Estrogen receptor
BCR–ABL (gene translocation)	CML	Imatinib, dasatinib, nilotinib	BCR–ABL
EGFR ± *KRAS* (*KRAS* mutation)	CRC	Cetuximab, panitumumab	EGFR
EGFR (kinase domain mutation)	NSCLC	Erlotinib, gefitinib	EGFR
PML–RAR (gene translocation)	APL	*All trans* retinoic acid	PML–RAR
BRCA1/2 (mutation)	Breast	Olaparib, veliparib	PARP
BRAF V600E (mutation)	Melanoma	Vemurafenib	BRAF
ALK (rearrangements)	NSCLC	Crizotinib	ALK

Abbreviations: APL, acute promyelocytic leukemia; CML, chronic myeloid leukemia; CRC, colorectal cancer; NSCLC, non-small-cell lung cancer.

Derived from La Thangue and Kerr (2011)

Given the early termination of the ADVIGO trials investigating figitimumab in NSCLC, the search of predictors of therapeutic efficacy of IGF1R inhibitors is underway (Basu et al., 2011).

Two of the key mechanisms shown to be involved in the activity of other growth factor TKRs are receptor mutations that lead to increased activity of receptor (such as EGFR) (Paez et al., 2004) or overexpression and thus increased receptor activity (e.g. HER2) (Slamon et al., 2001). Although IGF1R amplification has been detected in some tumour types, for example, Wilms, occasional luminal breast cancers and GISTs respectively (Natrajan et al., 2006, Adelaide et al., 2007, Tarn et al., 2008), it does not seem to be a common event as with other tyrosine kinases. Measurement of receptor activity using routine clinical laboratory tests is not currently possible.

Potential biomarkers include screening for IGF1R receptor expression or its downstream signalling activity using immunohistochemical techniques. Gene expression signatures of IGF1R activity may help better characterise those patients with tumours that actively use the IGF1R to mediate increased survival. To date, circulating IGF-1 levels have been investigated in patient serum samples from many of the clinical trials.

In MCF-7 breast cancer cell line stimulated with IGF-1 profiling of the genome displayed alterations in gene expression associated with cell proliferation, metabolism and DNA repair. This gene expression signature was investigated in 3 independent panels of breast tumour samples - most of whom were ER-negative tumours and was associated with poor prognosis. It was highly correlated with other poor prognostic factors, independent of proliferation and was independently predictive on multivariate analysis alongside ER status, HER2 status, age, nodal status (Creighton et al., 2008).

Genes from the signature overlapped with those found in cells overexpressing EGFR and ERBB2. Removal of the set of genes associated with PI3K activity did not decrease its predictive ability. An insulin/IGF-1 gene signature was correlated inversely with recurrence free survival in hormone-receptor positive breast cancer treated patients – indicating that this signature may predict hormone resistance (Fox et al., 2011).

It is not known whether these gene expression signatures are applicable across the range of tumour types nor whether it is a robust prognostic predictor nor whether they could inform decisions on the use of targeted agents. However, *in vitro* evidence suggests that the IGF gene expression signature in cell lines and xenografts is reversed following treatment with anti-IGF1R therapies and this is associated with reduced proliferation, increased apoptosis and mitotic catastrophe (in a triple negative breast cancer cell line). Sensitivity of cells to a dual IGF1R/InsR inhibitor also correlated with the IGF gene signature. Therefore, there appears to be preliminary evidence that this gene expression signature is a predictor of IGF1R clinical activity and perhaps, response to its inhibition (Litzenburger et al., 2011).

In a phase I expansion cohort study of figitumumab in 31 patients with relapsed sarcoma, which suggested that pre-treatment plasma levels of IGF-1>110ng/ml conferred a significant treatment advantage compared with lower levels (10.5 v 4.5 months overall survival, $p<0.001$) (Olmos et al., 2010).

In the ADVIGO trial of figitumumab in combination with paclitaxel and carboplatin for treatment of advanced NSCLC there were objective response rates in patients with higher IGF1R expression (correlating with the squamous subtype) and improved progression free survival (PFS) in patients with high pre-treatment free IGF-1 levels compared to low levels (PFS 6 v 3 months, $p=0.007$) (Jassem J, 2010). Low IGF-1

levels have previously been associated with prolonged survival in NSCLC (Han et al., 2006). However, in this trial elevated baseline fIGF-1 was found in female patients with adenocarcinoma, which may indicate this rather than predicted therapeutic efficacy, it is merely a prognostic biomarker. However, in the chemotherapy only treatment arm fIGF-1 was not a prognostic biomarker, which may indicate that it is of relevance in predicting response to figitumumab (Gualberto et al., 2011). In addition, fIGF-1 levels correlated with tumour vimentin expression – a mesenchymal marker and inversely with E-cadherin expression – indicating that fIGF-1 may play a role in EMT (Gualberto et al., 2011).

The utility of using pre-treatment circulating biomarkers depends on a number of factors including assay reproducibility and whether the biomarker changes in response to treatment. For example, figitumumab treatment has been reported to lead to increases in circulating IGF-1 levels and decreased soluble IGF1R from baseline. (Lacy et al., 2008, Molife et al., 2010)

Variability of IGF1R expression across different cell lines representing the same histological tumour type and across clinical tumour samples may be an indicator of variability of response. For example, in rhabdomyosarcoma receptor number seems to predict sensitivity to anti-IGF1R antibody (Cao et al., 2008)

Immuno-SPECT imaging using a radiolabelled IGF1R monoclonal antibody has been used in a proof of principal setting involving mice with three different types of human bone sarcoma xenografts: those with high, moderate and low responses to the targeting MAB. Tumours with a high response to the drug had homogenous uptake versus heterogenous and nonspecific uptake in the moderate and low response tumours respectively (Fleuren et al., 2011).

In a number of clinical trials, patients with epithelial-mesenchymal transition tumours (defined by E-cadherin and high IRS-1 expression) have a higher response rate (Karp et al., 2009a, Gualberto et al., 2008a). This finding has also been reported *in vitro*,(Buck et al., 2008) whereby epithelial but not mesenchymal tumour cells are sensitive to IGF1R inhibition. fIGF-1 levels correlate with the mesenchymal marker vimentin and inversely with E-cadherin in pre-treatment tumour samples of patients in a clinical trial of patients treated with figitiumumab (Gualberto et al., 2011).

Reductions in IGFBP3 expression following EGFR treatment were associated with an IGF1R-mediated resistance to EGFR and this may be a biomarker of activity of IGF1R inhibition in this context (Guix et al., 2008).

1.5 Aims and objectives

1.5.1 General aim

There is consistent epidemiological evidence indicating a link between the presence of obesity and the development of oesophageal adenocarcinoma(Renehan et al., 2008b, Calle et al., 2003). The insulin-like growth factor pathway has been implicated previously in mediating the systemic alterations associated with obesity and thus, the hypothesis under examination herein is that the insulin-like growth factor axis mediates some of the effect of visceral obesity on oesophageal adenocarcinoma progression and may therefore, be a therapeutic target in this disease.

The overall aim of this thesis is explore the relationship between the insulin-like growth factor-1 axis and visceral adiposity in oesophageal adenocarcinoma.

1.5.2 Specific objectives

The overall aim of this thesis will be explored by the:

- Investigation of the association between circulating, tumour mRNA and tumour protein expression of the IGF-1 family members and visceral adiposity in patients with oesophageal adenocarcinoma.
- Assessment of the functional role for a novel IGF1R tyrosine kinase inhibitor, picropodophyllin, on *in vitro* oesophageal adenocarcinoma cell proliferation, viability, apoptosis and cell cycle progression.
- Investigation of a potential mechanism of resistance to IGF1R tyrosine kinase-induced inhibition via vascular endothelial growth factor (VEGF) production *in vitro*.

- Exploration of Stathmin 1 (STMN1) as a proxy indicator of PI3K activity in oesophageal adenocarcinoma *in vitro*, its relationship to IGF1R activity and its functional role in oesophageal adenocarcinoma cell line epithelial mesenchymal transition. To investigate STMN1 protein expression in oesophageal adenocarcinoma tumour samples with respect to visceral obesity status, tumour characteristics, IGF1R expression and disease-specific survival.

Chapter 2

Materials and Methods

2.1 Reagents

All laboratory chemicals and reagents were stored according to manufacturers' instructions. They were of analytical grade and were purchased from Sigma Chemical Company (M), USA) and BDH Chemicals (Poole, UK) unless otherwise stated. Solid chemicals were weighed using a Scout Pro electronic balance (Ohaus corporation, NJ, USA) and made up in sterile distilled water (dH_2O). The pH of all solutions was measured using a pH 211 microprocessor pH meter (Hanna instruments, RI, USA). Solutions were autoclaved following preparation. Gilson pipettes were used for transfer of liquid volumes up to 1ml (Gilson S.A., France). For volumes greater than 1ml, an electronic pipette aid (Drummond, PA USA) and disposable plastic pipettes (Sarstedt Ltd., Wexford, Ireland) were used. All cell culture reagents were purchased from Lonza (Basel, Switzerland) unless otherwise stated. All cell culture flasks and plates were purchased from Sarstedt (Wexford, Ireland), unless otherwise stated.

2.2 Drugs

Cisplatin and 5-Fluorouracil chemotherapy drugs were prepared in saline and dimethyl sulfoxide (DMSO), respectively. Picropodophyllin (PPP) was purchased from proteinkinase.de and resuspended in DMSO. LY294002, rapamcyin and temsirolimus were all purchased from LC labs (MA, USA) and resuspended in DMSO. VEGF neutralising antibody and recombinant IGF-1 were resuspended in PBS and purchased from R&D systems (MN, USA). R1507 was a generous gift from Roche (Hoffmann-La Roche, Basel, Switzerland). All experiments included a vehicle control where DMSO

was used for resuspension to ensure any effects observed were related to drug treatment specifically and overall concentrations were limited to <0.1%.

2.3 Study population

Tissue samples were obtained from a cohort of consecutive patients who underwent oesophagectomy for oesophageal and junctional adenocarcinoma (Siewert type I and II) at St. James's Hospital, Dublin between July 2003 and December 2009. Patients with type III tumours were excluded (Rudiger Siewert et al., 2000). Samples were biobanked in accordance with the ethically-approved standard operating procedure of the department (Ennis et al., 2010). One hundred patients had circulating IGF-1 levels measured and a subset of these had total IGF-1 (n=24) and tumour mRNA levels of IGF-1 and IGF1R measured (n=78; the remainder did not have pre-treatment diagnostic samples biobanked). One-hundred and sixty one patients with a minimum 36 months of clinical follow-up for survival data had paraffin-embedded tumour sections arrayed on a tissue microarray, which was used to quantify protein expression of IGF1R. Of the 100 patients who had circulating IGF-1 and tumour mRNA levels measured, there were 41 patients included on the tissue microarray.

2.4 Anthropometry

Patient's anthropomorphic data were measured pre-operatively by a single observer. Waist circumference was measured to the nearest 0.5cm at the midpoint between the lower border of the rib cage and the iliac crest following gentle expiration. Central obesity was defined as a waist circumference greater than 80cm in females and 94cm in males (Alberti et al., 2009). Weight was measured to the nearest 0.1kg with the patient

dressed but without shoes or heavy outerwear. Height was measured to the nearest 0.5cm with the patient barefoot. Body mass index (BMI) was calculated as weight/height^2. All patients were asked about their baseline bodyweight at 12 months prior to diagnosis to allow an estimation of weight loss at diagnosis. Visceral fat area (VFA) was calculated by computerised tomography staging scans of cross-sectional transverse images at the level of the third and fourth intervertebral discs are previously described (Beddy et al., 2010). Visceral obesity was defined as VFA exceeding 130cm^2 (Despres and Lamarche, 1993).

2.5 Biological samples

Blood samples were collected pre-operatively following an overnight fast, between 7 and 9am and serum was stored at -80°C. Multiple tumour tissue samples were taken at the time of resection and the resection specimen was then formalin fixed and paraffin embedded for immunohistochemical staining. Tumour tissue was immediately snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was isolated from 20mg of each tumour sample using a standardised protocol (RNeasy®Mini Kit; Qiagen, Crawley, UK) by a single operator. The amount of extracted RNA was quantified by measuring the absorbance at 260nm using a Nanodrop® ND-100 spectrophotometer (Nanodrop Technologies Inc., DE, USA). Purity was checked by the ratio between the absorbance values at 260 and 280nm.

Tissue microarrays (TMAs) were created from the paraffin-embedded resection specimens. Areas containing viable tumour cells were marked by a pathologist. Three 2mm size cores were taken from tumour containing areas of the paraffin-embedded tissue blocks and arrayed on a paraffin block. Where present in the block, the invasive edge of the tumour was marked and included in the TMA.

All patients receiving a diagnosis of oesophageal cancer (including junctional tumours) in St James' Hospital are recorded prospectively in a database. Patient details were extracted by a dedicated database manager who liaised closely with clinical teams and attended multidisciplinary conferences in order to maximise the validity of the database. Data pertaining to patient demographics, clinicopathological stage and outcomes were reviewed for the study population. Tumour, node and metastasis descriptors and the staging classification used for this analysis were those defined in the seventh edition of the American Joint Committee on Cancer staging manual (AJCC, 2010).

2.6 ELISA

2.6.1 ELISA protein quantification from patient samples

Circulating free and total IGF-1 levels in 100 and 24 patients respectively, was determined using commercially available ELISA kits, following manufacturer's instructions (R&D systems, MN, USA). 100µl of undiluted patient serum was added in triplicate to wells. To determine total IGF-1 levels, a pre-treatment acid-ethanol step was carried out using reagents supplied in a commercial kit, to disassociate IGF from its binding proteins. All assays were performed in triplicate with duplicate samples between plates to ensure reproducibility.

2.6.2 ELISA protein quantification from *in vitro* cell culture samples

Enzyme-linked immunosorbent assay (ELISA) assays (R&D systems, Inc., MN, USA) were used to determine cytokine concentrations of free IGF-1 and VEGF in cellular supernatants.

In order to assess cytokine protein production in cellular supernatants, 2.5 x 10^4 cells were plated in a 96 well plate and allowed to adhere overnight at 37°C in 5% CO2.

Cells were treated and after the relevant time period of treatment the supernatants were harvested and stored at -20°C. 100µl of PBS was added to the cells and the plate was frozen at -80°C. The plate was freeze-thawed for 3 cycles in order to lyse the protein content which was then measured by BCA assay. Cytokine production, as measured by ELISA, were corrected for the total protein content of cells – to account for treatment induced apoptosis.

2.6.3 ELISA protocol

The high binding 96 well ELISA plate (Greiner-Bio, Frickenhausen, Germany) was coated with 50µl capture antibody overnight at 4°C and washed three times in washing buffer (PBS with 0.05% Tween 20). Samples were added to each well in triplicate. A standard curve was created by serially diluting the appropriate recombinant protein supplied with the kit and 50µl was added to the plate in triplicate. A set of wells were incubated with 1% BSA only as a background control. Samples were incubated overnight at 4°C. The plate was washed three times in washing buffer. 50µl of secondary antibody was added to each well and incubated at room temperature for 2hours, followed by 5 washes with washing buffer. A 50µl volume of streptavidin-HRP (1:200 dilution in 1% BSA in PBS) was added to each well and incubated for 20 minutes at room temperature in the dark. 50µl of substrate solution tetramethylbenzidine (TMB) was added to each well and covered with tinfoil until the colour developed (2-15 minutes). 25µl of stop solution (1M H_2SO_4) was added to each well to stop the reaction and the plate read immediately at 450nm using an Alpha Fluor Plus spectrophotometer (Tescan Trading AG, Switzerland). Protein concentrations were determined by interpolating from a standard curve of known concentrations. All assays were performed in triplicate with duplicate samples between plates to ensure reproducibility.

Table 2.1 ELISA kits used

ELISA kit	Capture Antibody	Top standard	Detection antibody
VEGF	1μg/ml	2000pg/ml	100ng/ml
IGF-1	4 μg/ml	2000pg/ml	80ng/ml

2.7 Quantitative Polymerase Chain Reaction (qPCR)

2.7.1 Isolation of RNA from cell lines

RNA was extracted using TriReagent (Molecular Research Center (MRC), Montgomery Road, OH, USA). Cells were washed once in PBS (Lonza) and 1 ml TriReagent was added. Cell lysate was scraped into a tube and kept at – 80 ° C until extraction was carried out up to one month later. Extraction was carried out according to MRC recommendations as follows: sample was defrosted and kept at room temperature for 5 minutes. Following this 100 μl 1-bromo-3-chloro-propane (Sigma) per ml TriReagent sample was added, vortexed thoroughly and kept at room temperature for 15 minutes. The sample was then centrifuged at 12,000 g for 15 minutes at 4 ° C to separate the sample into layers, the clear supernatant containing RNA was carefully removed to a clean tube and 0.5 ml isopropanol per ml TriReagent was added and sample was vortexed thoroughly. Centrifugation was carried out at 12,000 g for 8 minutes at room temperature. The pellet was washed with 75 % ethanol and resuspended in 25 – 40 μl RNase free water.

2.7.2 Isolation of RNA from Tissue

RNA from tumour tissue was extracted by a dedicated biobank manager (Dr Sarah McGarrigle) using RNeasy mini kit (Qiagen) using kit instructions.

2.7.3 RNA quantification

RNA purity was tested using a Nanodrop 1000 spectrophotometer (version 3.1.0, Nanodrop technologies, DE, USA) and a 260/280 ratio of between 1.8 and 2 was determined high purity and used for cDNA synthesis. A ratio 260:230 ratio greater than 1.7 indicated the samples were free from phenol contamination. RNA was quantified using 1µl of each sample of isolated RNA was loaded onto the instrument and concentration was measured in ng/µl.

2.7.4 cDNA synthesis

All cDNA synthesis was carried out in a sterile hood environment using between 100 ng and 1 µg RNA. The reverse transcriptase enzyme and buffer were purchased from Bioline (Bioline, Kilkenny, Ireland), all other reagents were purchased from Invitrogen (Invitrogen Corp., CA, USA). RNA was diluted in RNase free water and 0.5 µg/µl random primers (40 ng/µl, Invitrogen) was added to 1µl RNA to a total volume of 12µl. The sample was heated to 70 ° C for 10 minutes and then chilled on ice for 1 minute. Following this, a master mix was added consisting of RNaseOUT recombinant ribonuclease inhibitor (1 U/µl, Invitrogen), dNTPs (10mM, prepared as a 1:1:1:1 ratio of dATP, dGTP, dTTP and dCTP), Bioscript reverse transcriptase (200units/µl) in 5X Bioscript reaction buffer and this mixture was incubated at 37 ° C for 1 hour. The resulting cDNA was stored at – 20 ° C.

Table 2.2. Reagents and volumes used in cDNA synthesis

Reagent	Volume (µl) (per sample)
Random Primers	1
250ng of RNA + RNase free H_2O	11
Heated for 10 min at 70°C and cooled on ice for 1 min then following mix added:	
5X Bioscript reaction buffer	4
H_2O	2
DNTPs	1
RNase out	0.5
Bioscript Reverse Transcriptase	0.5
Total Volume	**20**

2.7.5 Reverse-transcription polymerase chain reaction (RTPCR)

cDNA was used as a template for PCR. The following PCR master mix was added to each sample: Mangomix® Master mix (Bioline, Kilkenny, Ireland) forward primer (20µM), reverse primer (20µM) and RNase free water. 1µl cDNA was used per reaction and a non template control was included for each primer set by replacing cDNA with 1µl of RNase free water.

2.7.6 Quantitative Reverse transcription polymerase chain reaction

Quantitative PCR was used to quantify mRNA expression in samples relative to the 18s ribosomal RNA endogenous control. This was carried out using a probe based method, which works as follows: sequence specific probes are labelled with a flourescent reporter molecule at the 5' end, and a fluorescence quencher molecule at the 3' end. The

close proximity of the reporter to the quencher prevents detection of fluorescence. Upon transcription, the 5-3 exonunclease activity of the Taq polymerase removes the quencher molecule and allows fluorescence emission, detected following laser excitation of the sample. All reagents were purchased from ABI Biosystems (CA, USA). A master mix containing primer/probe and Taqman® Gene Expression Master Mix, was added to 1µl of cDNA template (see table below). A final volume of 20µl was pipetted into a well of a MicroAmp™ Optical 96 well reaction plate in duplicate(ABO Biosystems, CA, USA) and a real time PCR was performed on the ABI Prism 7500 (ABI Biosystems, CA, USA) real time thermal cycler. The plate was heated to 95°C for 15 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The FAM-labelled IGF1R probe set (assay ID Hs01547656_m1; amplicon length 68 base pairs) was used for the IGF1R receptor and FAM-labelled IGF-1 probe set (assay ID Hs00609566_m1; amplicon length 64 base pairs) for IGF-1. Taqman® ribosomal RNA VIC-labelled probe for the 18S gene served as an endogenous control for RNA quality and quantity. A non-template control was included in every experiment. All samples were assayed in triplicate.

The relative quantification of target gene expression was performed using the comparative cycle threshold (CT) method. This method is based on the use of a calibrator sample which permits quantification in the unknown tumour samples. A sample with a median expression of IGF1R was used as the calibrator sample. The relative target expression was given by the formula: $2(-\Delta\Delta CT)$, where $\Delta\Delta CT = \Delta CT$ patient sample- ΔCT calibrator sample; with the ΔCT=CT target-CT 18S.

Table 2.3. Reagents and volumes used for qPCR

Reagent	Volume (µl)
Master Mix	10
Forward Primer	1
Reverse Primer	
Probe	
RNase Free Water	8
cDNA	1
Final Volume	**20**

2.7.7 Human cancer pathway profiler arrays

For Human Cancer Profiler Arrays, cDNA synthesis was generated using First Strand cDNA synthesis kit (Super Array BioScience Corporation, Frederick, MD, USA). 250ng of each RNA sample was combined with 1ml of random primers in a sterile PCR tube and made up to a final volume of 10µl with RNase free water. The contents were vortexed briefly and centrifuged before being placed in a thermal cycler at 70°C for 3 min, followed by 37°C for 10 min. 10µl of reverse transcription cocktail (4µl of 5x RT-Buffer, 4µl of RNase free water, 1µl of RNase inhibitor and 1µl of reverse transcriptase) was added to each sample, briefly vortexed and centrifuged before incubating at 37°C for 1 hr. Samples were then heated to 95°C for 5 min to degrade the RNA and deactivate the reverse transcriptase. 80µl of RNase free water was added to each sample and mixed well to give a final volume of 100µl.

Changes in the expression profile of a panel of genes representative of molecular pathways of tumourigenesis were examined using RT^2 profiler™ Human Cancer Pathway Finder PCR Array technology (Super Array Bioscience Corporation, Frederick, MD, USA). This array is based on a 96 well plate format, containing primer sets for 84 genes involved in six biological pathways of tumourigenesis: apoptosis, cell

cycle, angiogenesis, invasion and metastasis, signal transduction and adhesion, as previously described (Hanahan and Weinberg, 2000). Five housekeeping genes were included to normalise the data and two negative controls were included to estimate the level of genomic DNA contamination in the PCR system. A full list of genes measured on this array are listed in Appendix II.

Samples were prepared for loading onto the qPCR array by adding 1225µl of 2X SuperArray PCR master mix and 1127µl RNase free water to 98µl of the diluted cDNA synthesis reaction to give a final volume of 1127µl. 25µl of reaction mix was added to each well except the non template control, to which the same mix was added with RNase free water instead of cDNA. Real time PCR detection was performed on an ABI Prism 7500 (ABI Biosystems, CA, USA) real time thermal cycler. The plate was heated to 95°C for 15 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The threshold cycle (Ct) for each well was calculated using the instrument software and data analysis was carried out using a Microsoft Excel based data analysis template. Data analysis was based on the ΔΔCt method with raw data normalised by the house keeping genes included on the plate (18S). Gene changes were only reported if the transcript was amplified before 35 cycles.

2.8 Immunohistochemical staining

Specificity of antibodies was determined by Western blotting. Briefly, oesophageal adenocarcinoma cell lines (JH-Eso-AD1 and OE33) were lysed and protein samples were run on 10% polyacrylamide gel and transferred onto biotrace polyvinylidene fluoride (PVDF) membrane. The western blot was performed as described in section

2.17. Single bands were obtained at the appropriate size. The immunohistochemistry staining for IGF1R was also optimised by staining 50 full-face sections of resected oesophageal cancer paraffin-embedded blocks. The sections were reviewed to ensure tissue integrity, specific staining and minimal background (non-specific) staining.

Positive control tissue sections with known protein expression were included. Tissue microarrays (TMAs) were generated using a Beecher Microarrayer. Tissue microarrays (TMAs) using an automated staining system on a Bond III automated immunostainer (Leica Microsystems, Wetzlar, Germany). Briefly, TMA blocks were then cut into 4μm sections, mounted and baked overnight at 37°C. Slides were deparaffinised, rehydrated and antigen epitope retrieval steps were carried out using a sodium citrate buffer solution pH 6.0 at 100°C for 20 minutes. Sections were blocked using diluted serum from the animal in which the secondary antibody was raised. The primary antibody was added at the appropriate dilution and then incubated at the temperature and for the time indicated in Table 2.2. A secondary biotinylated antibody was incubated on the sections, followed by an avidin-biotin complex reagent and then diaminobenzidine (Sigma, MI, USA). Sections were counterstained with haematoxylin (Sigma, MI, USA), dehydrated through methanol and xylene and mounted under coverslips (VWR International, West Chester, PA, USA) using DPX mountant (B.D.H. Ltd., Poole, Dorset, UK.

Table 2.4 List of antibodies used in immunohistochemisty

Antibody	Supplier	Isotype	Positive control	Conditions
Anti-IGF1R	R &D systems	Goat; polyclonal	Placenta	1:150, o/n 4°C
Anti-STMN1	Cell signalling	Rabbit; monoclonal	TAC*	1:3000, o/n 4°C
Anti-VEGF	Millipore	Rabbit; Polyclonal	Placenta	1:500, RT 2 hrs

*TAC is a composite block with tonsil, normal appendix and carcinoma tissue

Slides were digitally scanned using Scan Scope (Aperio Technologies, CA USA) and images taken with Image Pro-plus 4.1 software (Media cybernetics, Gelichen, Germany). Staining was graded by three independent observers. The intensity of staining of tumour areas on the section was scored between 0-3, where zero was negative, one was weak, two was moderate and three represented strong staining.

The quantity of positive staining within the core was scored between 0 and 100%. The intensity score and percentage positivity were multiplied giving an overall score for each core of 0-300. The average overall score for each of the three cores per patient was calculated and the mean score for each patient was collated with clinicopathological and survival data from the prospective departmental database. Analysis of data on protein expression of IGF1R included all patients – patients with negative staining were scored 0 and included in the analysis.

2.9 Cell culture

All cell culture work was carried out in an aseptic manner within a grade II laminar air flow cabinet. The cabinet was allowed to run for at least 20 minutes before use. The

cabinet was made sterile with 70% (v/v) ethanol. All equipment and reagents were sanitised in a similar manner before being brought into the cabinet. A clean lab coat with elasticated cuffs and sterile disposable gloves were worn at all times. The cabinet was cleaned in a similar manner following completion of the work and air was once again allowed to circulate for 20 minutes.

2.9.1 Oesophageal cell lines

The following oesophageal cells lines were used during this study: OE33 (oesophageal adenocarcinoma) and JH-Eso-Ad1 (oesophageal adenocarcinoma). OE33 cells were obtained from the American Collection of Cell Cultures (ATCC). JH-Eso-AD1 were obtained under materials transfer agreement from Drs. Anirban Maitra and James R Eshleman in John Hopkins University.

OE-33 and JH-Eso-Ad1 were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium which was supplemented with 10% (v/v) foetal bovine serum (FBS) and penicillin streptomycin (50 units penicillin, 50 units/ml streptomycin). Cells were incubated in 25cm^2 or 75cm^2 vented flasks at 37°C in a humidified atmosphere with 5% CO_2.

Cells were examined daily using an inverted phase-contrasted Nikon microscope (Nikon Corp., Tokyo, Japan). Sub-culturing was performed when the cells reached 80-90% confluency. Adherent cells were detached for sub-culturing by trypsinisation. The growth medium was decanted and the cells were washed with 10ml of 0.01M phosphate buffered saline (PBS) (13.8mM NaCl, 2.7mM KCl, pH 7.4) to remove residual FBS. 1ml (25cm2 flasks) or 2ml (75cm^2 flasks) of trypsin ethylene-diamine tetra-acetic acid (EDTA) (0.05% (w/v) trypsin, 0.02% (w/v) EDTA) was added to the flasks. Flasks

were incubated at 37°C for approximately 5 minutes to allow the cells to detach from the flask surface. 10ml of complete medium (medium containing 10% FBS) was then added to the flasks to inactivate the trypsin. Cells were transferred to a sterile tube and pelleted by centrifugation. (Thermo IEC, Needham heights, MA, USA) at 1300 x g for 3 minutes. The supernatant was then discarded and the cell pellet re-suspended in 10ml of complete medium. This suspension was used to seed fresh flasks at a number of different ratios. Complete medium (5 or 10ml was added to the cells in $25cm^2$ and $75cm^2$ flasks respectively. All culturing experiments were carried out within 20 passages. Representative images of the cancer cell lines in their exponential growth phase are shown in Figure 2.1.

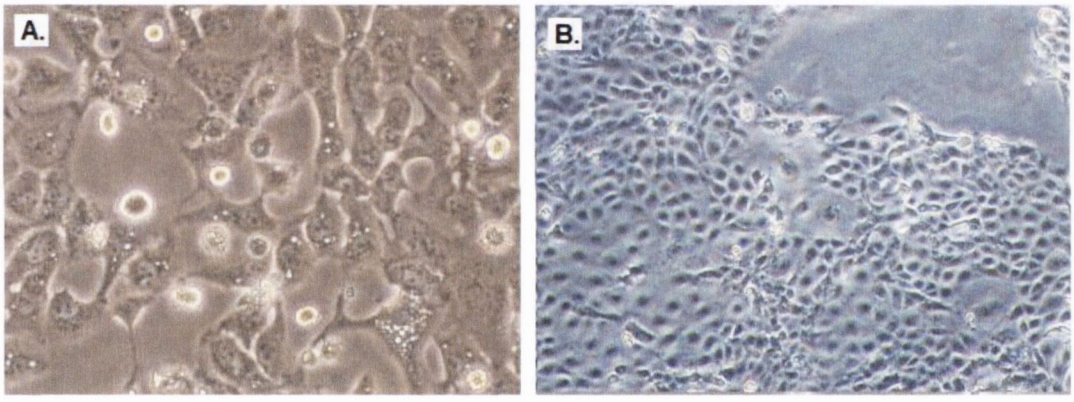


Figure 2.1 Representative images of (A). OE33 and (B). JH-Eso-AD1 cell lines in expontential growth phase. 10x magnification, light microscope

Cell stocks were stored frozen under liquid nitrogen in a cryofreezer (NuAire Corp., Plymoth, MN, USA) or at -80°C. Stocks were prepared from cells growing in the exponential phase at less than 80% confluency. To prepare frozen stocks, cells were washed in 10ml PBS and trypsinised as above (section 2.9). 5-10ml of complete medium was added to the trypsinised cells and the cells were re-suspended 5% (w/v)

dimethyl sulphoxide (DMSO) in complete media. 750µl of DMSO solution was added for each cryovial of cells to be frozen down. The solution was applied in a drop-wise manner to the cells during re-suspension. Cryovials were placed at -20°C overnight before being transferred to -80°C for short term storage. For long-term storage, vials were transferred to liquid nitrogen.

Cryovials were removed from liquid nitrogen and placed at 37°C to thaw rapidly. Cells were transferred to a fresh $25cm^2$ flask, to which 5ml of complete media had been added. Cells were incubated at 37°C for 24 hours. Media was replaced the following day to remove any dead cells and residual DMSO. Flasks were maintained and passaged as previously described (2.9).

2.9.2 Cell counting and viability

Cell counting was carried out using a bright-line haemocytometer (Hausser Scientific, PA, USA). Cell viability was examined using a trypan blue dye exclusion assay. 180µl 0.4% (v/v) trypan blue was mixed with 20µl of this suspension was added to the counting chamber of the slide. Viable cells were stained due to their active exclusion of trypan blue, whereas dead cells stained blue due to their disrupted membranes. The number of viable cells was counted in each of the four corners of the grid. The number of cells/ml was then calculated using the following equation:

$$\text{Cells/mL} = (N / 4) \times 10 \times 10^4$$

Where,

N = total cell number counted

4 = number of fields counted

10 = dilution factor

10^4 = constant

When necessary, all densities were adjusted using complete medium, unless otherwise stated.

2.9.3 Mycoplasma testing

Upon receiving the cells and every 4-6 months following, cells were tested for mycoplasma infection using the MycoAlert® mycoplasma detection assay (Lonza). This assay exploits the activity of certain mycoplasmal enzymes, which react with the absence of mycoplasma and can then be detected by measuring the ratio of the level of ATP in sample before the addition of the substrate, to that following addition of the substrate. Cells were passaged in antibiotic free media for two passages before carrying out the assay. Briefly, 2ml of culture medium was transferred into a centrifuge tube and centrifuged at 200 x g for 5 minutes to pellet floating cells. 100µl of the cleared supernatant was then transferred into a luminescence compatible plate. 100µl of MycoAlert® reagent was added to each sample for 5 minutes and a 1 second integrated reading was taken on the luminometer (reading A) (Victor^{TM}, Perkin Elmer, Columbus, OH). 100µl of MycoAlert® substrate was then added to each sample for 10 minutes and a second reading was taken (reading B). The ratio of reading B to reading A was used to determine the presence of mycoplasma in the cell culture medium. Cells infected with mycoplasma produced ratios greater than 1. Cells that tested for the presence of mycoplasma and frozen aliquots were discarded and the incubator in which they were stored was cleaned thoroughly.

2.10 VEGF neutralisation

The reported ND_{50} neutralising dose for a 50% effective neutralisation of 10ng/ml VEGF from cell lines in culture according the datasheet for VEGF neutralising antibody (R&D systems, MN, USA) is 1000ng/ml. VEGF production in the supernatant of both cell lines as measured by ELISA (section 2.5) was no greater than approximately 0.5ng/ml). Thus the neutralising dose of VEGF used for all experiments was 50ng/ml. Supernatants from cells treated with neutralising antibody alone were assayed for VEGF production and confirmation of neutralisation to below assayable levels was confirmed (<12.5pg/nl).

Cells were treated in a 6 well format with 3.0 $x10^6$ cells per well and treated with media only (control); VEGF neutralising antibody (50ng/ml); PPP (relevant IC50 dose (Table 2.5) and both the IC50 dose of PPP and VEGF neutralising antibody.

2.11 MTT assay

Cells were seeded in 96 well plates at a concentration of 2.5 x 10^4 cells/ml for 24hr treatments and allowed to adhere to the plate for at least 6 hr (overnight). Cells were then subsequently grown in media as a control and in media with drug treatment for 24hrs. MTT reagent was dissolved in RPMI media was added to the wells to a final concentration of 0.5mg/ml and incubated for 2 hr at 37°C. The media was subsequently removed, the cells washed with PBS, and 100µl of DMSO was added to each well to lyse the cells. The 96 well plate was incubated in the dark for 5 min to allow colour development and read immediately at 450nm using an Alpha Fluor Plus Spectrophotometer (Tescan Trading AG, Switzerland) to determine viable cell number.

2.11.1 MTT assay to calculate IC_{50} dose

For experiments to determine the IC_{50} dose, a range of drug concentrations were chosen based on the usual IC_{50} dose reported in the literature. After 24 hours of drug treatment, cell viability was determined by MTT assay. Experiments were repeated in triplicate. The percentage of cell growth inhibited was calculated as viability relative to control cells (%age absorbance versus control, where control absorbance =100%). The dose-response curve (expressed as a % of cell viability observed in unntreated controls) was used to determine the IC_{50} by fitting a line using logisitic regression analysis.

The IC_{50} dose was then used in further experiments using the relevant drug in each cell line (as reported in Table 2.5).

Table 2.5 IC_{50} doses calculated by MTT of drugs used *in vitro* for each cell line.

IC_{50} dose calculated per cell line	OE33	JH-Eso-Ad1
Picropodophyllin	12.79nM	3.14nM
LY294002	0.1064μM	0.05118μM
Rapamycin	32.96 μM	19.04 μM
Temsirolimus	132.7 μM	49.19 μM

2.12 BrdU proliferation assay

Cells were seeded at a dilution of 2.5 x 10^4/ well in 96-well plates in the appropriate complete media and allowed to adhere overnight at 37°C. Following the overnight incubation in serum-depleted media (0.5% FBS), cells were treated for 24 hours with the appropriate inhibitors. Cell proliferation was then assessed using a BrdU cell proliferation ELISA (Roche Diagnostics Ltd., Sussex, UK). The basis of this assay is as

follows: following culturing of the cells in 96-well plates, BrdU is added to the cells, and the cells reincubated. During this labelling period, the pyrimidine analogue BrdU (5-bromo-2'-deoxyuridine) is incorporated in place of thymidine into the DNA of proliferating cells. The anti-BrdU POD antibody then binds to the BrdU incorporated in newly synthesised cellular DNA. The immune complexes are then detected by subsequent substrate reaction. 10µl of a 1:1000 dilution of BrdU labelling solution was added to each well for 4 hours at 37°C. The media was then removed and the cells fixed and denatured with 200µl of a fixative solution for 30 minutes at room temperature (RT). 100µl anti-BrdU-POD (mouse monoclonal antibody, peroxidise conjugated) working solution was then added to each well for 5-10 minutes (or until colour change was sufficient for photometric detection). 25µl of 1mM H_2SO_4 was then added to stop the reaction. Absorbance was measured on an Alpha Flour Plus plate reader (Tecan Trading AG, Switzerland) at 450nm with the reference set to 690nm. Wells containing cells but no BrdU label were used to subtract back ground absorbances and percentage increase/ decrease in proliferation was calculated relative to untreated cells.

2.13 Propidium iodide cell cycle analysis

Cell cycle analysis was performed by Propidium iodide (PI) staining and flow cytometry (Darzynkiewicz and Juan, 1997). PI is a fluorescent dye, which passes through a permeabilised membrane and intercalates into cellular DNA. The intensity of the PI signal is proportional to DNA content, and the relative content of DNA indicates the distribution of a population of cells throughout the cell cycle. Cells in the G0/G1 phases of the cell cycle have a DNA content of 2n, cells in the G2/M phases of the cell cycle have a DNA content of 4n, while cells in the S phase of the cell cycle have a DNA

content of less than 4n but greater than 2n. Cells in the exponential growth phase were harvested by trypsinisation as previously described (section 2.3.2). Cells were seeded at a density of 3.7×10^5 cells in T25 cm^2 flasks, and allowed to adhere overnight at 37°C in 5% CO_2/95% humidified air. Cells were then treated with the relevant treatment, whilst control flasks were mock treated. At 6 h, 10 h, 24 h and 30 h post irradiation, cells were collected by trypsinisation as described previously (section 2.3.2) and transferred to 5 mL falcon tubes (BD Biosciences). Cells were then centrifuged at 180 × g for 3 min, and the supernatant decanted. The cell pellet was then fixed and permeabilised by drop-wise addition of 4.5 ml ice-cold ethanol (70% v/v in PBS) (Merck) while vortexing, to avoid the formation of aggregates. Permeabilisation aids the access of PI to cellular DNA. Cells were stored in the fixative for at least 2 h at 4°C. Fixed cells were then centrifuged at 180 × g for 3 min, and the supernatant decanted. Cells were then washed with 1 ml PBS and centrifuged as before. Each sample was resuspended in 0.5 mL Triton X-100 (0.1% v/v in PBS), containing PI (0.02 mg/mL) and RNase A (0.2 mg/mL), except for appropriate controls. Addition of RNase A ensures any RNA present is digested, preventing any interference with the DNA signal. Samples were incubated at 37°C for 30 min and then at RT°C for 1.5 h in staining solution. Unstained control samples used for instrumental setup were incubated in 1 mL PBS. DNA content was then measured using a $CyAn_{ADP}$ (Dako, Glostrup, Denmark) flow cytometer. A minimum of 10,000 events were collected, and doublets were excluded from analysis using doublet discrimination. Data were analysed by histogram plot using Summit v4.3 software (Dako, Glostrup, Denmark). The X-axis of the histogram plot represents PI fluorescence or DNA content, whilst the Y-axis represents cell number.

2.14 High content screening: Apoptotic morphology imaging

Morphological changes consistent with apoptosis were assessed using a multiparameter apoptosis assay to individually analyse changes on an individual cell level using high content screening. 1.8 X 10^4 cells/well were seeded in a 96 well plate and allowed to adhere overnight at 37°C in 5% CO_2/95% humidified air. Cells were washed with 1 ml warmed PBS and supplied with 100µl warmed complete medium. The IC_{50} dose of cisplatin (0.01µM, previously determined in-house (Allott, 2010)) was used to treat cells as a positive control. The IC_{50} dose of PPP was the treatment of interest. Both treatments were applied for a period of 24 hours. A mitotracker red/Hoeschst solution was prepared (5.5µl of 1mM of MitoTracker Red + 11µl 1mM Hoechst 3334 to 5.5ml of fresh cell culture medium). Thirty minutes before the end of the incubation period 50µl of the mitotracker red/Hoescht solution was added to the cells and they were incubated for a further 30 minutes at 37°C. 100µl of warmed fixation solution (1.1ml of 37% formaldehyde+ 9.9ml PBS) was added to the cells and cells were incubated for 10 minutes at room temperature. Wells were aspirated and cells washed once with PBS. Wells were aspirated and 200µl permeabilisation buffer was added to each well (0.5% Triton-X-100 in PBS). Cells were incubated for 90 seconds. Wells were aspirated and 50µl of Alexa Fluor 488 Phalloidin solution (27.5µl stock solution to 5.5ml PBS, made up fresh for each assay) was added to each well. Cells were incubated for 30 minutes at room temperature and then washed twice with PBS. Cells were stored in 200µl PBS at 4°C for up to one week prior to analysis using the InCell analyser. Experiments were performed in triplicate.

2.14.2 InCell analysis

Morphological changes were objectively analysed using the IN Cell Analyzer 1000 (GE Life Sciences, Buckinghamshire, UK) which is a modular lamp based cellular and subcellular imaging system for automated imaging in cells. It is an automated process, using algorithms to extract and analyse quantitative data from cell images so that changes in cell morphology including the localisation of intra-cellular organelles can be objectively measured and compared. Phalloidin is a green fluorescent label which binds to f-actin in the cytoskeleton; MitoTracker is a red label for the mitochondria and Hoescht is a nuclear stain. Mitochondria in apoptotic cells fragment, become leaky and lose their membrane potential thus the In Cell analyser can measure increases in mitochondrial number, decreases in intensity and the area per object become heterogenous (large SD). On induction of apoptosis cells may undergo changes in shape and in proximity and spacing within colonies. The spreading of cytoplasm fluorescence intensity decreases upon induction of apoptosis indicating cytoplasm becomes concentrated around the nucleus. Within the nucleus, intensity of nuclear DNA increases as does the total area per cell of DNA as the nuclear DNA fragments and granulates. Representative images were taken of each treatment.

Table 2.6 Morphological changes consistent with apoptosis (Häcker, 2000)

Cellular target	Phenotypic change
Nucleus	Condensation, disintergration
Cell shape and structure	Detachment, blebbing, disassembly into apoptotic bodies
Mitochondria	Swelling, condensation

2.15 Annexin-V-FITC/Propidium Iodide Apoptosis assay

Apoptosis was measured using annexin V-FITC and propidium iodide staining and assessed by flow cytometry. The annexin V-FITC conjugate facilitates rapid fluorimetric detection of apoptotic cells. Annexin V is an anti-coagulant protein, which preferentially binds to negatively charged phospholipids. Early in the apoptotic process, phospholipid asymmetry is disrupted, leading to the exposure of phosphatidylserine (PS) on the outer leaflet of the cytoplasmic membrane. Annexin V binds to PS phospholipid monomers in a ratio of approximately 1:50. PI is a fluorescent dye that intercalates into cellular DNA. PI is membrane impermeant, and therefore only stains the DNA of necrotic cells and dead cells. Thus, the combination of annexin V and PI allows for differentiation between early apoptotic cells (annexin V-FITC$^+$), late apoptotic (secondary necrotic) and/or necrotic (annexin V-FITC$^+$ and PI$^+$), and viable cells (unstained).

Cells in the exponential growth phase were harvested by trypsinisation as previously described (section 2.9.2). Cells were seeded at a density of 3.0×10^6 cells/well in 6-well plates, and allowed to adhere overnight at 37°C in 5% CO_2/95% humidified air. Cells were washed with 1 mL warmed PBS and supplied with 1.5 mL warmed complete medium. Cells were treated with the relevant drug treatments for a 24 hour period and supernatants (containing non-adherent cells) were collected in 5 mL falcon tubes (BD Biosciences, San Jose, CA, USA). Adherent cells were harvested by trypsinisation, as described previously (section 2.3.2), combined with their respective supernatants and pelleted by centrifugation at 180 × g for 3 min. Supernatants were discarded, and the cell pellets were washed with 0.5 mL PBS, and centrifuged as above. Supernatants were discarded, and pelleted cells were resuspended by vortexing in 1 mL 1X binding buffer

(0.01 M HEPES (Gibco BRL, Paisley, UK), NaOH, pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$). Cells were then pelleted by centrifugation at 180 × g for 3 min and the supernatants discarded. A 3 μL volume of annexin V-FITC (IQ products, Groningen, The Netherlands) was added to each sample, with the exception of appropriate controls. Samples were vortexed, and incubated for 15-20 min in the dark at 4°C. Cells were then resuspended in 500 μL PI (1/4000 dilution of 1 mg/mL stock in 1X binding buffer) (Invitrogen, Carlsbad, CA, USA), with the exception of appropriate controls, which were resuspended in 0.5 mL 1X binding buffer. Tubes were vortexed, and apoptosis was measured using a FACSCalibur flow cytometer (BD Biosciences). Instrumental controls were set up in parallel with the samples in order to define the boundaries of each population during analysis on the flow cytometer. These controls included: unstained cells, single-stained cells with annexin V-FITC only and PI only. A minimum of 10,000 events were collected and the data was analysed by dot plot using CellQuest software (BD Biosciences). The X-axis (FL1 channel) of the dot plot represents the log Annexin V-FITC fluorescence, whilst the Y-axis (FL2 channel) represents the log PI fluorescence. The flow cytometer was calibrated using the instrumental control. Data analysis was carried out using Cell Quest software by a trained user of the instrument Dr Joanne Lysaght. The x axis (FL1) of the dot plot represents the log Annexin V-FITC fluorescence while the Y axis (FL2) represents the log PI fluorescence. The percentage of apoptosis in treated cells was expressed relative to untreated control cells from three independent experiments.

2.16 Transfections

Small interfering RNA (siRNA) is a class of double-stranded RNA molecules, 20-25 nucleotides in length, which may be used to silence gene expression of a target gene of

interest. siRNA inhibits gene expression through sequence-specific dsRNA-mediated degradation of the target messenger RNA. This silencing may be observed through several cell generations. In order for siRNA knockdown to be successful the following conditions must be met: 1. Target sequence selection and siRNA design. 2. Suitable cell line; 3. Optimised delivery conditions (using a transfection reagent); 4. Abundance and turnover rate of the target mRNA of interest; 5. Ability to assay gene or protein of interest using RT-PCR or Western blotting as appropriate.

Dharmacon siRNAs were used throughout for IGF1R knockdown experiments (Thermo Scientific, Waltham, MA, USA). A SMARTpool reagent was purchased. SMARTpool technology incorporate a multi-component algorithm that identifies siRNAs with a very high probability of potent and specific silencing. SMARTpool reagents combine four SMARTselection-designed siRNAs into a single pool to increase the probability of potent silencing.

The delivery of siRNA into cells requires the use of transfection reagents. Lipid-based transfection reagents facilitate transfection by complexing into aggregates with the negatively charged nucleic acid complexes. These nucleic acid-transfection reagent complexes are then efficiently taken up by cells. The following transfection reagents were trialled during optimisation steps included: Dharmafect 1; Lipofectamine, Lipofectamine 2000; Fugene; TransIT (Table 2.7).

For best results, at least 80% of the initial cells must be viable meaning that there is a maximal transfection reagent and siRNA concentration, individual to each cell type, beyond which excessive toxicity will result in too few viable cells remaining for analysis.

Cells were trypsinised and counted and plated at a density of 3.0 x 10^6 cells / well of a 6-well plate in antibiotic free complete medium. This plating density was empirically altered during attempts to optimise knockdown (Chapter 4, section 4.3.1.3). Cells were incubated and allowed to adhere overnight at 37°C with 5% CO_2. The recommended initial final concentration of siRNA was 5-50nM for optimisation steps. A 5µM solution of siRNA was made up in sterile PBS. The appropriate amount of siRNA was diluted in 200µl serum-free media. A 200µl solution with the appropriate concentration of transfection reagent was made in serum free media. These solutions were mixed gently by pipetting and incubated for 5 minutes at room temperature. One solution was then added to the other for a total volume of 400µl and incubated for 20 minutes at room temperature. 1.6ml of antibiotic free complete media was then added for a final volume of 2ml. Media was removed from the cells, cells were washed twice in PBS and it was replaced with the siRNA-transfection reagent solution. The length of incubation was varied from 24 to 72 hours. To limit toxicity, media was replaced after the 24 hour timepoint. The following controls were used: untreated cells; mock transfection (transfection reagent only, no siRNA); Positive control (siRNA targeting an endogenous gene, GAPDH); non-specific/scramble control siRNA (siCONTROL TM non-targeting siRNA) and the test siRNA. In some experiments, siGLO® transfection indicators was added instead of scramble siRNA to assess transfection efficiency. siGLO® green has a FITC fluorophore label and was resuspended in RNase-free PBS. After 24 hours delivery and detection of siGLO® was assessed using a 400x magnification with an exposure time of 2.5-5 seconds and a gain of 8 using a green fluorescence filter.

All experiments were performed in triplicate. Following an appropriate time period (24-48 hours for mRNA and 24-72 hours for protein) either RNA was harvested and mRNA

expression measured with RT-PCR (section 2.7) or protein was harvested and protein measured using Western Blotting (section 2.17).

The toxicity on OE33 from a number of different transfection reagents was assessed. The concentrations used per well of a 6-well plate for each transfection reagent are reported in table 2.7.

Table 2.7 Transfection reagents tested in OE33 cell line

Transfection reagent	Company	Concentration (µl)	?>20% toxicity
Dharmafect 1	ThermoScientific	0.5	Yes
		1	Yes
		1.5	Yes
Lipofectamine	Invitrogen	3.75	No
		5	No
		7	Yes
		10	Yes
Lipofectamine 2000	Invitrogen	2.5	No
		5	No
		7.5	No
Fugene	Promega	2	No
		6.4	No
		13.4	Yes
TransIT	Mirus Bio	1.67	Yes
		2.5	Yes
		3.33	Yes

2.17 Protein electrophoresis

2.17.1 Protein isolation from cell cultures

After decanting culture medium from flasks, cells were washed in cold 1xPBS. If a large number of non-adherent cells were observed in the culture medium, the medium was collected and centrifuged at 1300 rpm x 3 minutes. The recovered cells were re-suspended in ice cold-lysis buffer (RIPA buffer (5mM Tris HCl, pH 7.4, 150m NaCl, 5mM EDTA, 0.5% (v/v) Triton-X 100, 0.5% (w/v) sodium dodecyl sulphate (SDS), 0.5% (w/v) deoxycholic acid)+ 1mM phenylmethylsulphonyl fluoride (PMSF)+ 1mM protease inhibitor cocktail (5µg/ml aprotinin, 5µg/ml leupeptin) + 100µM sodium orthovanate). 100µl lysis buffer was used to re-suspend the cells per $75cm^2$ flask. The re-suspended cells were then combined with adherent cells in the appropriate flasks and left on ice for 30 minutes. Lysed cells were collected using cell scrapers and cell lysates ere passed through a 1ml U-100 syringe (Becton Dickinson, Franklin Lakes, NJ, USA) 10 time to shear genomic DNA. Isolated protein lysates were stored at -80°C.

2.17.2 Nuclear and cytoplasmic extracts

Cells were trypsinised as described in section 2.9.2. The pellet was resuspended and washed in PBS and then centrifuged at 1300rpm for 3 mins. 400µl of Buffer A was added to the cell pellet (10mM HEPES (pH 7.8); 10mM KCL; 0.1mM EDTA; 2mM DTT; 1mM PMSF). Cells were left on ice for 15 minutes. 25µl of 10% Nonide + P-40 (NP40) and the solution was vortexed vigorously for 10 seconds. Cells were then centrifuged for 30 seconds at 10,000rpm. The supernatant was removed and stored at -20°C – this is the cytoplasmic component. 50µl of Buffer C (20mM HEPES (pH 7.8); 420mM NaCl; 5mM EDTA; 5mM DTT; 1mM PMSF; 10% glycerol) was added to the

nuclear pellet. Cells were rocked at 4°C for 30 minutes and then centrifuged at 10,000rpm for 10 minutes. The supernatant is the nuclear component and was stored at -20°C. The pellet was discarded.

2.17.3 Protein quantification

The Pierce bicinchoninic acid (BCA) protein assay (Thermo Scientific, IL, USA) was used for the determination of protein concentration in samples. The principle behind the assay is as follow: in the presence of protein, Cu^{2+} is oxidised to Cu^{+}, which reacts with the BCA reagent under alkaline conditions, yielding a coloured product. The commerically available kit contains two reagents; an alkaline bicarbonate solution and a copper sulphate solution, which were mixed in a 50:1 ratio respectively, prior to use. 200µl of this working reagent was added to 10µl of protein standard or 2µl of sample in a 96-well plate, which was then incubated at 37°C for 1 hour. The absorbance of each well was read on an Alpha Flour Plus plate reader (Tecan Trading AG, Tokyo, Japan) at 562nm. Protein concentrations of BSA between 0 and 1000µg/ml were used (representative standard curve shown in appendix 1).

2.17.4 Denaturing polyacrylamide gel (SDS-PAGE)

A 12% SDS-PAGE gel was used or resolution of proteins smaller than 100kDA while an 8% SDS-Page gel was used for resolution of proteins larger than 100kDA. A 12% separating gel was prepared as follows for a 30ml gel mould: 9.2ml 30% acrylamide:bisacrylamide (37.5:1), 4.5ml 1.875 M Tris HCL (pH 8.8), 8.3ml dH_2O, 176µl 10%(w/v) SDS, 120µl 10% (w/v) fresh ammonium persulphate , 10µl N,N,N',N'-tetramethylethylenediamine (TEMED).

The solution was gently mixed and the gels were cast between upright glass plates (Bio-Rad Laboratories, Hercules, CA, USA). Isopropanol was layered onto of the gels to exclude air and aid polymerisation; this was removed once polymerisation occurred. A 5% (w/v) stacking gel was prepared containing 1ml 30% (w/v) acrylamide:bisacrylamide (37.5:1). 2ml 0.6M Tris HCl (pH 6.8), 6ml water, 100µl 10% (w/v) SDS, 150µl 10% (w/v) ammonium persulphate, 10µl TEMED. The stacking gel was added to the top of the polymerised gel and a 12-well comb was inserted before allowing the gel to set.

2.17.5 Protein electrophoresis and transfer

Protein samples were diluted to a final volume of 30µl with 2X Laemmli buffer (2ml Tris HCl pH 6.8, 5ml 10% (w/v) SDS, 1ml 2-β2mercaptoethanol, 2ml glycerol, 0.05 g bromophenol blue) and denatured by heating to 95°C for 10 minutes. Samples were separated by electrophoresis at 50mA per gel in the electrolyte buffer (50mM Trizma base, 384 mM glycine, 0,1% (w/v) SDS) for 90minutes or until the dye front reached the bottom of the gel. 10µl of a tri-chrom pre-stained protein marker (Pierce, Rockford, IL,USA) was also loaded onto each gel and all gels were run in duplicate. Separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Pall Corp., Pensacola, FL, USA) using semi-dry transfer apparatus (Sigma Chemical Company, MO, USA). PVDF membrane was pre-activated in 100% methanol for one minute and soaked alongside Whatman filter paper (Whatman Laboratory Division, Maidstone, Kent, UK) in transfer buffer before transfer. The transfer apparatus was covered in ice-cold transfer buffer (0.15M glycine, 20mM Tris, 0.1% SDS, 20% (v/v) methanol). Proteins were transferred for 1 hour at 100V and 400mAmps.

2.18 Western blotting

Following the transfer of proteins, membranes were blocked with 5% (w/v) non-fat dry milk (Marvel®) which was reconstituted in Tris-Buffered saline (25mM Tris HCl (pH 7.6), 150 mM NaCl containing 0.1% Tween-20 (TBST) on a shaker (Bibby Sterilin., Staffordshire, UK) for one hour at room temperature. The membrane was then incubated in the primary antibody, diluted in 5% (w/v) Marvel in TBST on a shaker overnight at 4°C. Membranes were washed three times for 5 minutes each in TBST before incubation in horseradish peroxidase (HRP) conjugated secondary antibody (1.3g/l; 1:2000 dilution in TBST) (Dako, Glostrup, Denmark) for one hour on a shaker at room temperature. Six further 5 minute washes in TBST were then carried out. The Supersignal® West Pico Chemoiluminescent substrate kit (Thermo Scientific, Rockford, Il, USA) was then used to detect bound antibody complexes. Working reagent was prepared just prior to use by mixing two solutions in equal volumes and adding to the membrane for a further 1 minute. Membranes were then exposed to scientific X-Ray film (Fuji Photo Film Co Ltd., Tokyo, Japan), which was developed using a medical film processor (Agfa-Gevaert, Mortsel, Belgium). Exposure times ranged from 1-30 minutes depending on the signal intensity.

Where necessary, membranes were stripped of antibody complexes for re-probing using Restore Western Blot stripping buffer (Pierce, Rockford, IL, USA). The membrane was placed in 10ml striping buffer on a shaker at room temperature for 15 minutes, followed by three washes with TBST.

Densitometric analysis was carried out using TINA (version4.0) software (Raytest, Straubenhardt, Germany). Values were represented as a ratio of sample band intensity to that of corresponding β-actin control.

Table 2.8 List of antibodies used in Western blotting

Antibody	Supplier	Isotype	Type	Block	Primary Ab
Anti-IGF1R	R&D systems	Goat	polyclonal	5% Marvel, 1h RT	1:500, o/n 4°C
Anti-pAKT Ser 473	Santa Cruz	Rabbit	polyclonal	5% BSA, 1h RT	1:1000, o/n 4°C
Anti-pERK	Santa Cruz	Mouse	monoclonal	5% BSA, 1h RT	1:1000, o/n 4°C
Anti-STMN1	Cell signalling	Rabbit	monoclonal	5% BSA, 1h RT	1:1000, o/n 4°C
B-actin	Calbiochem	Mouse	monoclonal	5% Marvel, 1h RT	1:20,000, 1h RT

2.19 Statistical analysis

Statistical analysis was performed using SPSS® (version 16.0) software (SPSS, Chicago, IL, USA). The normality of the data was assessed and non-parametric test used were appropriate. Continuous variables were compared using unpaired t tests (Mann-Whitney test for non-parametric data). Association of categorical variables (differences for dichotomous variables between groups) was assessed using chi-square test. Correlations between variables were assessed using the Spearman and Pearson correlation coefficients as appropriate. All data are expressed as mean ± the standard error of the mean (SEM), which is the standard deviation of the distribution of sample mean. When the SEM is small, it indicates that the distribution of the sample means has less error estimating the true mean. SEM is calculated as the standard deviation of the original sample divided by the square root of the sample size. Significance was determined by analysis of variance (ANOVA), or Student's *t*-test, depending on the

number of groups to be analysed or their non-parametric equivalents where the distribution was not known to be normal. A probability (p) of ≤ 0.05 was considered to represent a significant difference between groups. The *t*-test was used to compare the means of two groups. In cases where data was paired (i.e untreated versus treated), a paired *t*-test was used for statistical analysis. Otherwise, an unpaired *t*-test was used. When population variances were unequal, Welch correction was applied. For statistical analysis involving 3 or more groups, the ANOVA test was used. Tukey *post-hoc* analysis was performed following ANOVA to determine statistical significance between individual groups. Survival statistics were calculated using Kaplan-Meier method and the log rank test was used to assess differences in survival between groups. Survival time was measured from the date of diagnosis to the date of an event or last follow-up. Disease-specific survival was assessed in all cases. A significance level of 0.05 was used for all analyses and all p values reported are two-tailed.

Chapter 3

Role of the insulin-like growth factor I axis and visceral adiposity in oesophageal adenocarcinoma

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3.1 Introduction

There has been a change in the epidemiology of oesophageal cancer in the Western world in recent decades, with an increasing incidence of adenocarcinoma which is now the most prevalent subtype of oesophageal cancer (Enzinger and Mayer, 2003). This parallels an increasing prevalence of obesity that may fuel this association (Donohoe et al., 2010, Engel et al., 2003). Numerous epidemiologic studies have demonstrated that obesity is a strong independent predictor for both incidence of and deaths from oesophageal adenocarcinoma (Calle et al., 2003, Renehan et al., 2008b). The risk of developing oesophageal adenocarcinoma rises with increased body mass index and a systematic review and meta-analysis estimated an odds ratio in males with BMI $\geq$30kg/m^2 of 1.52 (1.33-1.74) compared with patients with a normal BMI (Renehan et al., 2008b). Furthermore, Barrett's oesophagus, a precursor of oesophageal adenocarcinoma (Solaymani-Dodaran et al., 2004), is associated with adiposity (Ryan et al., 2008, Cook et al., 2008). Thus oesophageal adenocarcinoma may represent an exemplar model of obesity-associated cancer, and an appropriate experimental paradigm to uncover potentially important pathways underpinning this link.

Adipose tissue is a complex endocrine and metabolic organ. Current concepts suggest that centrally deposited fat, so-called visceral adipose tissue, is more metabolically active than peripheral subcutaneous fat, and hence a more significant fuel for the association of fat with dysmetabolism and related problems, including cancer.(Kershaw and Flier, 2004) Amongst the mechanisms which may play a role in the link between obesity and cancer is the insulin-like

growth factor (IGF) axis (Donohoe et al., 2010). The observation that insulin resistance is associated with an increased risk of development of cancer provoked the hypothesis that this may be mediated via activity of the IGF axis (Pollak, 2008). Insulin resistance leads to reduced insulin like growth factor binding protein (IGFBP-1 and -2) and results in a subsequent increase in free IGF-1 (Lukanova et al., 2002). Prospective studies provide evidence of a relationship between circulating IGF-1 and the risk of developing prostate, breast, colorectal and other cancers (Pollak et al., 2004b). It has been found that IGF1R plays a role in the establishment and maintenance of cellular transformation (Sell et al., 1993) and that the receptor or its ligands may be over-expressed in human tumours (Hellawell et al., 2002, Law et al., 2008). Moreover, its action may protect against apoptosis and favours invasion and metastasis (Frasca et al., 2008, Samani et al., 2007).

Studies *in vitro* support a role for the IGF axis in oesophageal adenocarcinoma progression, with blockade of the IGF1R leading to apoptosis (Piao et al., 2008) and IGF-1 stimulating proliferation (Liu et al., 2002). In oesophageal cancer, over-expression of IGF1R has been associated with the malignant progression of Barrett's oesophagus to adenocarcinoma (Iravani et al., 2003). No previous studies have investigated the IGF axis in oesophageal adenocarcinoma in man, and its association with visceral adiposity, and this represented the primary focus of this study.

3.2 Specific aims

The aims of the present study are to:

- Investigate the association between visceral adiposity and serum levels of free and total IGF-1
- Explore the association between tumour expression of IGF1R at mRNA and protein level and visceral obesity
- Examine the relationship between disease-specific survival from oesophageal adenocarcinoma and IGF1R protein expression in tumour tissue

3.3. Results

3.3.1 Patient demographics

The study population consisted of 220 patients, 178 (81%) were male. The mean age was 64.5 years, with a range of 30.0 to 86.0 years. Patient and tumour characteristics are summarised in Table 3.1. Information on population selection and characteristics are described in detail in section 2.3

Table 3.1 Demographic details of study population (n= 220)

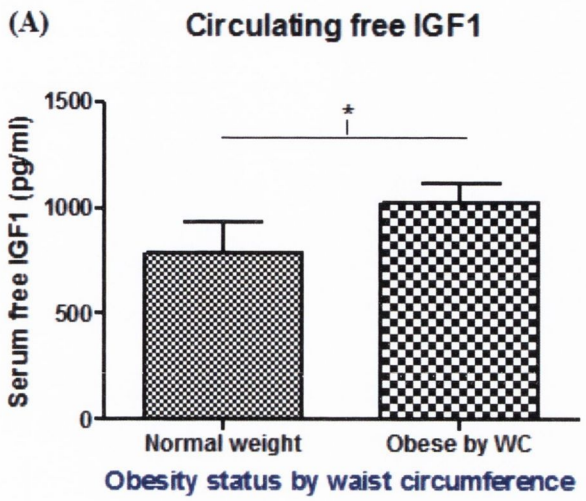
		Mean (SD) or n (%) as appropriate
Male: female		178:42 (80.9%:19.1%)
Age (years)		64.5 (11.1)
Waist circumference (cm)		96.3 (12.8)
Body mass index (kg/m^2)		26.3 (4.7)
Total fat area (cm^2)		347.3 (24.4)
Visceral fat area (cm^2)		171.5 (12.8)
Superficial fat area (cm^2)		175.7 (11.6)
Pathological T stage	T1	32 (14.6)
	T2	49 (22.3)
	T3	128 (58.1)
	T4	8 (3.6)
	Missing	3 (1.4)
Nodal status	N0	78 (35.5)
	N1	93 (42.3)
	N2	26 (11.8)
	N3	20 (9.1)
	Missing	3 (1.4)
Neoadjuvant treatment		72 (32.7%)

Anthropomoporhic measurements were made as delineated in section 2.4

3.3.2 Serum IGF-1 levels

The level of free IGF-1 in 100 patient's serum samples and total IGF in a subset of 24 patients were assessed using ELISA (section 2.5 and 2.6) and results were correlated with tumour type, gender and obesity status. Mean free IGF-1 levels were significantly higher in the serum of patients who were defined as obese by waist circumference (1026.0 vs 787.0 pg/ml, p=0.014) (Figure 1.3a) and visceral fat area (1136.5 vs 672.9 pg/ml, p=0.019) but not by BMI (984.6 BMI > 30 vs 958.3 pg/ml BMI < 30, p=0.584). Free IGF-1 levels correlated with waist circumference (Spearman's ρ=0.288, p=0.014). Mean total IGF-1 levels were also increased in those classified as obese by waist circumference (131.0 vs 83.73 ng/ml, p=0.002) (Figure 1.3b) and visceral fat area (123.9 vs 91.64 ng/ml, p=0.057).

No association was found between clinicopathological factors (including lymphatic, venous, perineural invasion, tumour stage or grade) and circulating IGF-1 levels. To analyse survival, patients were grouped into two groups around the median level of free IGF-1 (greater than and less than median free IGF-1). Median disease-specific survival was not significantly different between patients with above and below the median levels of free IGF-1 (p=0.976). Disease-free survival was 23.9 months for patients with below the median free IGF-1 versus 15.6 months for those with greater than median free IGF-1 (p=0.101) (Figure 3.2).



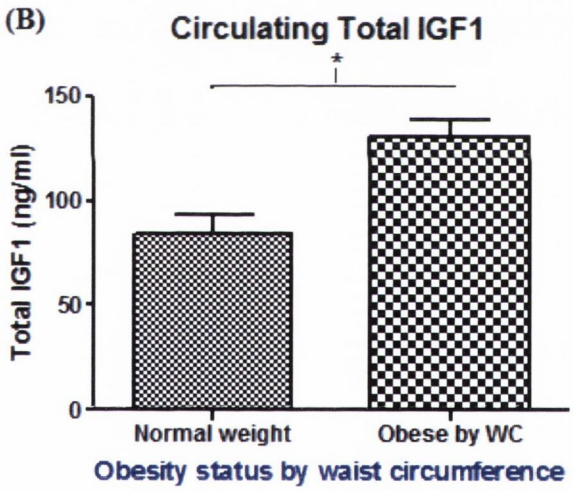


Figure 3.1: Circulating IGF-1 axis expression and visceral obesity (A). Mean free IGF-1 levels were significantly higher in the serum of patients who were defined as obese by waist circumference (787.0 vs 1026.0 pg/ml, p=0.014, n=100). (B). Total IGF-1 levels were also increased in those classified as obese by waist circumference (83.73 vs 131.0 ng/ml, p=0.002, n=25). All data are Mean ± SEM.

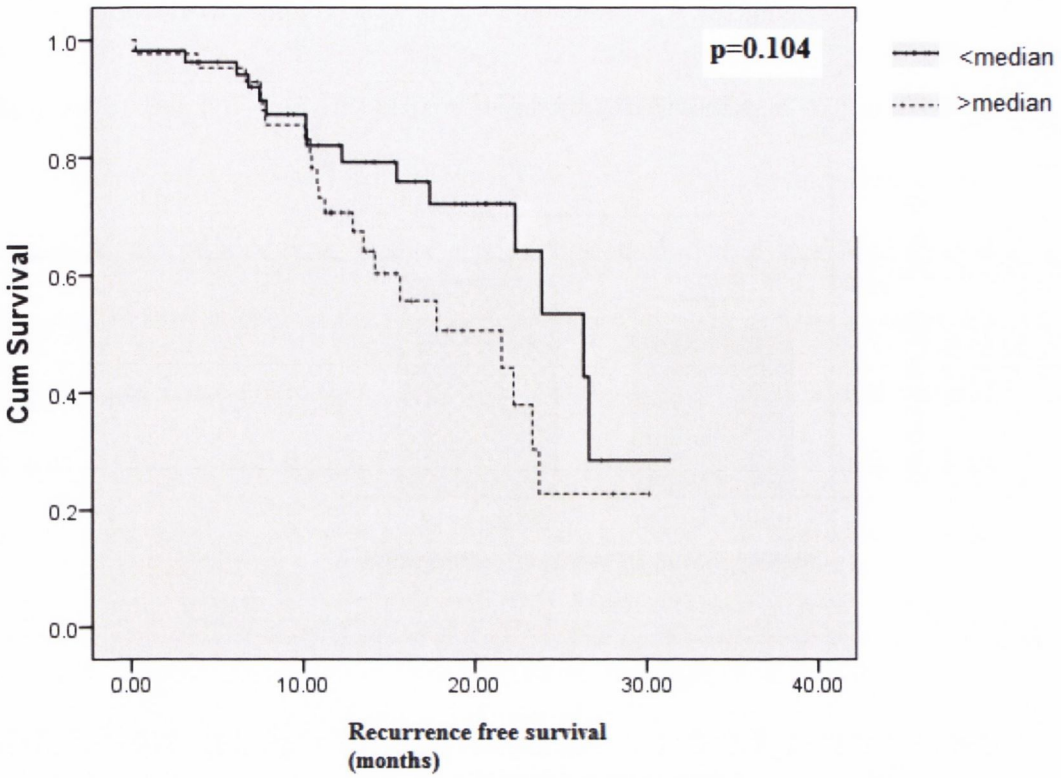


Figure 3.2. Increased serum free IGF-1 is associated with reduced disease-free survival. Disease-free survival (DFS) was 23.9 months for patients with below the median free IGF-1 versus 15.6 months for those with greater than median free IGF-1 (log-rank test, p=0.104).

This study included n=100 patients. A power calculation was performed using a proportional hazards calculator for time to failure analysis (available on-line at http://hedwig.mgh.harvard.edu/sample_size/time_to_event/para_time.html).With assumptions of an 80% probability of detecting a 20% difference (HR 1.2) in survival at a two sided 5.0% significance level, where the accrual and follow-up interval periods are set at 60 months and the median survival was that of the lowest time to failure as reported above (15.6 months) resulted in a requirement of 976 patients in order for the study to be adequately powered.

3.3.3 IGF1R and IGF-1 gene expression in resected tumour specimens

IGF1R and IGF-1 gene expression was investigated in 78 resected tumour specimens (section 2.5 and 2.7). mRNA expression of IGF1R correlated moderately with that of IGF-1 (Spearman's ρ=0.425, p=0.002). Tumour IGF1R or IGF-1 gene expression levels did not correlate with circulating concentrations of free IGF-1 (p=0.406 and 0.350 respectively). IGF1R mRNA did not correlate with protein IGF1R expression in tumours (ρ=0.309).

3.3.4 Association of tumour gene expression levels and obesity status

IGF1R RQ values of IGF1R and IGF-1 correlated moderately with all markers of obesity at the $p<0.05$ significance level (Table 3.2).

Table 3.2. Correlations between RQ values for mRNA expression of IGF1R and IGF-1 and measures of obesity status.

	BMI	WC	TFA	VFA	SFA
IGF1R (Spearman's ρ, p-value)	0.399, 0.005	0.335, 0.021	0.385, 0.011	0.204, 0.189	0.508, <0.001
IGF-1 (Spearman's ρ, p-value)	0.372,0.009	0.312, 0.031	0.363, 0.015	0.318, 0.035	0.333, 0.027

Patients were grouped according to their IGF-1 mRNA expression into quartiles and the group with IGF-1 expression in the lowest quartile were compared to those within the highest quartile. Patients with the lowest mRNA expression had significantly lower BMI (p=0.002), total fat area (p=0.013), visceral fat area (p=0.013), superficial fat area

(p=0.013) and waist circumference (p=0.003) than those with the highest quartile of expression of IGF-1 mRNA (Table 3.3).

Table 3.3 Differences in obesity status between tumours with lowest versus highest quartile IGF-1 mRNA expression.

(Mean (SD))	Lowest quartile IGF-1 expression	Highest quartile IGF-1 expression	p-value#
BMI (kg/m^2)	24.2 (3.3)	30.3 (4.9)	0.002
Waist circumference (cm)	88.7 (10.7)	108.3 (16.2)	0.003
Total fat area (cm^2)	290.6 (107.4)	491.1 (186.5)	0.013
Visceral fat area (cm^2)	148.6 (84.9)	257.6 (107.8)	0.013
Superficial fat area (cm^2)	142.0 (44.1)	234.0 (104.8)	0.013

Viscerally obese patients were 4.3 times more likely to be within the highest quartile of IGF-1 expression than the lowest (p=0.037) and 5.2 times more likely to be within the highest quartile of IGF1R expression than the lowest (p=0.022).

Patients classified as viscerally obese had significantly higher IGF1R mRNA expression in their tumours (mean RQ value: 0.14 versus 0.79; p=0.015) (Figure 3.3) and higher IGF-1 mRNA expression in tumours also (mean RQ value: 1.13 versus 4.91: p=0.101).

3.3.5 Tumour gene expression and clinicopathological data

A greater proportion of patients with greater than median IGF1R mRNA expression in their tumours had perineural invasion compared with those with less than median IGF1R mRNA expression (27.3% vs 39.1%, p=0.018). There was a higher proportion of lymphatic invasion in those with greater than median IGF1R expression (50.0% vs 77.3%; p=0.061). There was no difference in IGF1R or IGF-1 mRNA expression across tumour stage (T and N stage) or grade. No associations between IGF1R or IGF-1 mRNA expression and survival were found.

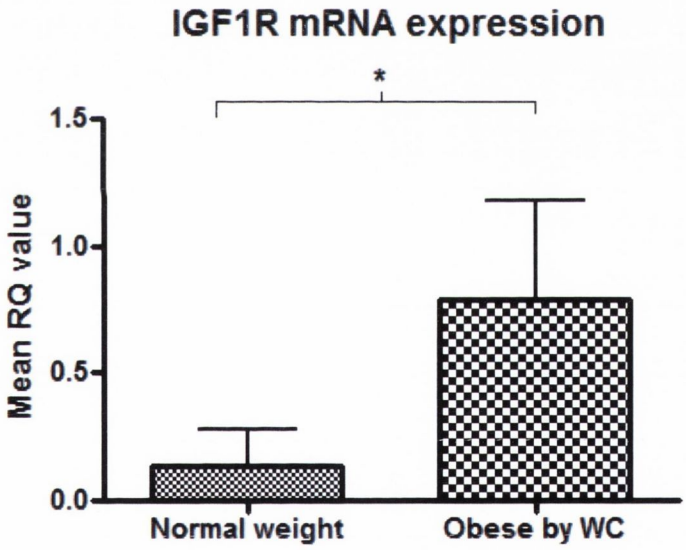


Figure 3.3: IGF1R mRNA according to visceral obesity status. Patients classified as viscerally obese by WC had significantly higher IGF1R mRNA expression in their tumours (mean RQ value: 0.14 versus 0.79; p=0.015). p<0.05, Data are mean ±SEM

3.3.6 Association of tumour protein levels with obesity status, clinicopathological details and survival

Resected tumour specimens (containing viable tumour tissue) from 161 patients with oesophageal adenocarcinoma were stained by IHC in tissue microarrays for IGF1R (described in section 2.8). Forty-two patients (26.1%) did not have any IGF1R expression on tissue microarray immunohistochemistry, the remainder had expression which was scored to a maximum score of 300 (Figure 3.4). Of those with positive IGF1R expression, 10/119 had membranous staining only, 35/119 had cytoplasmic staining only and 74/119 had both membranous and cytoplasmic staining. 50% of patients had positive IGF1R staining in the peritumoural stroma. Patients with positive stromal IGF1R expression had a higher mean superficial fat area than those with negative stroma (179.3 versus 143.8cm^2, p=0.029).

IGF1R protein expression in tumours correlated with obesity status: moderately with waist circumference (0.380, p=0.016) and weakly with BMI (0.18, p=0.031). Visceral fat area values were only available for 35 patients in the cohort of patients who underwent IHC analysis and there was no correlation of VFA with IGF1R. Mean IGF1R expression was higher in obese patients (as defined by waist circumference, p=0.023) (Figure 3.5).

Clinicopathological factors analysed included markers of invasion (lymphatic, venous and perineural), nodal status, T stage and differentiation and there were no statistically significant differences observed amongst these categorical variables and IGF1R expression.

3.3.7 Disease-specific survival and tumour IGF1R protein expression

Survival was assessed by grouping patients into groups: those who had no IGF1R expression (IGF1R negative) versus those with positive IGF1R expression and those with an IGF1R score of less than versus greater than the median. Disease-specific survival was not statistically significantly different (p=0.315) between patients with less than median IGF1R expression (44.5 months (95% CI: 17.5-71.5 months)) versus those with greater than median IGF1R expression (25.13 months (4.4-45.9 months). Disease-specific survival was significantly reduced in those with positive IGF1R staining (median: 23.4 months versus 60.0 months, p=0.027) (Figure 3.6).

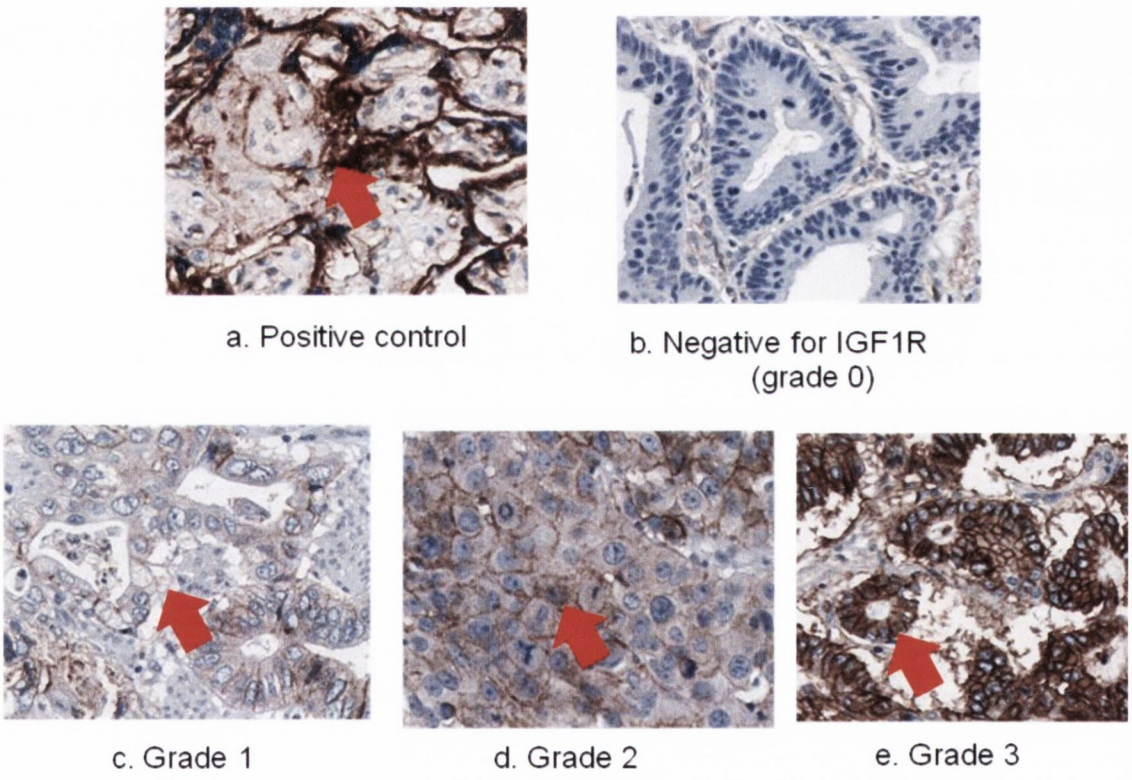


Figure 3.4 Tissue microarray staining: Paraffin-embedded tissue microarrays of oesophageal adenocarcinoma were stained using immunohistochemistry for IGF1R expression. All figures are 10X magnification. Placental tissue was used as a positive control. Red arrows indicate positive membranous staining for IGF1R. The intensity of membranous IGF1R staining of tumour-bearing areas on the tissue microarray cores was scored between 0-3, where zero was negative, one was weak, two was moderate

and three represented strong staining. In addition, the quantity of positive staining within the tissue microarray core was scored between 0 and 100%. The intensity score and percentage positivity were multiplied giving an overall score for each core of 0-300.

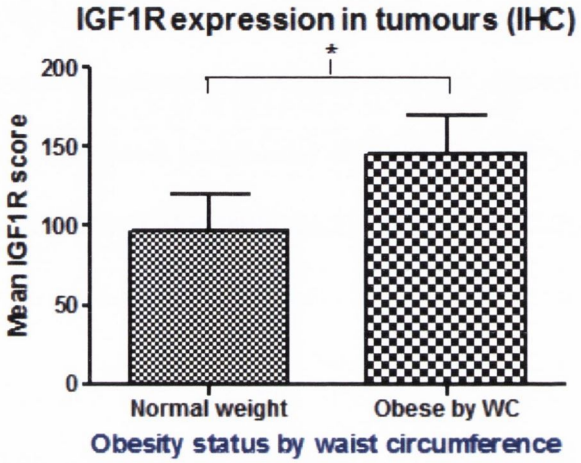
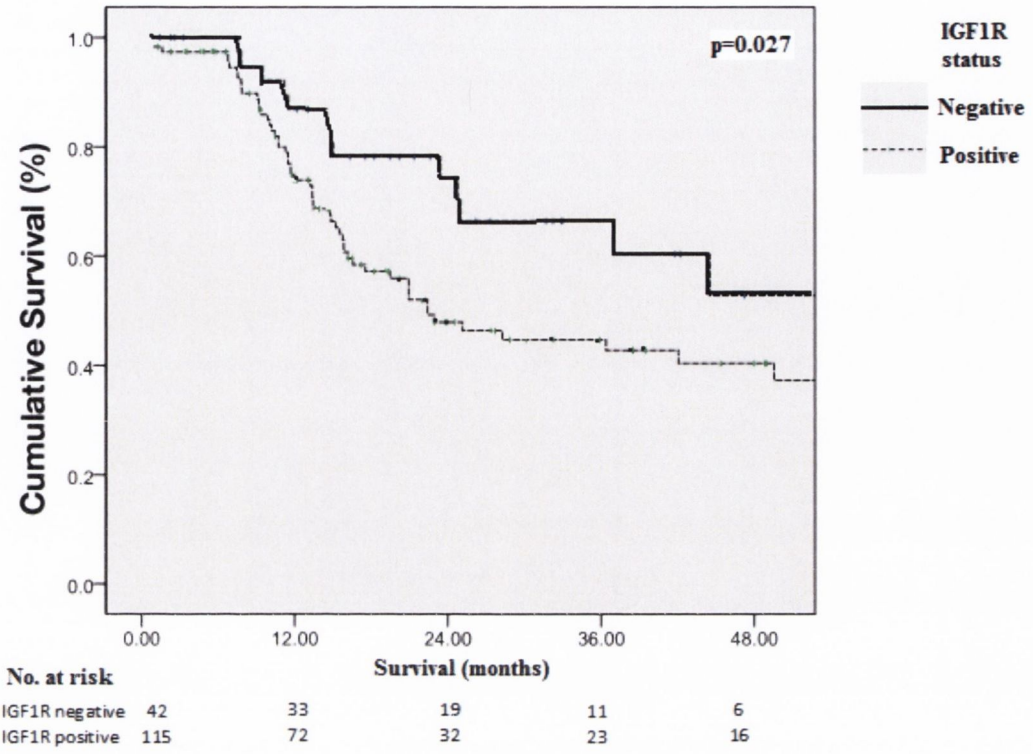


Figure 3.5. IGF1R protein expression in oesophageal adenocarcinoma tumours is elevated in viscerally obese patients. Mean IGF1R expression was higher in obese patients (as defined by waist circumference, $p=0.023$). $p<0.05$, Mean ±SEM



IGF1R protein	Median survival	5 year survival	p-value
Negative	**60.0 months**	**52%**	**0.027**
Positive	**23.4 months**	**32%**	

Figure 3.6. IGF1R protein expression in tumours and 5 year disease-specific survival. Patients with tumours negative for IGF1R have a significantly longer median survival than those with positive IGF1R tumoural staining (median survival: 60.0 v 23.4 months, p=0.027) with a corresponding increase in five year survival of 52% versus 32%.

On univariate cox regression analysis, positive tumour IGF1R staining was associated with death from oesophageal cancer compared with IGF1R negative tumours (OR= 1.703 (0.963-3.014), p=0.027). However, on multivariate analysis of factors independently associated with disease-specific survival only nodal status (positive versus negative) and resection margin involvement (R) status (R0 vs R1) were independently associated with survival (Table 3.4).

Table 3.4. Cox regression analysis:

Factor	Univariate analysis		Multivariate analysis	
	Hazard ratio (95%CI)	p-value	Hazard ratio (95%CI)	p-value
IGF1R positive	1.703 (0.963-3.014)	0.027	X^2=2.579	0.055
Node negative versus node positive	0.241(0.12-0.486)	<0.001	0.27(0.136-0.555)	**<0.001**
Early cancer (Tis-T2)	0.421 (0.24-0.738)	0.003	X^2= 2.452	0.088
R0 resection	0.414 (0.245-0.697)	0.001	0.473 (0.279-0.801)	**0.001**

3.4. Discussion

The treatment of oesophageal cancer historically is associated with high morbidity and mortality rates, a poor prognosis despite aggressive treatment, and an adverse impact on health related quality of life (Enzinger and Mayer, 2003). A strong association between obesity and oesophageal adenocarcinoma has emerged, and an understanding of the factors fuelling this association may uncover new understandings of the biology of this cancer, and potentially new therapeutic targets, and this study highlights the potential importance of the IGF-1 axis in this disease paradigm.

Obesity is associated with the development of insulin resistance (Kahn et al., 2006) which results in compensatory hyperinsulinaemia. Large quantities of adipose tissue affects the body's handling of glucose (Brochu et al., 2000) and adipocytokines are thought to be involved in the pathogenesis of insulin resistance (Fasshauer and Paschke, 2003). Cytokines produced by adipose tissue, including TNF- α, IL-6, IL-1β, and adipokines such as leptin, and low concentrations of adiponectin, have deleterious effects on glucose homeostasis leading to chronic hyperinsulinaemia and insulin resistance, which may manifest itself clinically as Type 2 Diabetes Mellitus (Greenberg and McDaniel, 2002a). The interaction between insulin, body fat and the IGF axis is less well understood. It has been proposed that the IGF system mediates the effect of hyperinsulinaemia. Due to the structural homology between IGF1R and insulin receptors, these receptors can form hybrid receptors (Pollak, 2008). The IGF1R binds both IGF-1 and insulin, albeit with higher affinity for IGF-1. It is known that circulating levels of IGF are

influenced by circulating insulin levels which alter the level of IGF binding protein 1 and 2 (IGFBP1 & 2) and thus increases bioavailability of IGF (Jones and Clemmons, 1995). Whether obesity status can also influence tumour receptor expression is heretofore unknown.

Previous studies have suggested that IGF-1 levels and BMI are correlated (Lukanova et al., 2004, Onder et al., 2006) and that BMI may influence IGF-1 secretion in response to growth hormone (Roman et al., 2009). However, it is not a linear relationship and appears to be maximal at a BMI of 24-26 (Lukanova et al., 2002). We hypothesised that, akin to insulin resistance (Despres and Lemieux, 2006, Despres et al., 1989), changes in the IGF-1 axis related more to visceral adiposity than BMI alone, and in this study, mean serum circulating (free) IGF-1 and total IGF-1 concentrations were higher in viscerally obese patients. Viscerally obese patients were also more likely to have increased IGF-1 and IGF1R mRNA expression and IGF1R protein expression in their tumours, and tumour IGF1R expression correlated more strongly with waist circumference than with BMI (ρ= 0.380, p=0.01 versus ρ= 0.18, p=0.03).

Elevated IGF1R expression has previously been demonstrated in oesophageal adenocarcinoma tumour tissue (Iravani et al., 2003, Kalinina et al., 2010). In some individuals, high levels of IGF-1 are associated with reduced IGF1R activation owing to variants of the IGF1R which are deficient in signalling activity. This results in a feedback mechanism which acts to increase serum IGF-1 levels

without any corresponding increase in IGF1R signalling (Suh et al., 2008). Additionally, increased circulating levels of IGF-1 may not reflect the concentration of IGF-1 within the tissue microenvironment or the effects of paracrine or autocrine IGF production – factors which may be of more relevance to tumour IGF axis activity. Therefore, tumour expression of IGF-1 and IGF1R were analysed at mRNA and protein levels, and using both methodologies the expression of IGF1R was significantly increased in the viscerally obese cohort but was not significantly associated with serum IGF-1 levels.

Further studies are required to determine the downstream impact of this altered receptor expression on pathways that impact on survival, proliferation and apoptosis. In this study the changes in the IGF-1 axis did not correlate with clinicopathological status, but IGF1R expression was associated with changes in disease-specific survival. The patient cohort is restricted to those undergoing surgical treatment, which is exclusive to patients undergoing treatment with curative intent, representing approximately 40% of patients referred to this Centre (Enzinger and Mayer, 2003) . Notwithstanding the lack of association with standard clinicopathological variables, the IGF-1 axis was associated with prognosis, with significantly ($p < 0.05$) reduced survival associated with IGF1R expression and a trend ($p=0.1$) towards longer disease-free survival in patients with lower circulating IGF-1 levels.

The role of circulating IGF-1 as a biomarker is an area of current research interest. Population based studies have provided evidence that relate circulating ligand levels as well as polymorphic variation of relevant genes to cancer risk and

prognosis (Ma et al., 1999, Giovannucci et al., 2000, Palmqvist et al., 2002, Chan et al., 1998, Chan et al., 2002, Harman et al., 2000, Stattin et al., 2000). An important question with respect to the role of circulating IGF-1 levels is whether they may be used as a biomarker of response to IGF1R targeted therapies in patients with cancer. In a phase I expansion cohort study of figitumumab in 31 patients with relapsed sarcoma, which suggested that pre-treatment plasma levels of IGF-1>110ng/ml conferred a significant treatment advantage compared with lower levels (10.5 v 4.5 months overall survival, $p<0.001$) (Olmos et al., 2010).

In the ADVIGO trial of figitumumab in combination with paclitaxel and carboplatin for treatment of advanced NSCLC there were objective response rates in patients with higher IGF1R expression (correlating with the squamous subtype) and improved progression free survival (PFS) in patients with high pre-treatment free IGF-1 (fIGF1) levels compared to low levels (PFS 6 v 3 months, $p=0.007$) (Jassem J, 2010). Low IGF-1 levels have previously been associated with prolonged survival in NSCLC (Han et al., 2006). However, in this trial elevated baseline free IGF-1 was found in female patients with adenocarcinoma, which may indicate that this rather than predicted therapeutic efficacy, influences survival and fIGF-1 is merely a prognostic biomarker. However, in the chemotherapy only treatment arm fIGF-1 was not a prognostic biomarker, which may indicate that it is of relevance in predicting response to figitumumab (Gualberto et al., 2011). In addition, fIGF-1 levels correlated with tumour vimentin expression – a mesenchymal marker and inversely with E-cadherin expression – indicating that fIGF-1 may play a role in EMT (Gualberto et al., 2011).

IGF1R positivity was significantly associated with survival on univariate analysis, and approached significance (p=0.055) on multivariate analysis. The X^2 value in the multivariate cox regression analysis was more closely associated with survival than that of T stage, which is a known independent predictor of disease free survival in oesophageal cancer (AJCC, 2010), indicating that sample size and the homogenous patient cohort may account for the result.

Data from this study support the hypothesis that obesity status influences IGF axis expression. Visceral obesity is related to the IGF-1 axis across the spectrum of disease from circulating levels, to tumour mRNA and protein expression. Understanding obesity-related tumourigenic mechanisms may reveal new insights into oesophageal carcinogenesis and uncover potential targets for future intervention. Various drugs which act to inhibit the IGF axis are under clinical trial at present (Gualberto and Pollak, 2009), both tyrosine kinase inhibitors and monoclonal antibodies, some of which are specific to IGF1R function and others with off-target inhibition of HER2, Ras and VEGF signalling pathways amongst others (Golan and Javle, 2011). Furthermore, IGF1R activity may be involved in resistance to targeted HER2 (Nahta et al., 2006) and EGFR therapies (Chakravarti et al., 2002). Data from this study implies that investigation of the activity of IGF axis inhibitors, alone or in combination, may be relevant to explore in this paradigm model.

3.5 Clinical relevance

What is already known: Five-year survival rates, even following curative treatment, for oesophageal adenocarcinoma remain low. There is a clear need to develop new insights into the biology of this aggressive disease in order to identify targets for treatment. Epidemiological evidence demonstrates a strong association between obesity and the development of oesophageal adenocarcinoma. Uncovering the mechanisms which underpin this association may provide relevant avenues of investigation. Previous studies of the IGF-1 axis and cancer have shown equivocal results in relation to modulation of IGF-1 axis in obesity, using measures of total body fat. This study investigates expression of the IGF-1 axis with respect to visceral adiposity in samples from patients with oesophageal adenocarcinoma.

What's new: This study demonstrates higher expression of circulating levels of IGF-1 in the serum of viscerally obese patients. Expression of the IGF-1 receptor (IGF1R) is also higher in the tumours of viscerally obese patients at both the mRNA and protein level. Oesophageal adenocarcinoma tumours with positive IGF1R expression had reduced disease specific survival (60 versus 23.4 months, p=0.027).

Potential impact on future practice: Thus, drugs currently under development which inhibit IGF1R function may prove useful targeted therapies in oesophageal adenocarcinoma patients whose tumours express IGF1R.

Chapter 4

Picropodophyllin, a tyrosine kinase IGF1R inhibitor, is a novel therapeutic agent *in vitro* in oesophageal adenocarcinoma.

4.1 Introduction

The challenge for 21st century oncology is handling the complexity of the carcinogenic process. As techniques have advanced, we increasingly recognise the complexity of genomic and proteomic changes which underpin the development of malignant cells. Genome-wide arrays have demonstrated that there is considerable heterogeneity within the patterns of gene expression of tumours which histomorphologically look identical. (Perou et al., 2000, Sørlie et al., 2003, Sawyers, 2004) Thus, the concept of personalised medicine arose – by uncovering the specific gene and protein changes unique to an individual patient's tumour, new therapeutic targets would be identified and treatment specific to the individual patient employed.

Traditional forms of chemotherapeutic agents have exploited features general to cancer cells, such as increased proliferation rate and altered DNA repair ability, to kill cancer cells. This crude, non-specific therapeutic approach leads to morbidity from interference with healthy tissues and resistance to treatment in tumours which do not utilise, or learn to overcome, the survival mechanisms affected by these drugs. Unlike standard chemotherapeutic agents, targeted therapies are chosen for their ability to interact with targets which are integral to the processes used by cancer cells to proliferate and metastasise.

The insulin-like growth factor 1 (IGF-1) axis comprises a transmembrane IGF-1 receptor and its primary ligand IGF-1, circulating levels of which are controlled by 6 binding proteins. This axis is known to have important physiological roles in the control of normal growth (Ohlsson et al., 2009, Pollak, 2008) (and perhaps lifespan (Holzenberger et al., 2003)); maintenance of tissue homeostasis (Sutherland et al., 2008) and a differentiated phenotype (Belfiore et al., 2009); alteration in the balance of

proliferation and apoptosis (LeRoith and Roberts, 2003); angiogenesis, cell adhesion, migration and wound healing (LeRoith and Roberts, 2003). IGF1R is a tyrosine kinase receptor which initiates intracellular signalling upon receptor activation by autophosphorylation and stimulation of tyrosine kinase activity, leading to recruitment and phosphorylation of the insulin-receptor substrate-1 (IRS-1). These receptor substrates activate two main signalling pathways: PI3K-AKT and RAS-Raf-MAPK which have multiple effects on gene regulation and protein expression, activation and translocation (LeRoith and Roberts, 2003). Critically, IGF1R is not a requirement for the growth of normal cells (Yu and Rohan, 2000).

There is a strong epidemiological association between the presence of obesity and oesophageal adenocarcinoma, with a number of studies reporting a higher incidence of and deaths from oesophageal adenocarcinoma in obese patients (Calle et al., 2003, Renehan et al., 2008b). Previous findings, reported in this thesis, have demonstrated that IGF1R is highly expressed in tumours of patients with oesophageal adenocarcinoma and is associated with decreased disease-specific survival.

Given the putative roles of IGF1R in cancer development and progression, many drug discovery efforts are ongoing to identify agents that selectively block the IGF1R pathway in tumour cells. The rationale for this, is the growing *in vitro* and *in vivo* evidence of involvement of the IGF axis in cancer development (Gualberto and Pollak, 2009). One such strategy is to identify low molecular weight molecules that inhibit the catalytic activity of the IGFIR by binding its active site and preventing phosphorylation of the receptor.

Picropodophyllin (PPP) is a cyclolignan tyrosine kinase inhibitor specific to the insulin-like growth factor receptor with little effect on insulin (IR), fibroblast growth factor

(FGFR), platelet derived growth factor (PDGFR) or epidermal growth factor receptors (EGFR) (Girnita et al., 2004). It is the first inhibitor reported to discriminate between IGF1R and IR (Girnita et al., 2004). In mouse models of uveal melanoma, myeloma and basal-like breast cancer, it has been shown to be well tolerated with oral dosing (Economou et al., 2008, Menu et al., 2006, Klinakis et al., 2009).

4.2 Specific aims

The aims of this chapter include:

1) Assessing the effect of PPP on cell viability in oesophageal adenocarcinoma cell lines *in vitro*.

2) Investigating the functional role of PPP on proliferation, cell cycle progression and apoptosis

3) Exploring alteration in IGF1R receptor localisation following PPP treatment

4.3 Results

4.3.1.1 Cell viability following PPP treatment

OE33 and JH-Eso-Ad1 cell lines were treated with a range of doses of PPP and cell viability was assessed using MTT assay. Non-linear logistic regression analysis was used to determine the inhibitory concentration required to reduce the viability of treated cells by 50% of the observed range of response (IC_{50} dose) for both cell lines studied (Figure 4.1) and this dose was used in further treatments. The 24 hour time point was chosen for all experiments, unless otherwise stated. The IC_{50} dose determined for PPP was 12.79 nM and 3.14 nM for the OE33 and JH-Eso-Ad1 cell lines respectively.

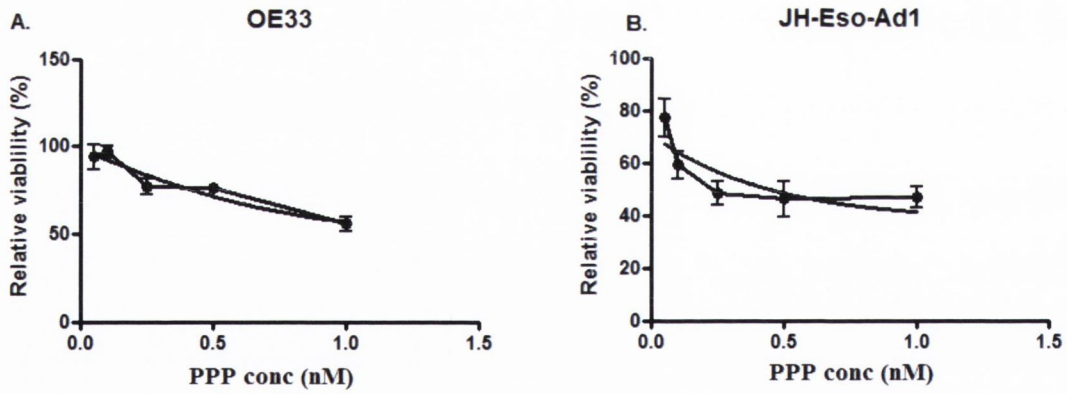


Figure 4.1 PPP treatment decreases OAC cell viability. (A). OE33 and (B) Jh-Eso-Ad1 cell lines were treated with a range of concentrations of PPP and cell viability was assessed by MTT assay using non-linear logistic regression analysis to determine the IC_{50} dose. The smooth line represents the best fit for dose calculation as determined by logistic regression analysis of n=3 experiments.

4.3.1.2 Autocrine IGF-1 activation following PPP treatment

Autocrine IGF-1 production following PPP treatment was assessed by measuring IGF-1 levels by ELISA in cellular supernatants following PPP treatment. PPP treatment led to a significant increase in autocrine IGF-1 production by the cells (Figure 4.2), with a 2.3-fold increase in IGF-1 in OE33 and 2.3–fold increase in JH-Eso-Ad1 lines. Since the potential concentration of IGF-1 may be higher near the cell surface, cells were then treated with saturating doses of recombinant IGF-1 (1000ng/ml – the dose which induces maximal proliferation in a panel of OAC cell lines) and cell viability was assessed in order to determine whether treatment with the receptor ligand could overcome the downstream effect of PPP on cell viability. There was no difference observed between cellular viability when cells were treated with both recombinant IGF-1 and PPP versus PPP treatment alone (Figure 4.3), with the viable cell count with each treatment being 60.2 ± 7.0% versus 59.1 ± 5.0%, p=0.96 respectively in OE33 and 50.5 ± 2.1% versus 61.3 ± 8.4%, p=0.163 respectively in JH-Eso-Ad1,versus untreated controls.

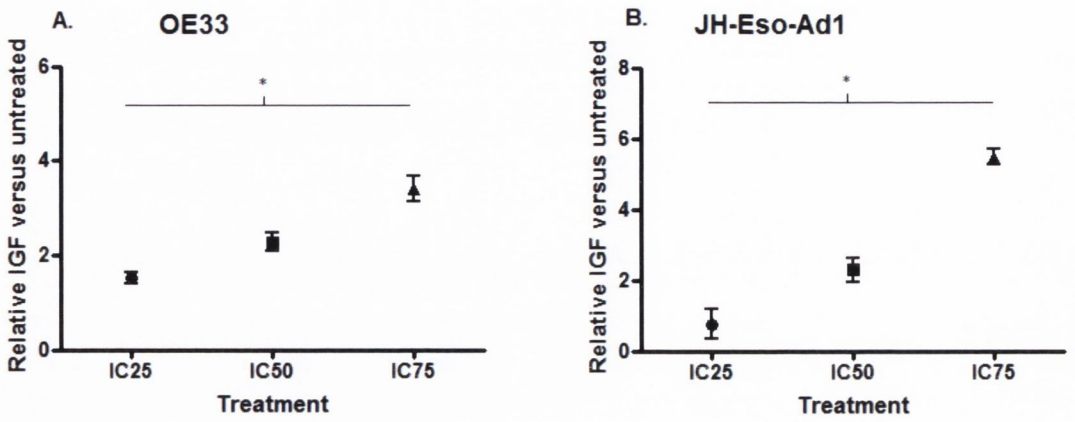


Figure 4.2 PPP treatment causes an increase in IGF-1 production by OAC cells. (A) OE33 and (B) JH-Eso-Ad1 cell lines were treated with IC_{25}, IC_{50} and IC_{75} doses of PPP and production of free IGF-1 in the cellular supernatants was assessed using ELISA. There was a significant increase in the autocrine production of IGF-1 by treated cells versus untreated controls, as analysed by ANOVA, $p<0.001$ with the fold increase with the IC_{25}, IC_{50} and IC_{75} treatments for OE33: 1.52 ± 0.13, 2.28 ± 0.20, 3.41 ± 0.28, respectively and for JH-Eso-Ad: 0.76 ± 0.41, 2.29 ± 0.25, and 5.48 ± 0.22 respectively. Data are mean ± SEM, n=6.

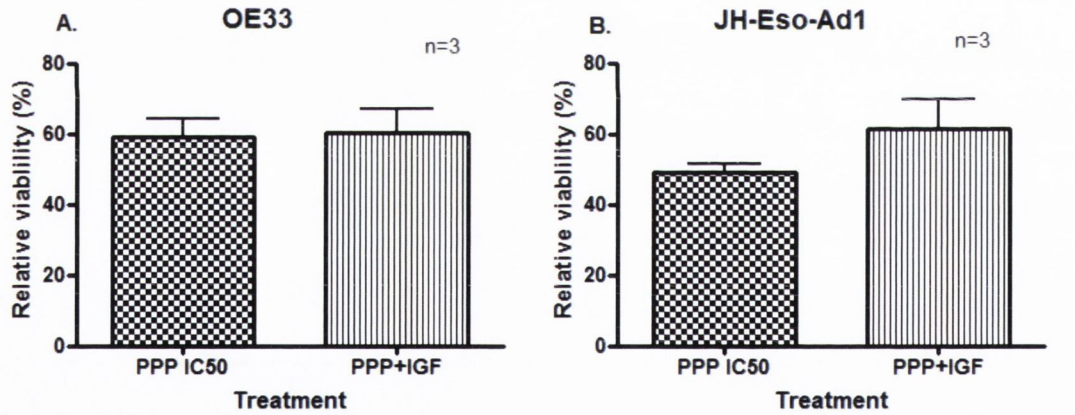


Figure 4.3 Addition of recombinant IGF-1 does not increase cell viability following PPP treatment. (A) OE33 and (B) JH-Eso-Ad1 cell lines were treated with a receptor saturating dose of IGF-1 (1000ng/ml) for 24 hours and cell viability was determined using MTT assay (section 2.11). There were no significant differences in cell viability between cells treated with IC_{50} dose of PPP alone versus those which were co-treated with IGF-1. Comparing the percentage of viable cells versus untreated controls, the mean viable cell percentage was (A) 60.2 ± 7.0% with PPP +IGF treatment versus 59.1 ± 5.0% PPP only, p=0.96 in OE33 and (B) 50.5 ± 2.1% versus 61.3 ± 8.4%, p=0.163 respectively in JH-Eso-Ad1. Significance was tested using the paired t-test. Data are mean ± SEM, n=3.

4.3.1.3 Specificity of PPP IGF1R activity

In order to assess whether PPP effects were mediated via IGF1R, an attempt was made to knockdown IGF1R expression in both OE33 and JH-Eso-Ad1 cell lines using siRNA technology. A summary of the various experimental set-ups and results thereof is reported in Table 4.1. The OE33 cell line was chosen for initial experiments as it has higher IGF1R expression at the protein level (Doyle et al., 2011).

Briefly the following parameters were varied: seeding densities and confluency at time of treatment; concentrations of targeting siRNAs (IGF1R and/or GAPDH-positive control); and treatment times in order to attempt to achieve at least 85% IGF1R knockdown as measured by qPCR (section 2.7).

Table 4.1 Results of initial experiments to optimise siRNA transfection for IGF1R knockdown in OE33 cell line

Expt	Seeding density	Con-fluence	Transfection reagent	Vol (µL)	SiRNA	Si RNA conc (nM)	Treat-ment time (hrs)	Result
1	400,000	80-90%	Dharmafect 1	2,4,6	GAPDH	25	24	51% knockdown GAPDH; toxicity observed
2	500,000	90%	Dharmafect 1	2,4,6	GAPDH	25	24	Toxicity observed; Knockdown to 75.5%
3	400,000	90%	Dharmafect 1	6,8,10	GAPDH	50	24	Cell lifted off - ?TF reagent too toxic
4	400,000	80%	Dharmafect 1	2,4,6	GAPDH	50	24	Cells lifted off - ?too confluent; Knockdown to 52%
5	100,000	80%	Dharmafect 1	2,4,6	GAPDH	50	24	Cells lifted off – TF too toxic at this seeding density

6	50,000	50-70-80%60%	Dharmafect 1	0.5, 1, 1.5, 2, 2.5				MTT assay: (% viability) 0.5: 117% 1: 110% 1.5: 31% 2: 31% 2.5:27%
7	100,000	60%	Dharmafect 1	0.5, 1, 1.5, 2, 2.5				MTT assay: (% viability) 0.5: 113% 1: 84.5% 1.5: 38.3% 2: 54.8% 2.5:34.3%
8	200,000	70%	Dharmafect 1	0.5-2.5			24 hours	Only cells at 0.5-1µl viable
9	200,000		Dharmafect 1	0.5-2.5			6 hours	Similar viability to cells treated for 24 hours

10	200,000		Dharmafect 1	0.5	GAPDH IGFIR IGFIR	25 25 50	24	Cells treated with SiRNA died
11	200,000	70%	Dharmafect 1	0.5	IGFIR	12.5, 25, 50	24	Viable
12	200,000	70%	Dharmafect 1	0.5	IGFIR	25, 50, 75	24	SIRNA treated cells died
13	200,000	70%	Dharmafect 1	1	IGFIR	12.5, 25, 50	24	SIRNA treated cells died
14	300,000	90%	Dharmafect 1	0.5	IGFIR	12.5, 25, 50	24	SIRNA treated cells died

All experiments in 6 well plate format. Reported results on mRNA expression are from qPCR

At this point, it was hypothesised that the transfection reagent, Dharmafect 1, especially when used in combination with IGF1R siRNA, was not optimal for the cell lines studied and a number of different transfection reagents were assessed for their toxicity across the range of their effective treating concentrations (section 2.17, table 2.7). From this experiment, the reagent Lipofectamine 2000 (Invitrogen Corp., CA, USA) was found to be the least toxic transfection reagent and this reagent was used in further experiments.

Transfection efficiency with Lipofectamine 2000 was assessed at 24 and 48 hours using siGLO® (ThermoScientific, Waltham, MA, USA), as described in section 2.17. This revealed that at both 24 and 48 hours approximately 80-90% of treated cells had fluorescing siGLO® visible (Figure 4.4). This indicates that toxicity observed following treatment with IGF1R knockdown is not related to the transfection reagent and that the poor knockdown results achieved were not due to suboptimal transfection efficiency.

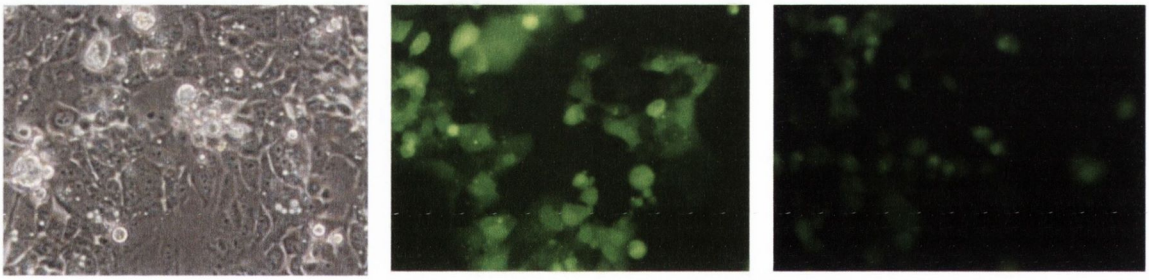


Figure 4.4 SiGLO® uptake in OE33 cell line after treatment with Lipofectamine 2000 (Invitrogen Corp, CA, USA). OE33 cells were plated at a density of 3.0 x10^5 cells per well of 6-well plate and treated with 25nM of SiGLO® transfection indicator (ThermoScientific, Waltham, MA, USA), mixed with 10µl Lipofectamine 2000 transfection reagent per well. (A). Cells were visualised under light microscopy and successful transfection with siGLO® was assessed using a microscope with a green fluorescent filter at (B). 24 and (C). 48 hours. Representative fields at 5X magnification.

Treatment with IGF1R siRNA and two different concentrations of lipofectamine 2000 revealed that while there was a decrease in IGF1R expression relative to mock transfected controls (transfection reagent only) there was only approximately 50% IGF1R knockdown at the mRNA level at 24 hours (Figure 4.5).

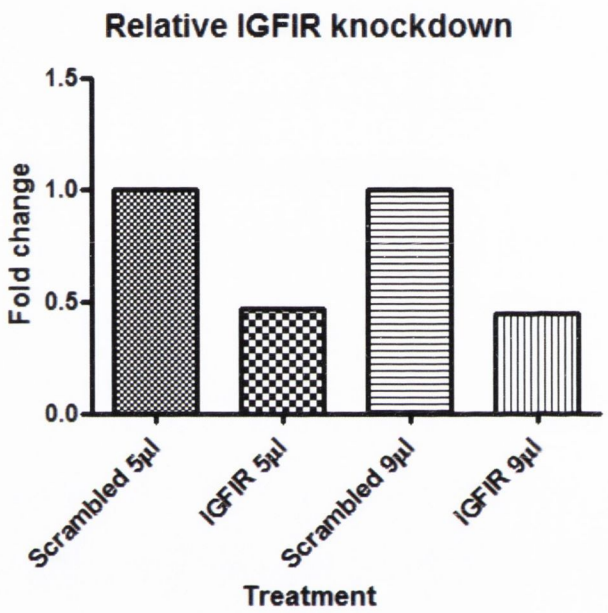


Figure 4.5 IGF1R knockdown in OE33 cells using two different volumes of Lipofectamine 2000 transfection reagent. IGF1R mRNA expression, as measured by qPCR, in cells treated with IGF1R siRNA was compared to a scramble control after 24 hours treatment. There was 53% and 55% knockdown of IGF1R with the 5µl and 9µl concentration of lipofectamine 2000 respectively.

A series of experiments were then performed across a 72 hour time period to assess knockdown at 24, 48 and 72 hours. The best rate of IGF1R knockdown achieved was 51% after 48 hours of treatment using 50nM siRNA. The positive control GAPDH

using the same conditions was knocked down by 78.5% (Figure 4.6). A similar process of optimisation in JH-Eso-Ad1 did not achieve any greater than 50% knockdown.

Using these conditions, IGF1R protein expression was measured by Western blotting (section 2.17) of protein from OE33 and JH-Eso-Ad1 cell lines but there was no decrease in IGF1R despite the approximately 50% decrease in IGF1R mRNA. Therefore, SiRNA knockdown on IGF1R was associated with cellular toxicity and despite, repeated attempts to optimise conditions for transfection, IGF1R knockdown was not achieved.

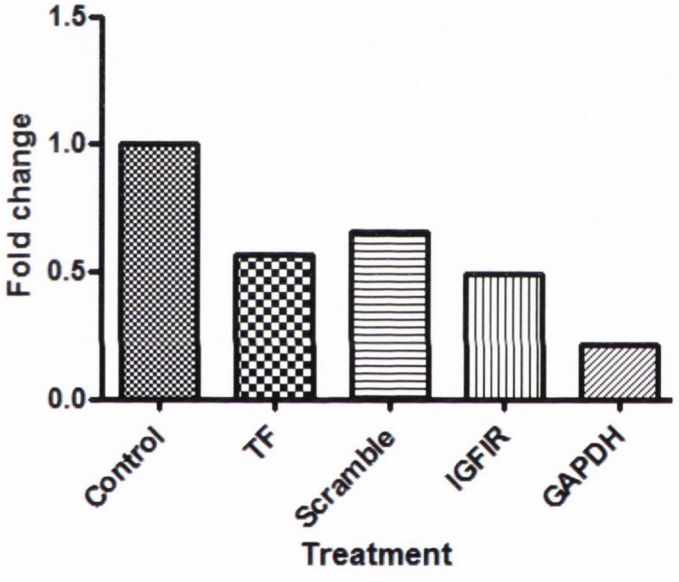


Figure 4.6 IGF1R knockdown at 48 hours. OE33 cells were seeded at a density of 3.0 x 10^5 cells per well of 6 well plate and treated at a confluency of 70%. All treatments were made up to 2ml/well. IGF1R expression mRNA expression by qPCR in control untreated cells was compared to the following treatments: versus mock transfection (TF; 10µl Lipofectamine 2000 only); 50nM scramble siRNA (non-targeting SiRNA); 50nM IGF1R siRNA. A positive control treatment well was included using 50nM GAPDH siRNA and expression of GAPDH in these cells was calculated relative to untreated controls. There was a 51% knockdown with IGF1R siRNA. Under the same conditions, there was 78.5% knockdown in GAPDH relative to scramble control.

4.3.2 Functional role of PPP treatment

The functional effect of PPP treatment *in vitro* in oesophageal adenocarcinoma was assessed by treating two oesophageal adenocarcinoma cell lines, OE33 and JH-Eso-Ad1 with the previously determined cell-line specific IC_{50} dose for a 24 hour period and assessing the effect on proliferation, cell cycle progression and apoptosis.

4.3.2.1 Proliferation

To determine whether treatment with PPP altered the proliferation of oesophageal adenocarcinoma cell lines, a BrdU cell proliferation assay was carried out. There was no difference in basal proliferation rates in either cell line after PPP treatment, however, there was a significant decrease in cellular proliferation in PPP treated cells versus controls after IGF stimulation (Figure 4.7). The relative decrease in proliferation in OE33 was 9.2% +/- 1.3%, $p<0.001$; in JH-ESO-Ad1: 28.4% +/- 4.2%, $p<0.001$.

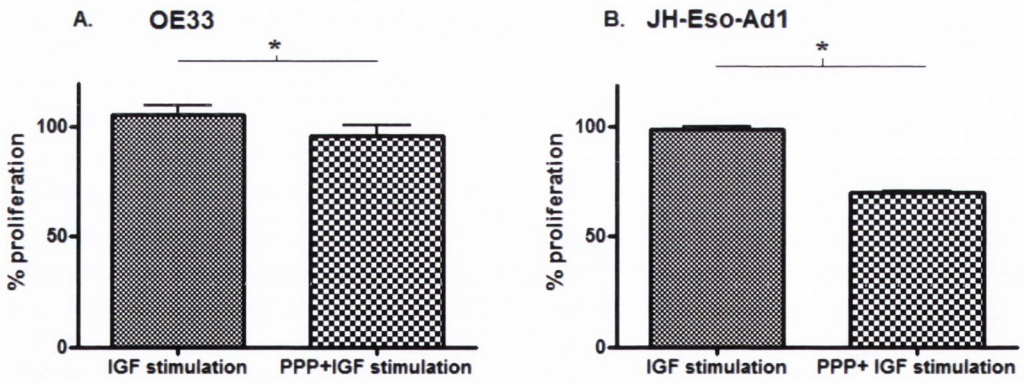
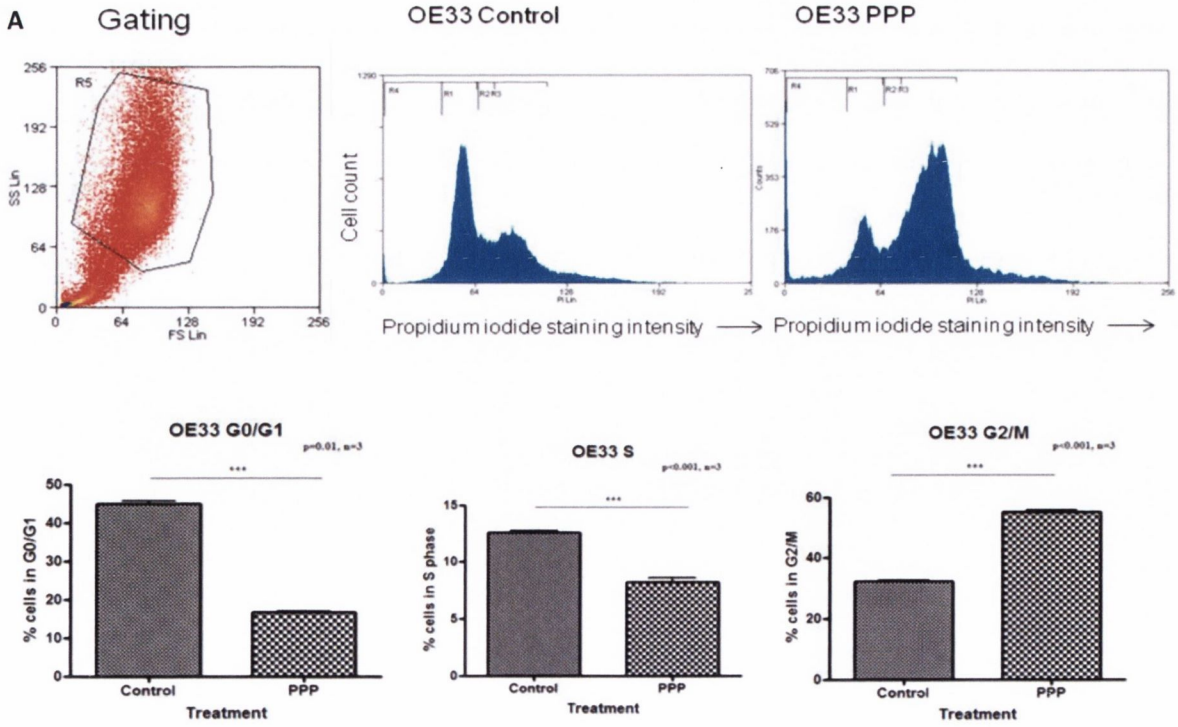


Figure 4.7 PPP inhibits cell line proliferation following IGF-1 stimulation. (A) OE33 and (B) JH-Eso-Ad1 cell lines were treated with receptor saturating doses of recombinant IGF-1 (1000ng/ml) and IC_{50} of PPP for 24 hours.). The relative decrease in proliferation in following treatment was (A) in OE33: 9.2% +/- 1.3%, $p<0.001$; (B) in JH-ESO-Ad1: 28.4% +/- 4.2%, $p<0.001$. Data are mean ± SEM, paired t-test, n=3.

4.3.2.2 Cell cycle progression

To investigate any alterations in cell cycle checkpoint operation, the effect of PPP treatment on cell cycle distribution in OE33 and JH-Eso-Ad1 cell lines was characterised by PI staining using flow cytometry as described in section 2.13 (Figure 4.8). Analysis was gated to exclude apoptotic cell populations. Treatment with the IC_{50} dose of PPP induced a significant accumulation of cells in G2/M phase at 24 hours (OE 33: 32.3 ± 0.75% vs. 55.2 ± 1.03%, p<0.0001; JH-Eso-Ad1: 20.25 ± 1.77% vs 27.56 ± 3.25%, p=0.02). There was a concomitant significantly reduced number of cells in G0/G1 phase (OE33: 45.0 ± 1.0% vs. 16.58 ± 0.62%, p<0.0001; JH-Eso-Ad1: 32.92 ± 2.39% vs 6.22 ± 0.58%, p=0.002) and in S phase (OE33: 12.59 ± 0.23% vs. 8.24 ± 0.6%, p=0.01; JH-Eso-Ad1: 13.09 ± 0.76% vs 4.87 ± 0.58%, p=0.002) in both cell lines. These data indicate that PPP blocks cells in the G2/M phase. There was also an increase in the sub G0 population following PPP treatment indicating that PPP induces apoptosis (expanded upon in section 4.3.2.3).



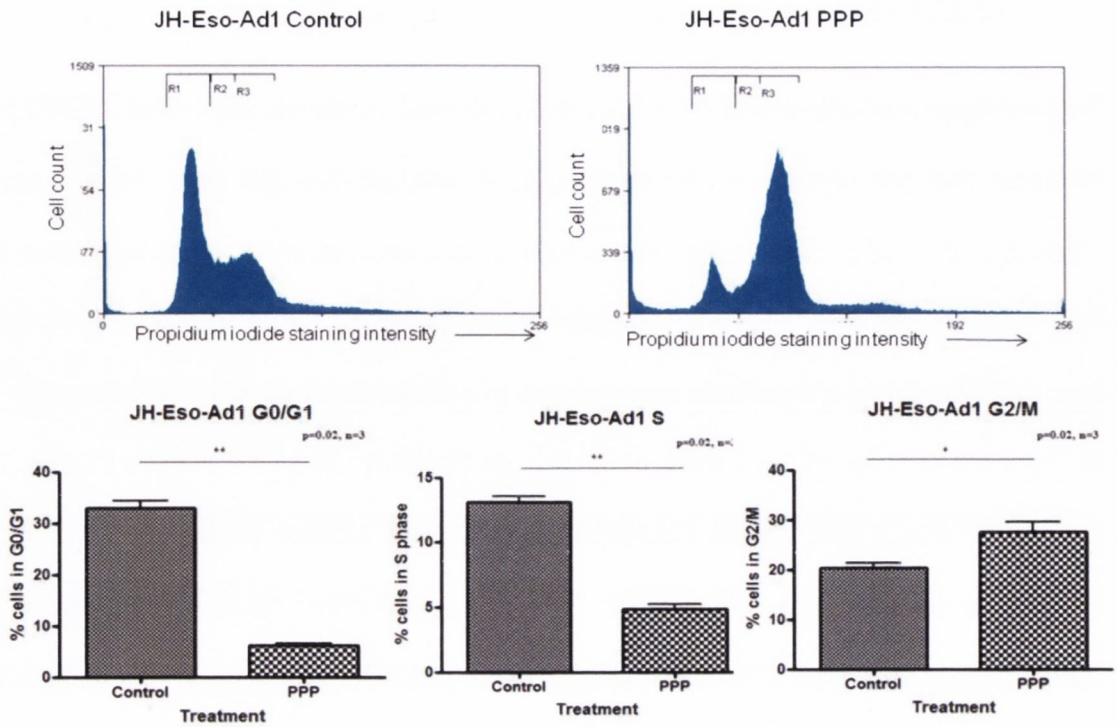


Figure 4.8 PPP treatment blocks cells in G2/M phase in (A) OE33 and (B) JH-Eso-Ad1. 24 hours after treatment with PPP, cells were stained with propidium iodide (PI) and cell cycle analysis was performed using flow cytometry (section 2.13). There were significant increases in the population in the G2/M phase (relative increase in (A) OE33: 28.42 ± 1.62%, $p<0.001$; (B) JH-Eso-Ad1: 24.68 ± 6.8%, $p<0.002$) with corresponding decreases in cell populations in the G0/G1 and S phases. Representative gating and PI staining plots are shown for each cell line. Data are mean ± SEM, paired t-test, n=3.

4.3.2.3 Apoptosis

The ability of PPP to induce alterations in apoptosis was initially assessed using high content screening techniques comparing morphologic changes consistent with apoptosis in both cell lines following PPP to controls and the current standard chemotherapy agent for OAC, cisplatin, as a positive control (Figure 4.9). These morphologic changes were

objectively measured using the In-cell analyser as described in section 2.14.2. Morphologic changes consistent with apoptosis include increases in nuclear and mitochondrial area and staining intensity and were analysed using automated InCell analysis of nuclear and mitochondrial area and staining intensity and cell count. Changes were compared in cells treated with PPP to control untreated cells in both OE33 and JH-Eso-AD1. Increases in nuclear and mitochondrial area and staining intensity represent early stage swelling, followed by condensation and disintegration. Cellular staining intensity decreases as the cells detach, bleb and disassemble into apoptotic bodies. The standard deviation of measurements increases when there is increased heterogeneity in cell populations of the relevant measurement.

Following PPP treatment, there were significant decreases in cell count and increases in nuclear area and intensity and mitochondrial area and staining intensity (Figure 4.10; Tables 4.1(a) and (b)).

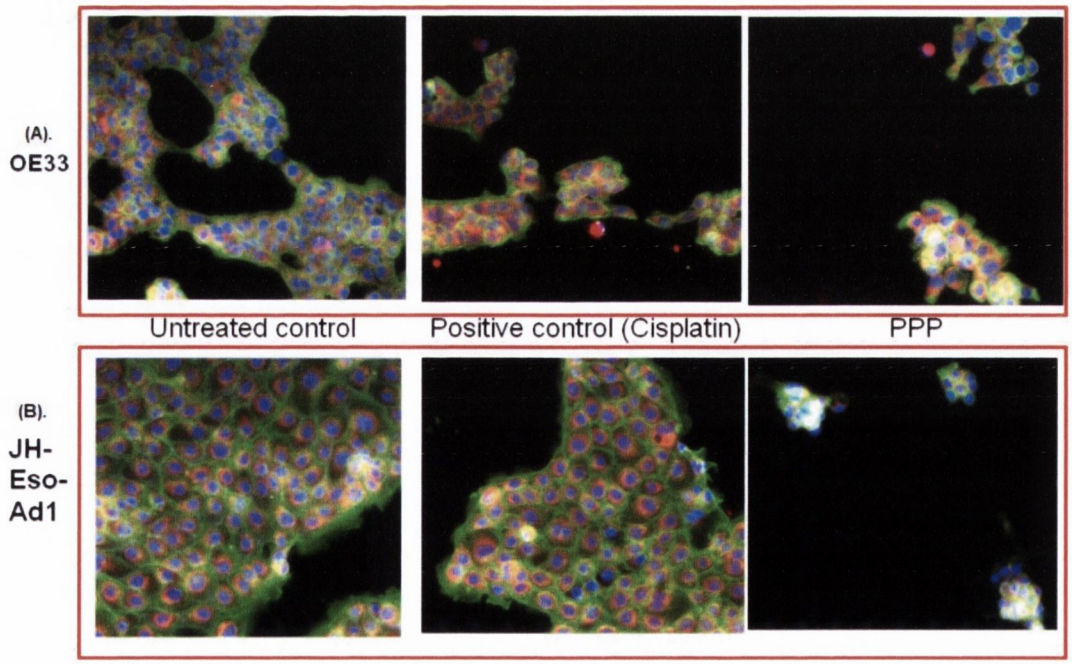


Figure 4.9 PPP induces changes associated with apoptosis using High content screening. Cells were stained with fluorescent markers for f-actin (Phalloidin; green);

the nucleus (Hoescht; blue) and mitochondria (MitoTracker; red). Morphologic changes consistent with apoptosis in both cell lines (A) OE33 and (B) JH-Eso-Ad1, were assessed following PPP treatment and compared to control untreated cells and a positive control, cisplatin (the current standard chemotherapeutic agent in OAC). Representative high power fields (20x magnification) for each treatment arm in both cell lines are displayed. There are substantial decreases in cell numbers following PPP treatment. There are visible increases in mitochondrial staining intensity with more diffuse staining and the nuclei have more diffuse staining visible after PPP treatment. These images were then analysed using Incell analysis techniques.

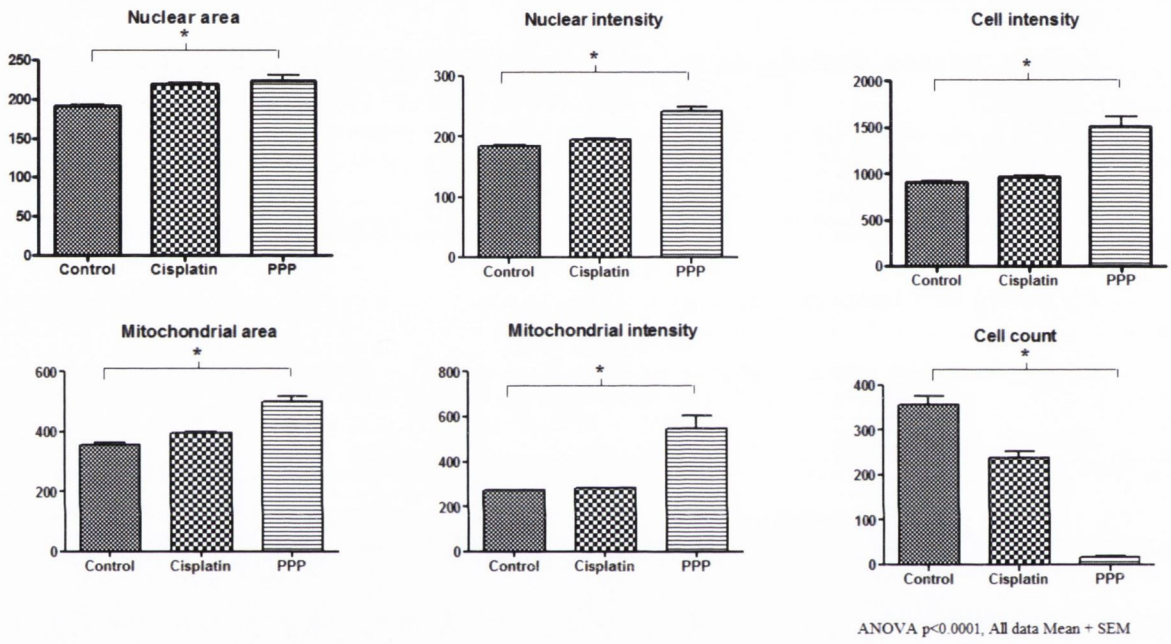


Figure 4.10: InCell analysis of changes visible using high content screening are consistent with induction of apoptosis. Morphological changes were automatically analysed using the INCell Analyzer 1000 (GE Life Sciences, Buckinghamshire, UK) which can assess changes in the number, intensity of staining, and area per object for each fluorescent stain. Changes consistent with apoptosis are thus numerically quantified and were subjected to statistical analysis using ANOVA. In the JH-Eso-Ad1 cell line, there were increases in both nuclear and mitochondrial area and staining intensity, as well as decreases in cell numbers. Data are reported in Table 4.1(b) Data are mean ± SEM; n=3, *=P<0.001.

Table 4.1 (a) InCell analysis of morphological changes after PPP treatment in OE33

Morphologic changes: OE33	Control (mean ±SD)	PPP (mean ±SD)	p-value, paired t-test
Nuclear area	199.42 ± 4.74	210.88 ± 8.86	0.07
Nuclear Staining Intensity	197.86 ± 7.9	205.76 ± 5.9	0.04
Cell intensity	1144.18 ± 90.7	1315.85 ± 17.93	0.06
Mitochondrial stain area	411.7 ± 16.4	391.29 ± 10.79	0.3
Mitochondrial staining intensity	334.55 ± 23.67	347.35 ± 10.0	0.25
Cell count	185.9 ± 6.11	119.57 ± 4.39	<0.0001

Table 4.1 (b) InCell analysis of morphological changes after PPP treatment in JH-Eso-Ad1

Morphologic changes: JH-Eso-Ad1	Control (mean ±SD)	PPP (mean ±SD)	p-value, paired t-test
Nuclear area	190.2 ± 10.06	214.24 ± 8.04	0.004
Nuclear staining intensity	184.84 ± 5.54	258.61 ± 11.74	0.006
Cell intensity	900.8 ± 44.95	1771.58 ± 88.13	0.0008
Mitochondrial stain area	350.65 ± 27.39	502.45 ± 87.64	0.01
Mitochondrial staining intensity	269.63 ± 10.34	679.24 ± 205.15	0.08
Cell count	357.2 ± 69.72	16.3 ± 3.34	0.014

Data are mean ± SD, n=3, paired t-test

In order to confirm that apoptosis was induced in both cell lines following PPP treatment, cells were stained with Annexin V-FITC and propidium iodide (PI) and the proportion of stained cells were assessed by flow cytometry (section 2.15). Early apoptotic cells was quantified as AnnexinV positive only cells and total apoptosis (apoptosis + necrosis) as cells with dual AnnexinV-FITC/PI staining. There was a significant induction of both early and total apoptotic events in both cells with PPP treatment. There appeared to be a ceiling effect with OE33 treatment as total apoptosis was not significantly different between the IC_{25}, IC_{50} and IC_{75} doses. However, no such effect was observed in JH-Eso-Ad1 with increasing concentrations associated with increasing levels of apoptosis. (Table 4.2 (a) and (b)). Representative dot plots and results are displayed in Figure 4.11.

Table 4.2 (a) Annexin-V-FITC/PI staining after PPP treatment in OE33

OE33 n=3	% of total cells in early apoptosis (mean ± SD, n=3)	p-value vs control (paired t-test)	% of total cells in late apoptosis (mean ± SD, n=3)	p-value vs control (paired t-test)
Control	15.5 ± 3.2	-	20.5 ± 4.63	-
PPP IC25	21.97 ± 3.0	0.0009	35.7 ± 3.7	0.007
PPP IC50	15.93 ± 2.62	0.9	35.2 ± 2.84	0.07
PPP IC75	14.13 ± 1.06	0.39	32.3 ± 1.67	0.03

Table 4.2 (b) Annexin-V-FITC/PI staining after PPP treatment in JH-Eso-Ad1

JH-Eso-Ad1 n=3	% of total cells in early apoptosis (mean ± SD, n=3)	p-value vs control (paired t-test)	% of total cells in late apoptosis (mean ± SD, n=3)	p-value vs control (paired t-test)
Control	5.94 ± 0.62	-	10.73 ± 1.58	-
PPP IC25	12.93 ± 2.15	0.03	19.06 ± 2.9	0.04
PPP IC50	10.02 ± 1.03	0.03	15.42 ± 0.97	0.06
PPP IC75	38.2 ± 12.45	0.048	46.07 ± 9.1	0.02

Data are mean ± SD, n=3, paired t-test.

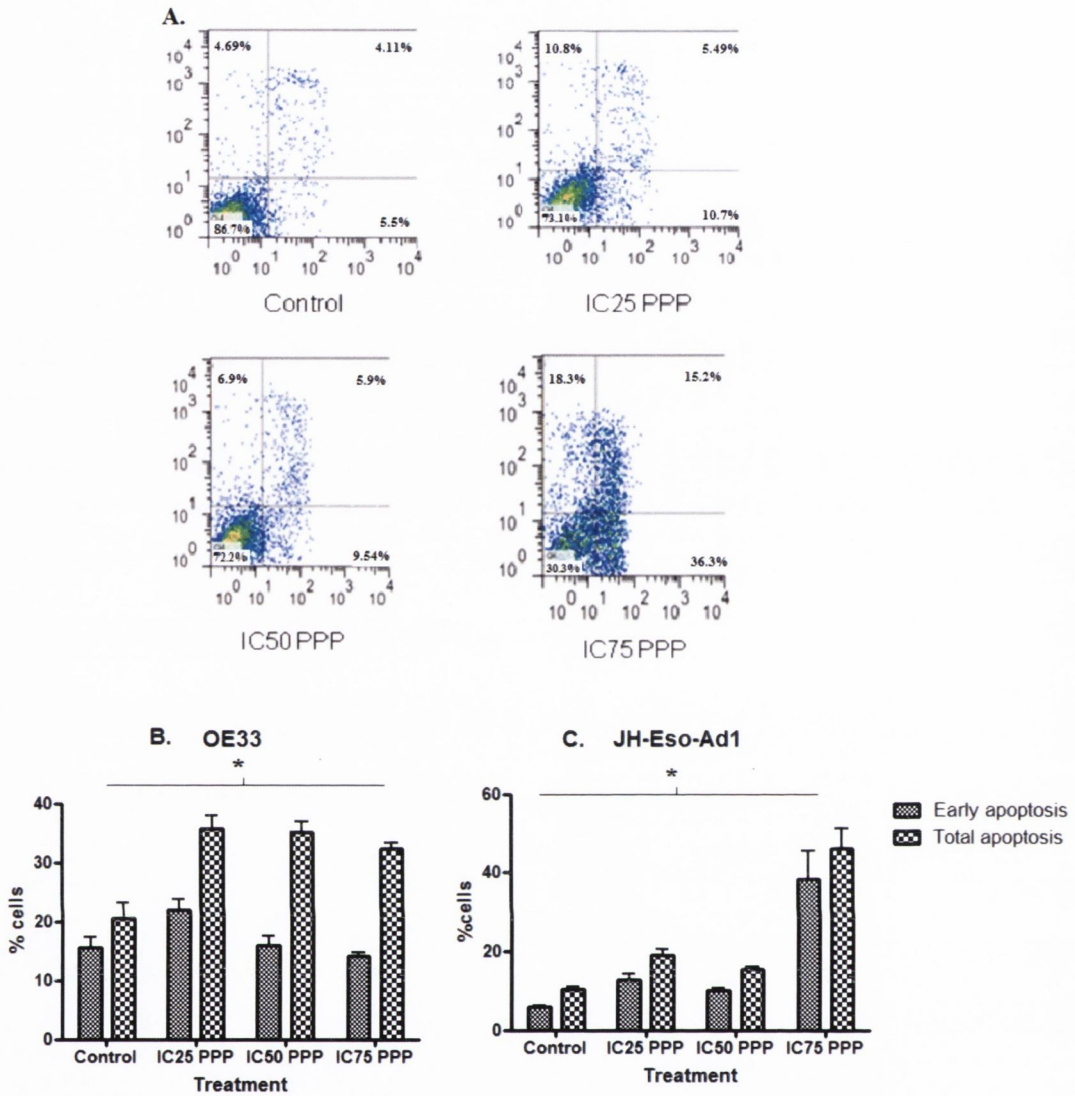


Figure 4.11 PPP induces apoptosis, measured by AnnexinV-PI staining. Representative dot plots from treatments with IC_{25}, IC_{50} and IC_{75} doses of PPP in JH-Eso-Ad1 cell lines are displayed in (A) Data from n=3 experiments of the various treatments in both cells line (B) OE33 and (C) JH-Eso-Ad1 indicate increases in early and late apoptosis with PPP treatment. There appeared to be a ceiling effect with (B) OE33 treatment as total apoptosis was not significantly different between the IC_{25}, IC_{50} and IC_{75} doses. However, no such effect was observed in (C). JH-Eso-Ad1 with increasing concentrations associated with increasing levels of apoptosis. Data are mean ± SEM, n=3, ANOVA, $p<0.001$.

4.3 Receptor localisation following PPP treatment

4.3.1 Total IGF1R expression is decreased following PPP treatment.

Total levels of expression of IGF1R after PPP treatment were assessed by Western blotting. There was a significant reduction in expression of IGF1R in OE33 and a trend towards reduced IGF1R in JH-Eso-Ad1 cells as measured by densitometry following PPP treatment (n=3), Figure 4.12. Following PPP treatment IGF1R (alpha subunit) expression was decreased by a mean of 46.32 ± 7.4% in OE33 and by 41.82 ± 9.2% in JH-Eso-Ad1.

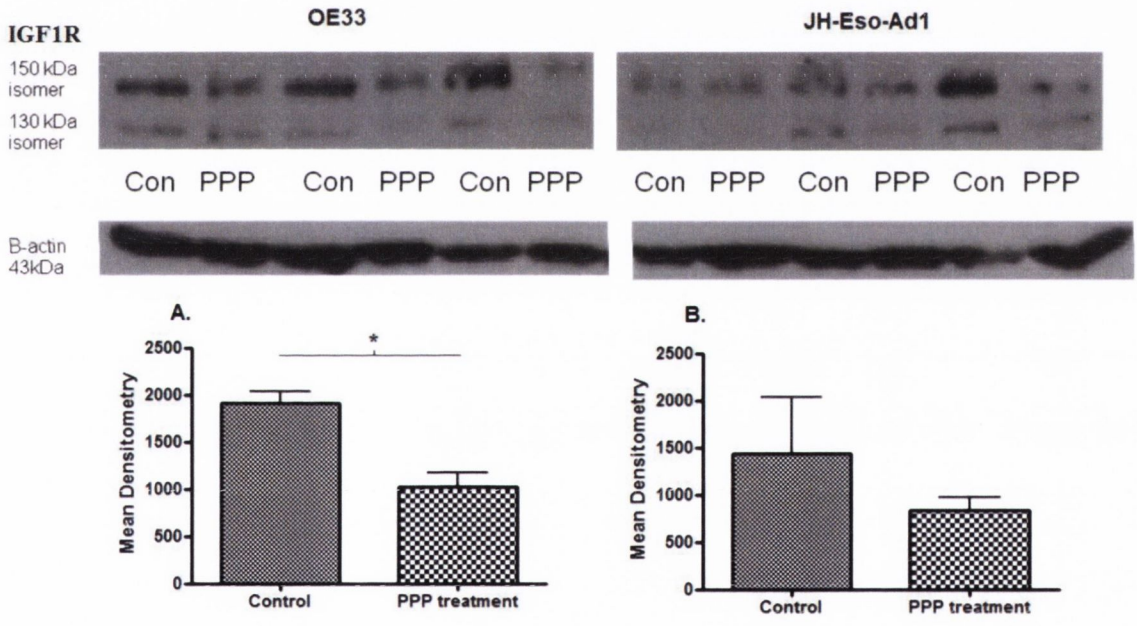


Figure 4.12 Treatment with PPP decreases total IGF1R expression. Following 24 hour treatment of cell lines with PPP, there was a decrease in the expression of both alpha and beta subunits of the total IGF1R cellular protein as determined by Western blotting (section 2.17). Protein expression was quantified by densitometry (section 2.19.4) and paired samples were compared using a paired t-test. (A). In OE33, following PPP treatment IGF1R (alpha subunit) expression was decreased by a mean of 46.32 ± 7.4%, p=0.01 and (B). by 41.82 ± 9.2% in JH-Eso-Ad1, p=0.4. Data are mean ± SEM, n=3. B-actin was used as a loading control.

4.3.2 Receptor localisation following PPP treatment

To determine the effect of PPP treatment on subcellular localisation of the receptor, cells were fractionated in a parallel experiment and the nuclear components were extracted and resolved by Western blotting and probed for IGF1R (section 2.17). In the nuclear extract, there was a trend towards decreased IGF1R expression in versus in untreated controls (100 ± 37.96 vs 49.82 ± 17.17% p=0.136).

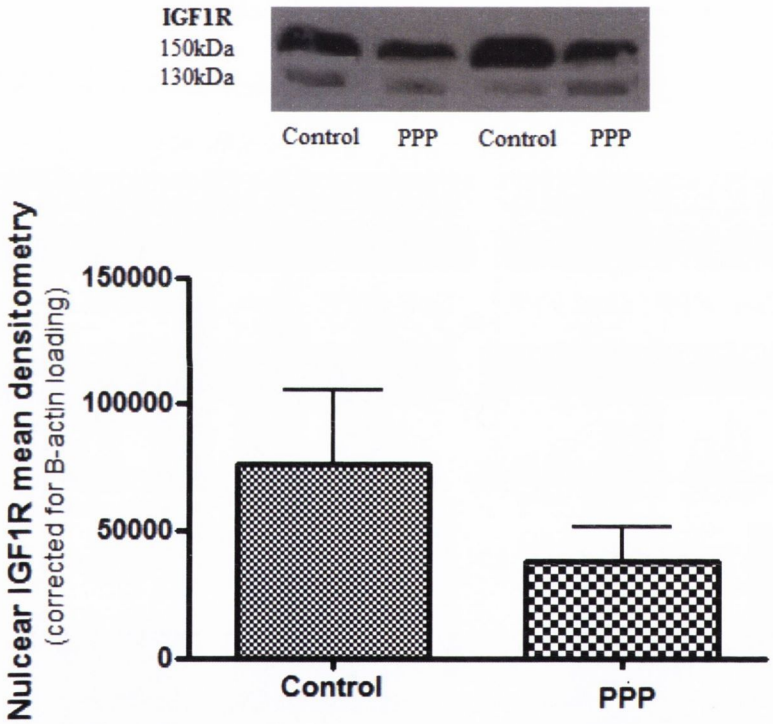


Figure 4.13. Treatment with PPP results in decreased nuclear IGF1R expression. OE33 cell line was treated with the IC_{50} dose of PPP for 24 hours and following this period nuclear extract was prepared from the treated cells and from control (untreated) cells. There was a trend towards an increase in the nuclear expression of IGF1R following PPP treatment versus controls (Control vs PPP: 100 ± 37.96 vs 49.82 ± 17.17% p=0.136). Data are mean ± SEM, n=3, paired t-test.

4.4 Discussion

The prognosis for oesophageal carcinoma is dismal with fewer than 20% of those diagnosed, alive at 5 years (Enzinger and Mayer, 2003). Most patients are unsuitable for treatment with curative intent, mainly due to the extensive nature of their disease at presentation (Reynolds et al., 2012). There is a clear requirement to explore targeted agents to expand treatment options for oesophageal adenocarcinoma. The physiological role of the IGF1R and the numerous *in vitro* studies demonstrating roles for IGF1R in metastasis and invasion make it a potentially attractive target for therapy (Roberts et al., 2010). Clinical evidence has been accruing that anti-IGF1R therapies have anti-tumour activity in patients with Ewing's sarcoma (Olmos et al., 2010), adrenocortical carcinoma and non-small cell lung cancer (Karp et al., 2009b).

Different methods of inhibiting IGF1R function include neutralising antibodies (both anti-receptor and anti-ligand), IGF-1 mimetics, dominant negative IGF1R and IGF1R siRNA. Whilst some of these approaches remain investigational, use of monoclonal antibodies and receptor tyrosine kinase inhibitors (TKI) have appeared in the clinical setting in early phase trials. Selective inhibition of IGF1R can be challenging as the receptor has 84% homology with that of the insulin receptor (Ullrich et al., 1986), with which it also heterodimerises. The potential clinical consequence is the development of insulin resistance and diabetes, secondary to excess growth hormone production, which may lead to unacceptable morbidity (Golan and Javle, 2011). Therefore, the ability to use an inhibitor specific to IGF1R and with anti-tumour activity is a potentially important clinical development. Clinical experience with TKIs has shown that *in vivo*, these drugs broadly inhibit both insulin and IGF receptors (Buck and Mulvihill, 2011). While this potentially increases theoretical hyperglycaemic and insulin resistance toxicities; this may help prevent insulin-mediated treatment resistance arising.

Furthermore, evidence suggests that these drugs do not accumulate well in muscle which is the main site for the development of insulin-resistance (Pollak, 2008).

Picropodophyllin is a cyclolignan agent with tyrosine kinase inhibitor activity against IGF1R (Vasilcanu et al., 2008). Treatment with PPP has previously been shown to decrease pAKT without affecting insulin-receptor activity (Girnita et al., 2004) and is thus thought to be relatively specific to IGF1R signalling and not that of the insulin receptor. It may be a potentially attractive targeted agent as it has shown activity in both *in vitro* and *in vivo* settings (Menu et al., 2006, Economou et al., 2008) but is not a licensed agent. Only cell lines which express IGF1R are sensitive to PPP (Girnita et al., 2004).

In this study, despite induction of increased autocrine production of IGF-1 in response to PPP treatment, there was no corresponding increase in cell viability and IGF-1 stimulated proliferation was significantly inhibited with PPP treatment. Attempts to artificially knockdown IGF1R using siRNA were unsuccessful due to large amounts of toxicity observed with relevant concentrations of siRNA. Numerous reports in the literature have reported difficulty with stable and transient knockdown of IGF1R or expression of inactive IGF1R mutants – as it results in the induction of apoptosis (Economou et al., 2008). This may indicate that IGF1R is a critical agent in the maintenance of cellular viability in oesophageal adenocarcinoma.

There were important functional consequences of IGF1R inhibition using PPP demonstrated in this study. PPP is a strong inducer of apoptosis and induction of apoptosis is now thought to be a critical component of effective chemotherapeutic agents (Pommier et al., 2004). PPP has been previously demonstrated to induce apoptosis by decreasing phosphorylation of Bad which leads to its dissociation from

Bcl-2, thus activating cytochrome c release from the mitochondria (Economou et al., 2008). Consistent with previous studies, there was a substantial increase in the fraction of cells in the G2/M phase, since signalling via IGF1R is necessary for late cell cycle phases (Adesanya et al., 1999, Sell et al., 1994).

Previous studies have demonstrated that PPP downregulates the IGF1R and that this is mediated via MDM2 E3 ligase which ubiquinates and causes degradation of IGF1R. The bridging molecule β-arrestin 1 links MDM2 and IGF1R and is necessary for the downregulation of IGF1R (Vasilcanu et al., 2007). β-arrestin 1 has also been shown to mediate HIF-1α induced VEGF expression in hypoxic conditions (Shenoy et al., 2012). Other IGF1R inhibiting antibodies lead to the downregulation of the IGF1R receptor (Sachdev et al., 2006, Huang et al., 2009, Kurmasheva et al., 2009) and this response to treatment may indicate clinical efficacy (Kurmasheva et al., 2009). In fact downregulation of IGF1R expression may be a critical step in the promotion of apoptosis, whereas inhibition of IGF1R phosphorylation only decreases proliferation (Hashemi et al., 2011). Re-biopsy following induction IGF1R inhibition for assessment of IGF1R expression may be a potential clinical application of this finding.

IGF1R activity may not only be altered by inhibition of phosphorylation but also SUMOylation and nuclear translocation, where modified IGF1R plays a role in transcriptional regulation (Sehat et al., 2010). SUMOylation is a post-translational modification whereby Small Ubiquitin-like Modifier (SUMO) proteins, a family of small proteins that are covalently attached to and detached from other proteins in cells, act to modify their function. The roles of nuclear IGF1R are only beginning to be characterised, however, it appears that nuclear IGF1R is found solely at the membrane receptor and that phosphorylation of the receptor is a requirement for nuclear translocation (Deng et al., 2011). Deng *et al* reported that nuclear IGF1R levels were

higher in breast cancer cell lines than in normal breast cells and that this correlated with the expression of Ubc9 a SUMO-conjugating enzyme. The consequences of increased amounts of nuclear IGF1R remain to be determined. In one study, the amount of nuclear IGF1R was associated with aggressive clinical outcome in renal cancer (Aleksic et al., 2010). In the present study, treatment with PPP resulted in a decrease in nuclear IGF1R expression as measured by Western blotting compared to untreated cells, although this was not statistically significant.

To conclude, this chapter demonstrates that IGF1R targeting is a viable option in this preclinical model of oesophageal adenocarcinoma – with important functional consequences - especially G2/M phase arrest and induction of apoptosis. Secondly PPP itself, may an attractive option for IGF1R targeting, especially given that it leads to the downregulation of expression of IGF1R, as well as functional results from inhibition of downstream signalling. An oral cyclolignan, AXL1717 with similar composition to PPP is undergoing clinical trials at the present time, with some favourable clinical responses reported in squamous NSCLC during the phase I study (Ekman et al., 2010b). Like all other forms of targeted therapy, prolonged treatment will lead to treatment resistance (Ellis and Hicklin, 2009). For example, a study of prolonged treatment with PPP of melanoma cell lines, lead to alteration in gene expression of genes involved in cell death such as PTEN and BCL2 (Hashemi et al., 2011). Acquired genomic copy number alterations in the regions corresponding to these altered mRNAs were also observed. Previous studies have also shown an inability to downregulate pAKT in response to PPP treatment (Strömberg et al., 2006, Vasilcanu et al., 2008). Mechanisms of resistance to PPP treatment will be explored in the next chapter. Understanding the effects of PPP will lead to a more rational approach for circumventing treatment resistance.

Results from the most recent set of phase III trials involving IGF1R inhibitors have been disappointing (expanded upon in chapter 1, section 1.4.4) (Gao et al., 2012). Most preclinical models of drug candidates which led to clinical trials of IGF1R inhibitors were based on experimental cancer models engineered to be IGF1R driven (Pollak, 2012b). In this study, there was no artificial overexpression of IGF1R and results from the previous chapter indicate that IGF1R protein expression may be prognostic factor in human oesophageal adenocarcinoma tumour samples.

4.5 Clinical relevance

What is already known: Personalised medicine using specific targeted therapies holds the promise of improving cancer survival rates. Identification of relevant targets for treatment of oesophageal adenocarcinoma is in its early stages. Epidemiological evidence suggests a link between obesity and the development of oesophageal adenocarcinoma. Data from this thesis support the hypothesis that the insulin-like growth factor axis may be a target in oesophageal adenocarcinoma.

What's new: Picropodophyllin (PPP), a cyclolignan specific inhibitor of IGF1R, has a number of functional effects on oesophageal adenocarcinoma cell lines, including G2/M phase arrest, induction of apoptosis and inhibition of proliferation. There was a decrease in the total amount of IGF1R present in the treated cells and also a decrease in the amount of nuclear expression of IGF1R.

Potential clinical implications: An oral cyclolignan, AXL1717 with similar composition to PPP is undergoing clinical trials at the present time. Data from this study indicate that this drug be of interest in the treatment of oesophageal adenocarcinoma.

Chapter 5

VEGF as a mechanism of treatment resistance to IGF1R inhibition in oesophageal adenocarcinoma

Presented at Patey Prize session of Society for Academic and Research Surgery (SARS) Annual meeting, Nottingham, UK, January 2012.

Awarded European Society of Esophagology Plenary prize, Annual Meeting, Newcastle, November 2011 and William O'Keefe Prize, October Surgical Meeting Plenary Session, Waterford, October 2011.

5.1.1 Oncogene addiction

The concept of oncogene addiction describes the apparent dependency of some tumours on one or a few genes to maintain the malignant phenotype (Weinstein, 2002). Clinical evidence of this is cited as the ability of therapies targeting specific genes or pathways to inhibit cancer cell growth or improve survival rates (Weinstein and Joe, 2006).

The poster child for targeted agents is imatinib and the dramatic clinical responses with its use led to anticipation of targeted agents acting as "magic bullets." It was the first drug which acts by specifically inhibiting oncoproteins characteristic of individual tumour types rather than non-specifically killing all rapidly dividing cells (Sawyers, 2004).

However, in clinical practice, targeted therapies have not proven to be the magic bullet once hoped (Daub et al., 2004). There may be primary or intrinsic treatment resistance – often due to inappropriate patient selection and use of targeted agents in patients whose tumours are not susceptible to inhibition of this pathway, or secondary resistance whereby after a period of treatment, a once effective treatment no longer prevents disease progression. Resistance mechanisms include increasing drug efflux, producing a drug-resistant variant of the targeted protein or substituting its cellular function by upregulating alternate pathways (Daub et al., 2004).

The phenomenon of compensation for targeted agent activity by enhanced activity in reciprocal RTKs has been described in a number of tumour types involving a number of RTKs. For example, resistance to EGFR and HER2 targeting agents may be mediated via a reciprocal upregulation in signalling via IGF1R pathway or by Met amplification (Bean et al., 2007, Engelman et al., 2007). In fact, in some patients, treatment with

tyrosine kinase inhibitors can lead to a disease flare, thought to be due to upregulation of alternate pathways in response to the targeted agent (Chaft et al., 2011).

Clinical phase III trials of anti-IGF1R agents have yielded disappointing results to date (Gao et al., 2012, Pollak, 2012b). Primary resistance appears to be an issue. Given the preclinical findings regarding widespread expression of IGF1R on a variety of different cancer types, it had been hoped that agents targeting this axis would have broad spectrum activity in unselected patient populations (Pollak et al., 2004a). Similar to most other targeted agents, anti-IGF1R agents may only have therapeutic activity in a subgroup of patients.

5.1.2 VEGF and IGF1R

There has been little exploration of the mechanisms underpinning resistance to IGF1R targeted therapies (section 1.4.4.2). One potential mechanism may be via upregulation of vascular endothelial growth factor (VEGF) production. VEGF is a critical growth factor for angiogenesis with a number of other proposed pro-tumourigenic mechanisms (Ellis and Hicklin, 2008). In other disease models, VEGF and IGF1R may be co-regulated with IGF1R activation leading to upregulation of VEGF production (Reinmuth et al., 2002, Akagi et al., 1998). Thus with inhibition of IGF1R activity, decreased VEGF production would be anticipated to occur. Both increases and decreases of VEGF have been reported in the literature following IGF1R inhibition. Increased VEGF production following treatment with an anti-IGF1R monoclonal antibody was attributed to weak agonistic effects of the monoclonal antibody (MAB) in one study (Warren et al., 1996). However, there is a complex and poorly understood regulation of VEGF production, especially with respect to how IGF1R may act to regulate VEGF. This interaction may potentially involve the mTOR pathway.

5.1.3 mTOR, VEGF and IGF1R

VEGF upregulation as a result of IGF1R inhibition may be mediated via mTOR activation. In response to growth factors and nutrients mTORC1 (mTOR complex 1) regulates cell growth by modulating protein synthesis, ribosome biogenesis and autophagy (Sarbassov et al., 2005). The exact mechanisms that regulate mTORC1 regulation remain poorly understood (Sabatini, 2006). PI3K-Akt activation leads to increased pAKT, which in turn decreases TSC1/2 (tuberous sclerosis protein 1/2), a negative regulator of the mTORC1 activating protein Rheb (Figure 5.1). mTORC1 then acts as a negative feedback regulator, i.e. an active mTORC1 pathway leads to suppression of PI3K-Akt signalling by inhibition of IRS1 (insulin receptor substrate 1) expression via increased S6K1 (Hay and Sonenberg, 2004). Therefore, mTOR inhibition leads to reciprocal increases in pAkt (Sun et al., 2005)

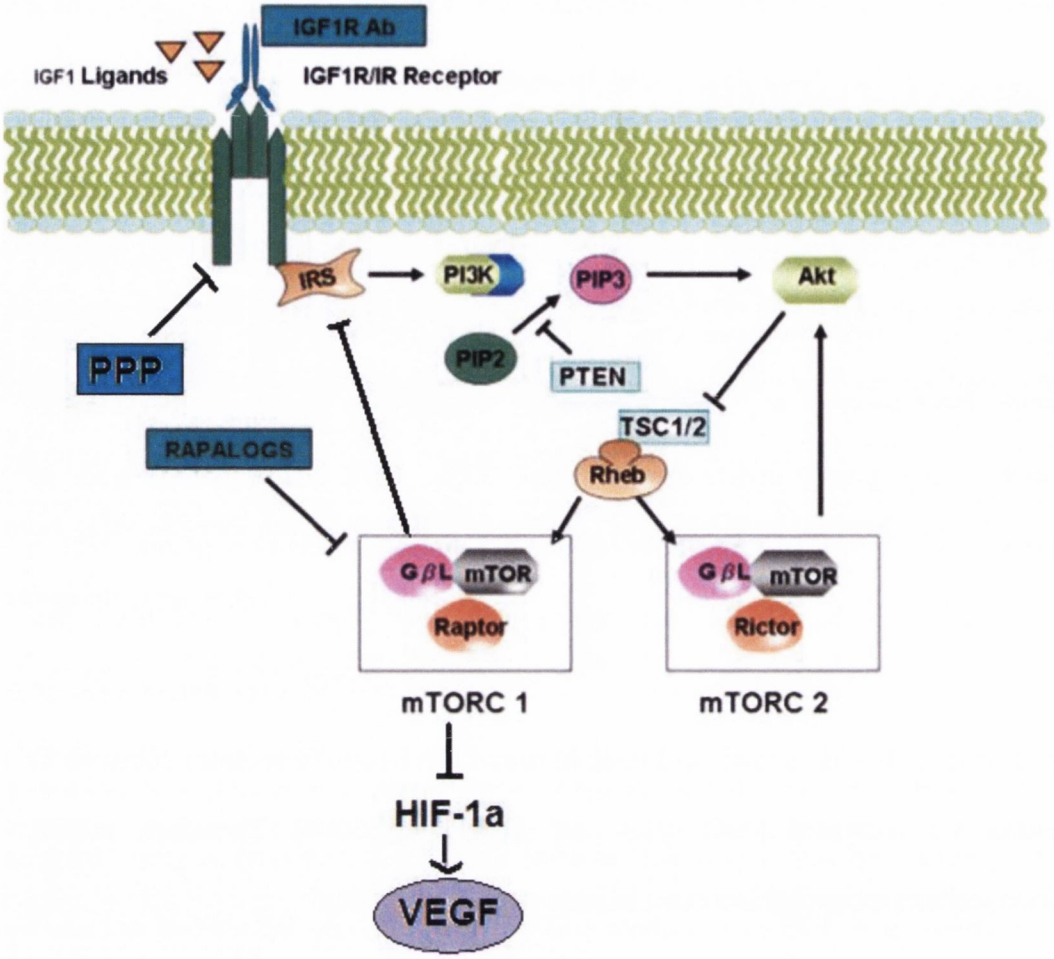


Figure 5.1 Simplified insulin-like growth factor 1 receptor and mTORC1 and mTORC2 signalling. Insulin Receptor substrate 1 (IRS1) and Phosphatidylinositol 3-kinases (PI3K) are activated by Insulin and/or IGF-1 signalling at the insulin/IGF1R receptor. PDK1 and Akt are recruited to the plasma membrane by products of PI3K, PIP2, PIP3 (phosphatidylinositol 3,4,5 trisphosphate and phosphatidylinositol 3,4 bisphosphate). After this event there is phosphorylation and activation of Akt by mTORC2 complex (mTOR + mLST8+ Rictor). This leads to a chain of activation of numerous targets by Akt. Now mTORC1 complex (mTOR + mLST8+Raptor) is activated by GTP bound Rheb and phosphorylates proteins like S6K. This starts a negative feedback loop to modulate auto-activity, through S6K-mediated pathway decrease in the activation of PI3K. There is some evidence that mTORC1 inhibition leads to activation of HIF-1α, which in turn leads to upregulation of VEGF mRNA and subsequent protein production.

There is some evidence that the signalling pathway which mediates the HIF-1α response is mTOR, whereby oxygen deprivation decreases mTOR activation resulting in activation of HIF-1α and therefore upregulation of VEGF and other hypoxic response genes (Majumder et al., 2004, Martin and Hall, 2005). In pancreatic cancer cells, mTOR inhibition leads to HIF-1α induced VEGF production (Wang et al., 2007c).

Since VEGF signals via complementary pathways, VEGF production may be an IGF1R inhibitor resistance mechanism and this hypothesis is explored in the present chapter.

5.2 VEGF and obesity status

Preliminary work carried out in this academic centre has implicated VEGF to be one of the factors involved in mediating the link between obesity and carcinogenesis. Using adipose tissue from a tissue bioresource within the St. James' Hospital biobank, centre the pro-tumourigenic factors produced by adipose tissue and which may, therefore, underpin the association between obesity, the inflammatory milieu and cancer were investigated (Lysaght *et al.*, 2011). The OE33 cell line was co-cultured with adipose tissue fragments and an Affymetrix gene expression array carried out. Utilising the KEGG database and pathway analysis techniques, the VEGF signalling pathway was found to be significantly upregulated after co-culture (Allott, 2010). Comparing subcutaneous and visceral adipose tissue depots, there were significantly increased amounts of VEGF in visceral adipose tissue (Figure 5.2). Significantly higher amounts of VEGF were observed in conditioned media from the visceral adipose tissue from centrally obese compared to non-obese patients. (Figure 5.3) Neutralisation of VEGF within this conditioned media from visceral adipose tissue significantly decreased

tumour cell proliferation following co-culture of cancer cells with adipose conditioned media (ACM) (Figure 5.4).

Therefore, similar to the IGF-1 axis, VEGF expression and activity may be one of the pathways of relevance to the link between obesity and carcinogenesis within the oesophageal adenocarcinoma model.

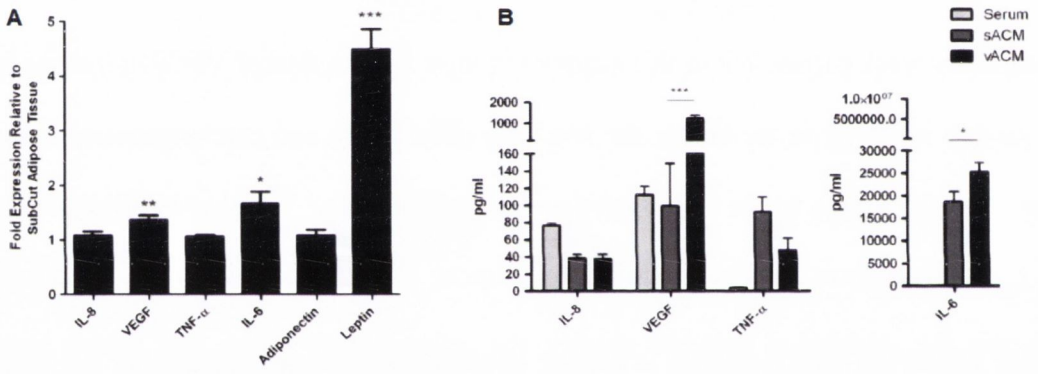


Figure 5.2: VEGF expression is higher in visceral adipose tissue relative to subcutaneous adipose tissue. (A). Increased VEGF, IL-6 and leptin gene expression in visceral adipose tissue relative to subcutaneous adipose tissue. Real-time PCR was carried out using specific primer probe sets for IL-8, VEGF, TNF- α, IL-6 and leptin. Results show gene fold expression in visceral adipose tissue relative to matched subcutaneous adipose tissue (n=25). (B). Adipokine screen of serum, sACM and vACM: Adipocyte conditioned media (ACM) was created by culturing adipose tissue from either patients' omentum (visceral ACM (vACM)) or from subcutaneous fat (sACM) in complete media for 72 hours. Protein levels of IL-8, VEGF, TNF-α were determined by ELISA. Significantly higher levels of VEGF were observed in vACM compared with sACM (n=45). Data are expressed as mean ± SEM, $p<0.05$ by Mann Whitney U test.

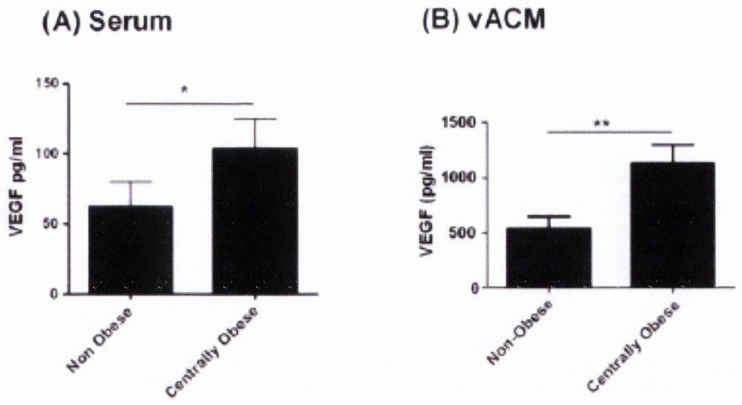


Figure 5.3: VEGF levels in serum and vACM from non-obese and centrally obese patients (A) Significantly higher levels of VEGF and leptin (data not shown) were observed in the serum of centrally obese patients (n=27) compared with normal weight patients (n=18) by ELISA. (B) VEGF in vACM was significantly higher in centrally obese patients compared with non-obese patients. Data are expressed as mean ± SEM, $p<0.05$ by Mann Whitney U test.

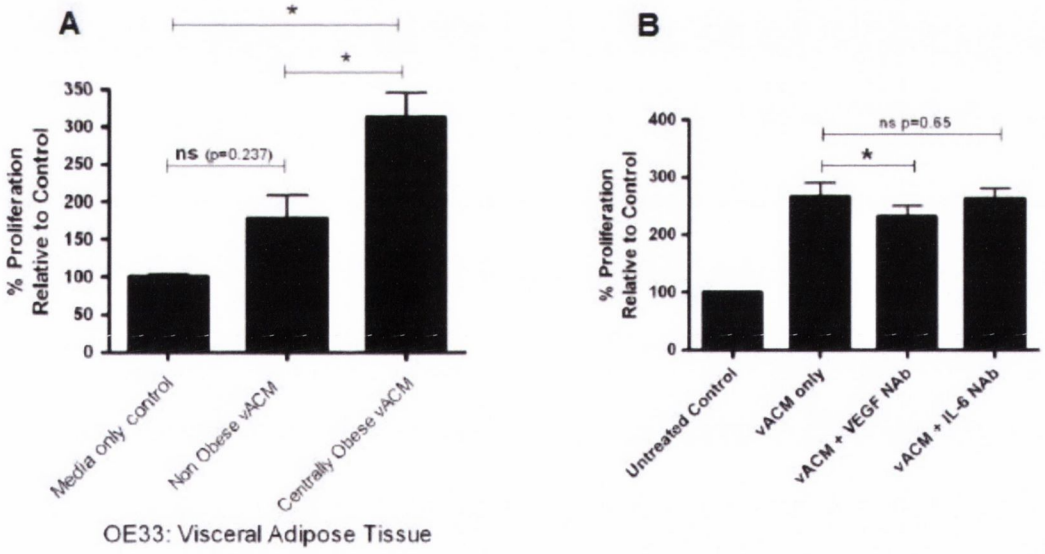


Figure 5.4: VEGF from vACM drives proliferation of OE33 oesophageal adenocarcinoma cells. (A). Proliferation of OE33 cells is increased following co-culture with vACM from obese patients and (B). Proliferation is decreased following neutralisation of VEGF within the vACM but not neutralisation of IL-6. Data are expressed as mean ± SEM, $p<0.05$ by Mann Whitney U test.

5.3 Specific aims

The overall aim of this chapter is to investigate mechanisms of resistance to IGF1R inhibition with the following specific aims:

1) Investigate whether VEGF is upregulated following PI3K pathway inhibition

2) Determine whether increases in autocrine VEGF production are of functional significance in oesophageal cancer cell lines

3) Explore whether combination of VEGF and IGF1R is of prognostic relevance in oesophageal adenocarcinoma patient tumour samples.

5.4 Results:

5.4.1 PPP treatment does not lead to decreased PI3K pathway inhibition

OE33 and JH-Eso-AD1 cells were treated with a specific IGF1R inhibitor, picropodophyllin (PPP) with functional effects on cell cycle progression, proliferation and apoptosis (Chapter 4). To confirm a decrease in downstream signalling following IGF1R inhibition, protein levels of phosphorylated AKT (pAKT), as a proxy indicator of PI3K pathway inhibition were measured by Western blotting (section 2.17). There was no statistically significant difference in pAKT expression at 24 hours following PPP treatment as measured by densitometry (OE33: 100 ± 0.99% vs 96.3 ± 2.9, p=0.25 and JH-Eso-Ad1: 100 ± 5.81 vs 104.23 ± 6.03%, p=1.0) (Figure 5.5). This indicates that signalling downstream of the IGF1R are not abrogated following IGF1R inhibition.

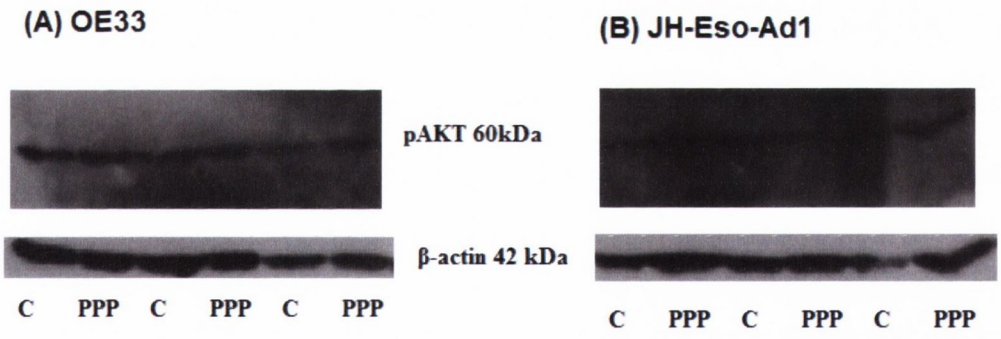


Figure 5.5 Treatment with PPP does not decrease pAKT levels. (A) OE33 and (B) JH-Eso-AD1 cells were treated for 24 hours with the relevant IC_{50} dose of PPP, a specific IGF1R inhibitor, and expression of pAKT protein was measured by Western blotting. β-actin levels were measured as a loading control. pAKT levels following PPP treatment were compared to control (n=3) and there was no significant differences observed between pAKT between treatment and controls. OE33: 100 ± 0.99% vs 96.3 ± 2.9, p=0.25 and JH-Eso-Ad1: 100 ± 5.81 vs 104.23 ± 6.03%, p=1.0. Data are mean ± SEM, n=3, $p<0.05$; Wilcoxon signed rank test.

5.4.2 PI3K pathway inhibition results in increased VEGF protein production

It was hypothesised that following treatment with PPP, maintenance of signalling via the PI3K pathway was via reciprocal increase in one of the cytokines which signals via receptor tyrosine kinases in turn leading to activation of the PI3K pathway. Given the preliminary findings outlined in section 5.2, indicating the relevance of VEGF to obesity-driven carcinogenesis and findings from other studies indicating co-regulation of IGF1R and VEGF, levels of VEGF were measured following treatment with LY294002. LY294002 is a non-specific inhibitor of PI3K pathway activation by inhibition of the catalytic subunit of phosphatidylinositol-4,5-bisphosphate 3-kinase (Vlahos et al., 1994). The IC_{50} dose of LY294002 for OE33 was determined by MTT assay (section 2.10).

Following treatment of OE33 with the IC_{50} dose of LY294002, protein levels of VEGF in cellular supernatants were measured by ELISA (section 2.6.2). VEGF levels in the supernatants of treated cells were significantly increased following PI3K inhibition (100 ± 4.5% vs 174.9 ± 16.5%, p=0.027, paired t-test) (Figure 5.6).

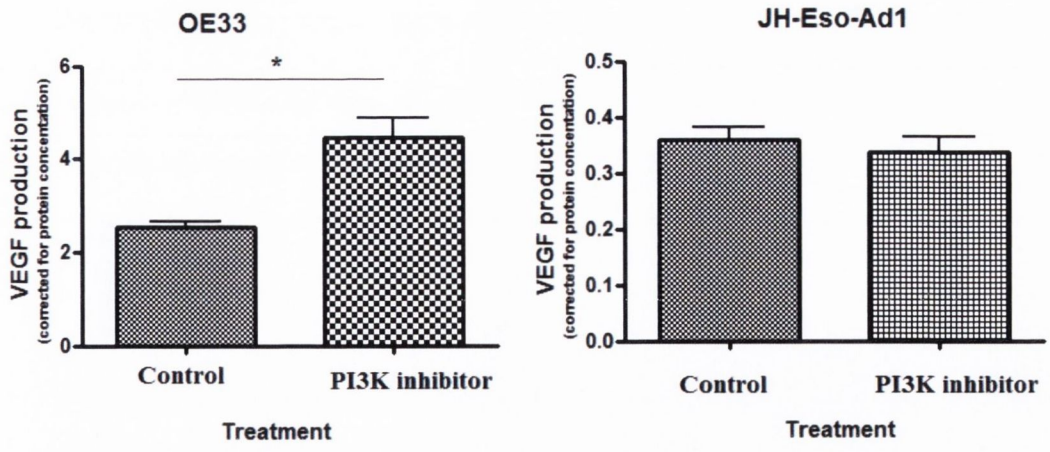
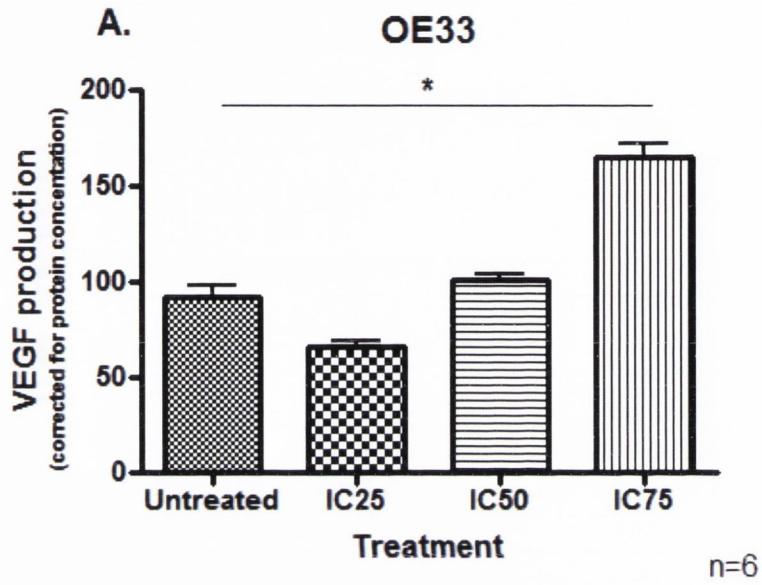


Figure 5.6 VEGF production is increased in OAC cell lines following PI3K inhibition. The OE33 cell line was treated with IC_{50} dose of LY294002, a non-specific PI3K inhibitor. VEGF protein in cellular supernatants was measured by ELISA and treatments compared to controls (n=6). Data are corrected for number of viable cells remaining by calculating the protein concentration of the remaining cells and correcting the VEGF concentration for protein concentration. VEGF in cellular supernatants was increased by 1.75 fold after treatment with LY294002 in the OE33 cell line, $p<0.05$ calculated by paired t-test; however, there was no significant alteration in VEGF production following PPP treatment in JH-Eso-Ad1 cell line. Data are mean ± SEM, n=3.

5.4.3 PPP treatment leads to increased autocrine VEGF production

VEGF protein in cellular supernatants was measured by ELISA following treatment of cell lines with PPP at IC_{25}, IC_{50} and IC_{75} doses. There was a significant increase in VEGF protein expression in both cell lines following IGF1R inhibition with PPP. In the JH-Eso-Ad1 cell line VEGF production was increased at 24 hours after treatment with both IC_{50} and IC_{75} doses (IC_{50}: 68.62 ± 1.8 vs 135.66 ± 5.1 pg/ml, p=0.0313; Wilcoxon matched pairs signed rank test and IC_{75}: 68.62 ± 1.8 vs 185.59 ± 18.2 pg/ml, p=0.0313). In OE33 cell line, there was an increase in VEGF production with the IC_{50} dose which achieved a borderline statistical significance (92.0 ± 6.19 vs 101.66 ± 2.8 pg/ml, p=0.09) and there was a significant increase after treatment with the IC_{75} dose of OE33 (92.0 ± 6.19 vs 165.4 ± 6.27 pg/ml, p=0.0313) (Figure 5.7).

This effect may be specific to either tyrosine kinase inhibitors of IGF1R or PPP alone as treatment with R1507 (Hoffmann-La Roche, Basel, Switzerland) a fully humanised monoclonal antibody against IGF1R did not significantly affect VEGF production in either cell line (Figure 5.8).



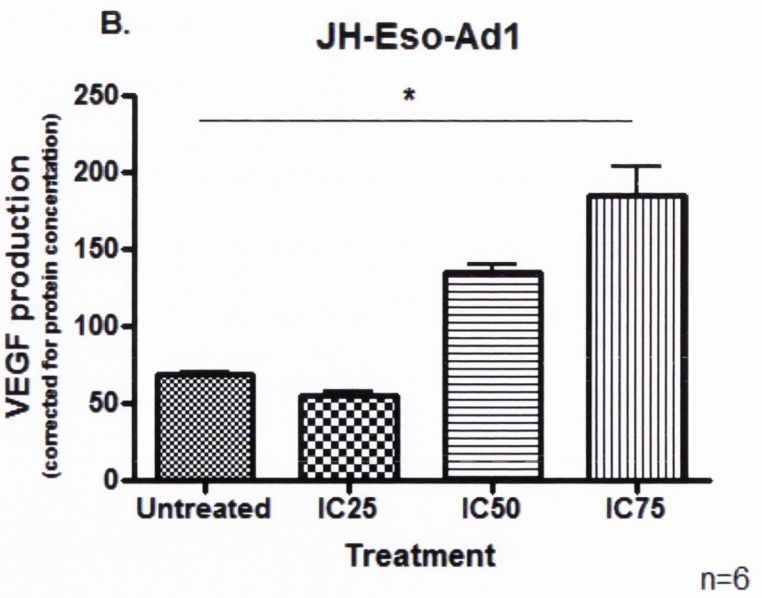


Figure 5.7 Treatment with PPP increases autocrine VEGF production. (A) OE33 and (B) JH-Eso-Ad1 cell lines were treated with the relevant IC_{50} doses of PPP. VEGF protein in cellular supernatants was measured by ELISA, corrected for protein production and treatments compared to controls (n=6). (A). In OE33 cell line, there was an increase in VEGF production with the IC_{50} dose which achieved a borderline statistical significance (92.0 ± 6.19 vs 101.66 ± 2.8 pg/ml, p=0.09) and there was a significant increase after treatment with the IC_{75} dose of OE33 (92.0 ± 6.19 vs 165.4 ± 6.27 pg/ml, p=0.0313) (B). JH-Eso-Ad1 IC_{50}: 68.62 ± 1.8 vs 135.66 ± 5.1 pg/ml, p=0.0313; Wilcoxon matched pairs signed rank test and IC_{75}: 68.62 ± 1.8 vs 185.59 ± 18.2 pg/ml, p=0.0313). Data are expressed as mean ± SEM, comparisons by Wilcoxon matched paired signed rank test.

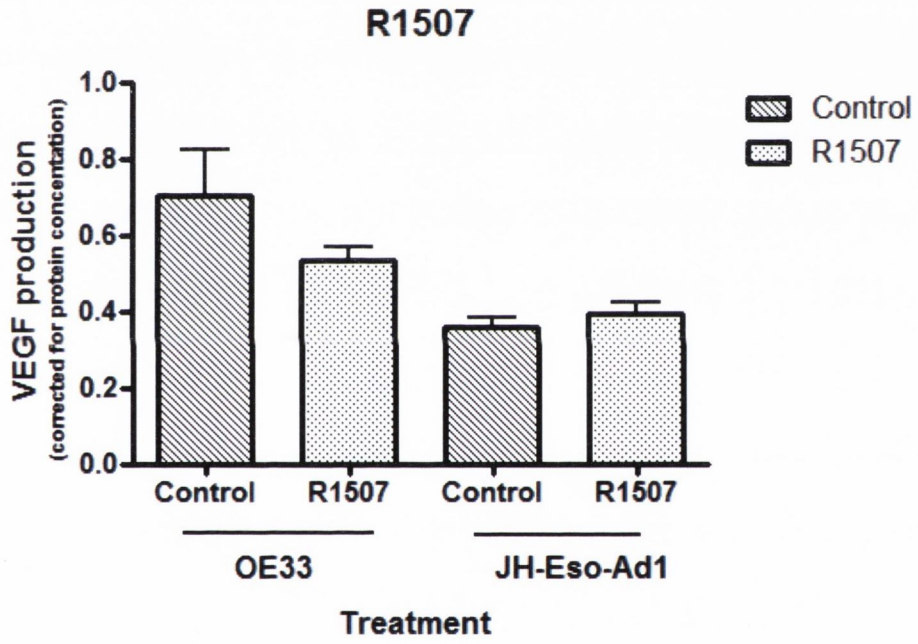


Figure 5.8 R1507 does not affect VEGF production in OAC cell lines at 24 hours. There was no significant difference in production of VEGF OAC cell lines following R1507 treatment (50μg/ml)) at 24 hours (OE33 p=0.31 and JH-Eso-Ad1 p=0.84). Data are mean ± SEM.

5.4.4 VEGF neutralisation leads to decreases in certain functional parameters.

In order to investigate whether VEGF production was relevant to cancer cell function a number of functional endpoints including viability, cell cycle progression, apoptosis and proliferation were investigated. VEGF protein excretion in supernatants was calculated using ELISA and the relevant concentration of a VEGF neutralising antibody (R&D systems, MN, USA) was calculated using the neutralising concentration (ND_{50}) curve provided in the product information sheet (section 2.10.1). The supernatants of cells treated with the VEGF neutralising antibody (50ng/ml) were screened for VEGF and neutralisation to below assayable levels was confirmed.

The experimental set-up is described in detail in section 2.10. To summarise, cells were treated with the VEGF neutralising antibody, IC_{50} dose of PPP and a combination of both treatments for a 24 hour time period.

5.4.4.1 Cell viability

Cell viability was investigated using MTT assay (section 2.11) at 24 hours. There were no statistically significant differences in cell viability following treatment with VEGF neutralising antibody alone (OE33: Control: 100% vs VEGF neutralising antibody 117.2 ± 32.4%, p=0.62; JH-Eso-Ad1: Control: 100% vs VEGF neutralising antibody 108.6 ± 19.9%, p=0.68). While a reduction in cell viability was observed following PPP treatment, addition of VEGF neutralisation did not significantly affect the overall cell viability at 24 hours (OE33: PPP: 74.8 ± 2.1% vs Both 59.1 ± 1.4%, p=0.22; JH-Eso-Ad1: PPP: 41.9 ± 10.3%, vs Both 50.1 ± 7.6%, p= 0.06) (Figure 5.9).

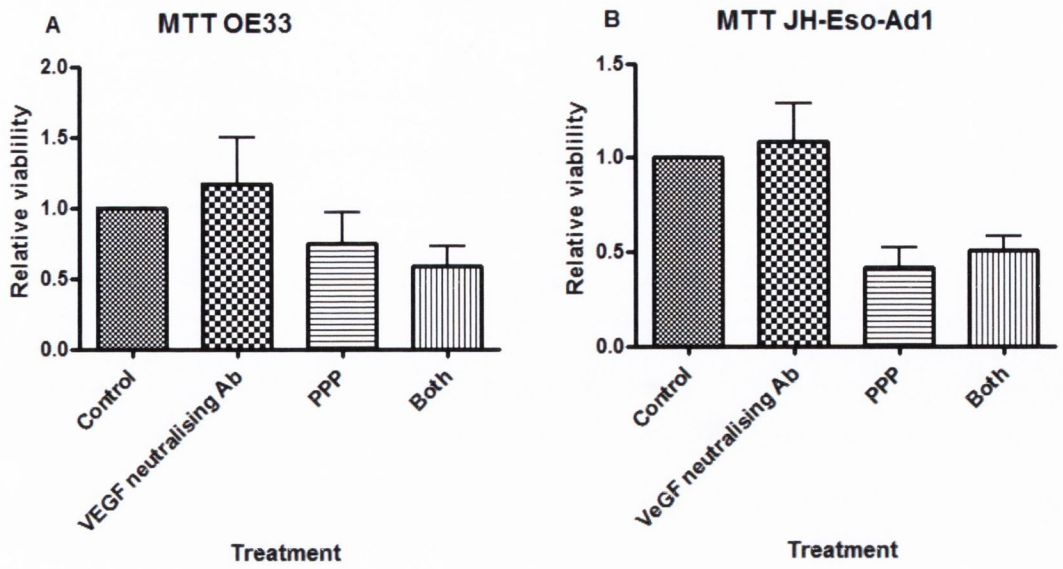


Figure 5.9 VEGF neutralising antibody treatment does not alter OAC cell line viability. A. OE33 B. JH-Eso-Ad1. There were no statistically significant increases in cell viability following VEGF neutralisation compared to controls nor when it was combined with PPP treatment compared to PPP treatment only. Data are mean ± SEM, n=3; $p<0.05$ paired t-test.

5.4.4.2 Apoptosis

Induction of apoptosis was measured using flow cytometric analysis of Annexin V-PI staining (section 2.15). There was no significant difference in the proportion of cells in either early or late apoptosis following VEGF neutralising antibody treatment only in either cell line. There was a substantial increase in both forms of apoptosis following PPP treatment as previously noted in chapter 4. However, the proportion of cells in apoptosis was not further altered with combination VEGF neutralisation and IGF1R inhibition (Table 5.1; Figure 5.10). It was noted that a large amount of cellular debris was noted following PPP treatment both alone and in combination with VEGF neutralisation. This was not included in the gated analysis, as per standard protocol and may represent cells which have already undergone necrosis by the 24 hour timepoint (Figure 5.10).

Table 5.1 AnnexinV-PI staining in OAC cell lines following treatment with VEGF neturalising antibody and PPP

	Control	VEGF Neut Ab	PPP	Both	p-value*
OE33					
Early apoptosis	13.4 (0.8)	9.94 (0.3)	20.8 (1.9)	18.83 (0.8)	0.25
Late apoptosis	9.47 (0.4)	11.67 (1.1)	22.13 (1.7)	22.5 (2.0)	1.0
JH-Eso-Ad1					
Early apoptosis	3.97 (0.3)	4.3 (0.5)	3.75 (0.3)	3.76 (0.26)	0.5
Late apoptosis	8.25 (1.0)	10.8 (1.26)	13.2 (0.6)	9.96 (0.67)	0.25

All data are mean ± SEM. P-value refers to comparison of PPP versus Both using the Wilcoxon matched-pairs sign rank test.

The relative absence of an effect of combination treatment on apoptosis may reflect the substantial induction of apoptosis with PPP treatment and which was not affected by VEGF autocrine upregulation, perhaps because it is an early event which once the cells are committed to cannot be affected. The process by which IGF1R inhibition can lead to apoptosis is poorly understood and it alternatively may be due to the fact that apoptosis is regulated via a different pathway to the PI3K-pAkt pathway (Peruzzi et al., 1999).

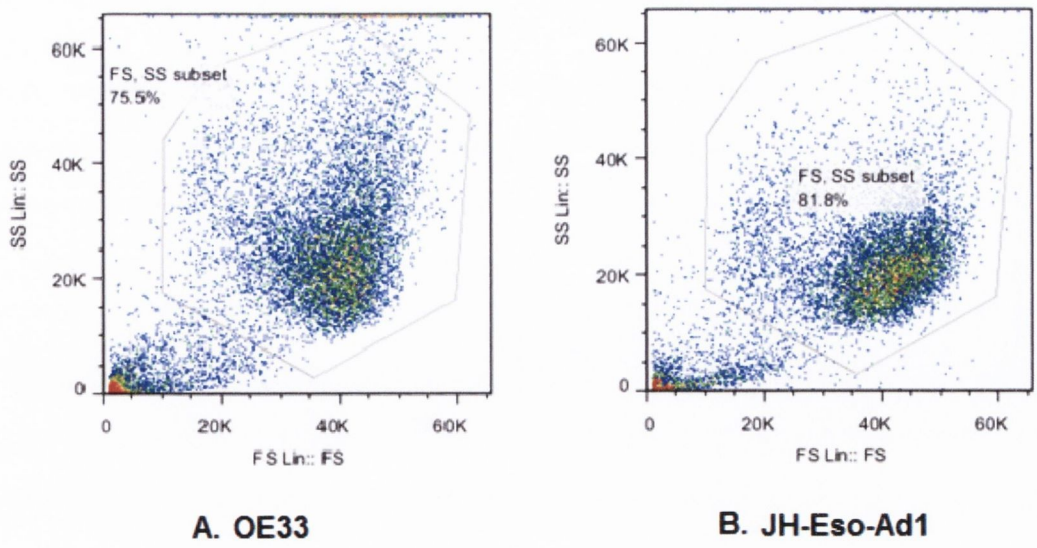
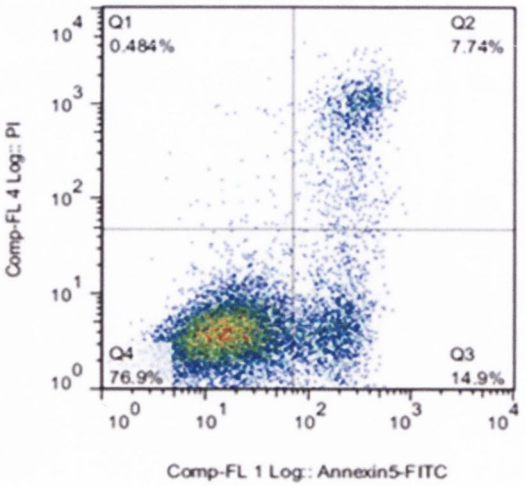
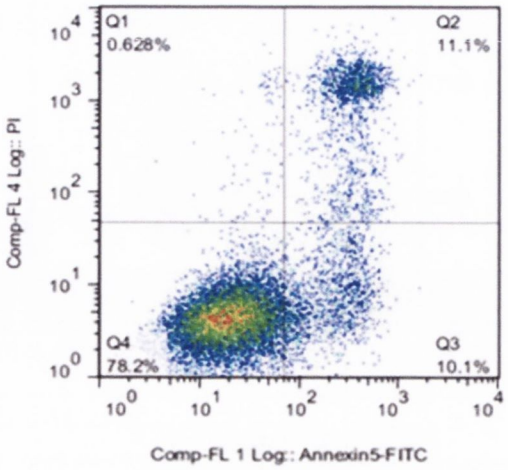


Figure 5.10 Cellular populations included in the gated analysis for AnnexinV-PI staining. Following treatment with PPP there was a large volume of cellular debris in both (A). OE33 and (B). JH-Eso-Ad1 cell lines which may represent cells which have already undergone cell death and is not included in the analysis by convention as they are not intact cells, have high levels of autofluorescence and would likely skew the data.

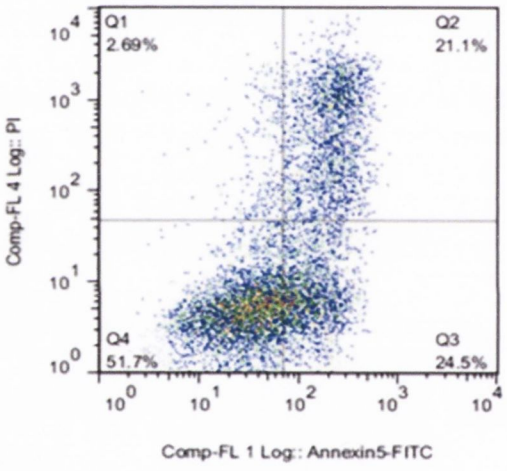
A. Representative dot plots



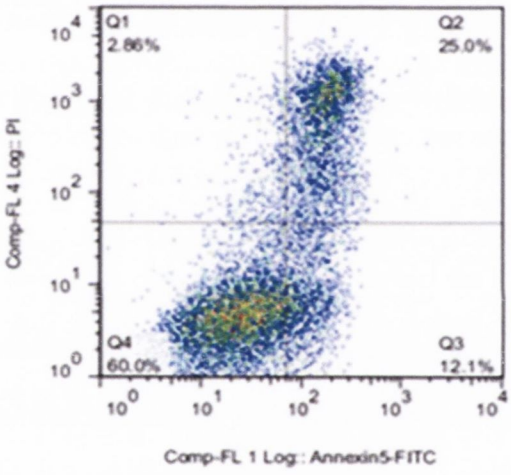
Control



VEGF neut Ab



PPP



Both

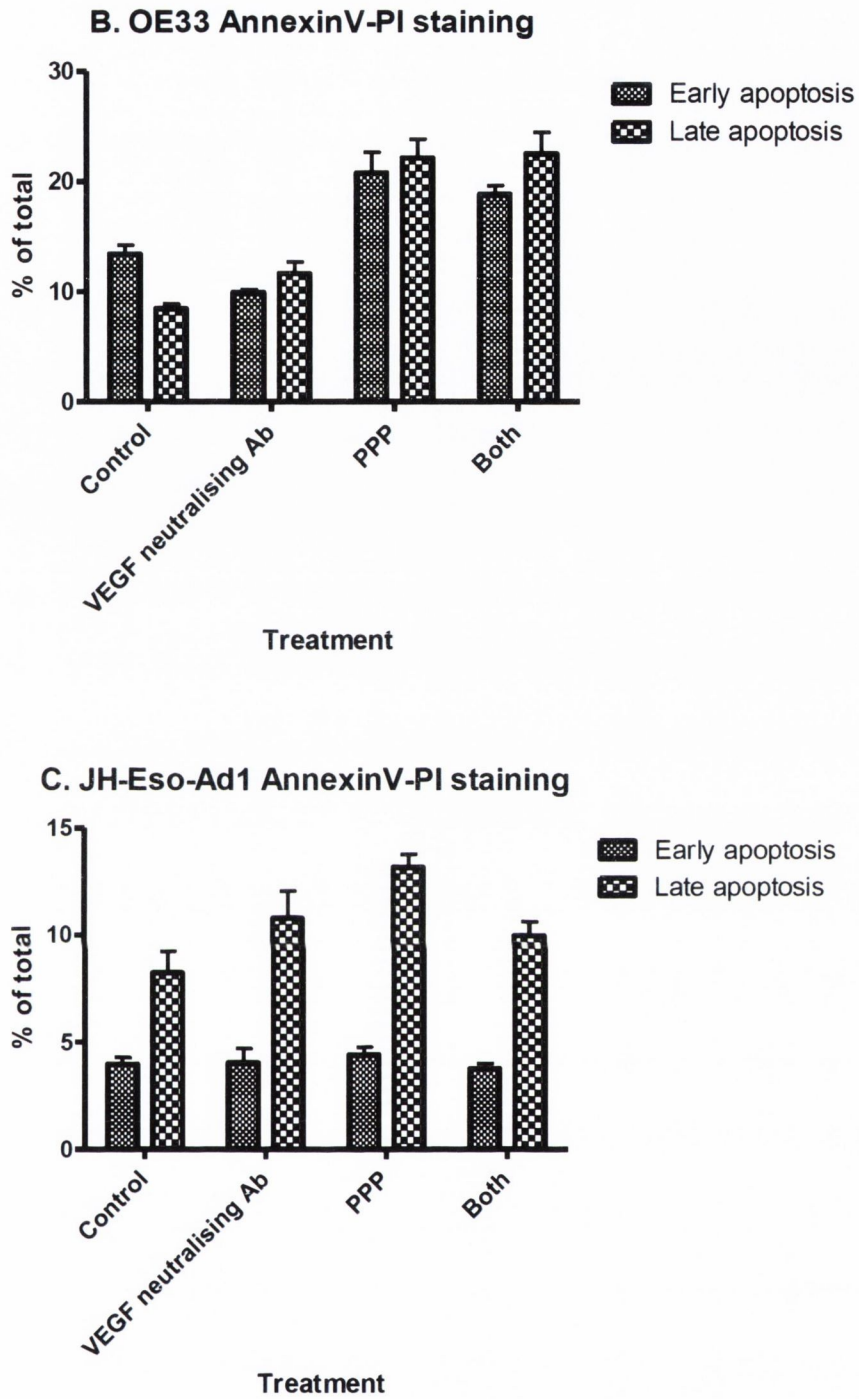


Figure 5.11 Treatment with both PPP and VEGF neutralising antibody does not alter the proportion of cells in apoptosis. (B). OE33 and (C). JH-Eso-Ad1 cell lines were treated with VEGF neutralising antibody in combination with PPP and induction of apoptosis was investigated using AnnexinV-PI staining. (A). Representative dot plots of OE33 cells are included. There was no significant alteration in the proportion of cells in both early and late apoptosis at the 24 hour time point using the different treatments. All data are mean ± SEM, n=3.

5.4.4.3 Proliferation

Proliferation was measured using a BrdU cellular proliferation assay (section 2.12). Combination treatment with both IGF1R inhibition and VEGF neutralisation resulted in a further decrease in cellular proliferation in the OE33 cell line (% proliferation versus controls following PPP treatment: 84.9 ± 10.0% compared to 59.8 ± 5.4% with both treatments, $p<0.009$ paired t-test). (Figure 5.12) This effect was not noted with the JH-Eso-AD1 cell line (% proliferation versus controls following PPP treatment: 62.95 ± 7.2% compared to 64.95 ± 8.2% with both treatments, $p=0.46$ paired t-test). This may reflect the greater proportional decrease in proliferation following PPP treatment in the JH-Eso-Ad1 cell line.

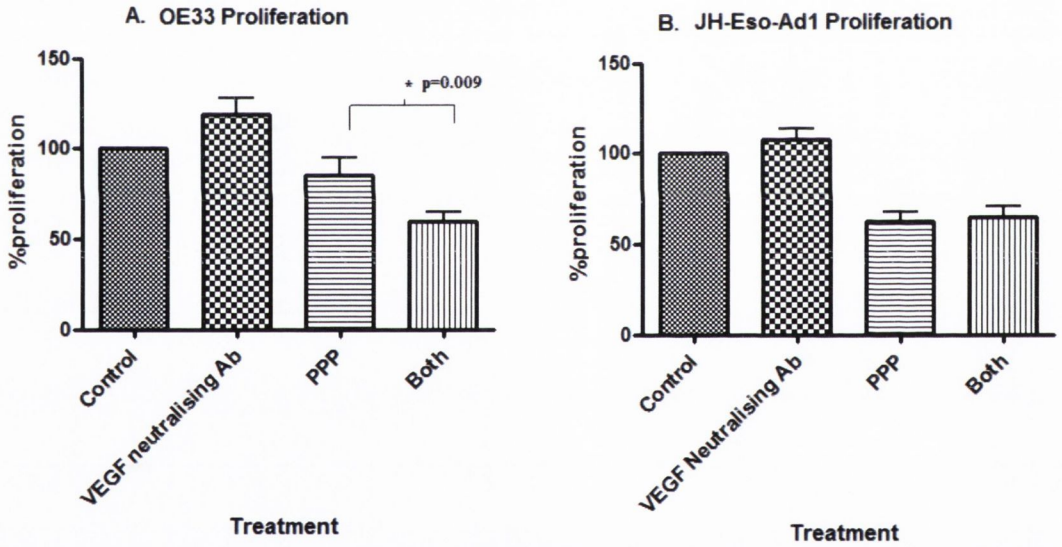


Figure 5.12 Treatment of OE33 with PPP and VEGF neutralising antibody in combination further inhibits cellular proliferation. Cellular proliferation was measured using BrdU proliferation assay. Proliferation of control cells was compared to cells treated with VEGF neutralising antibody only, PPP only and both treatments combined for a period of 24 hours. VEGF neutralising antibody did not affect cellular proliferation. A. In the OE33 cell line, proliferation was decreased further by adding VEGF neutralisation to PPP treatment (% proliferation versus controls following PPP treatment: 84.9 ± 10.0% compared to 59.8 ± 5.4% with both treatments, $p<0.009$ paired t-test). B. In JH-Eso-Ad1, there was a greater decrease in cellular proliferation following PPP treatment alone (62.95 ± 7.2%) and there was no significant decrease in proliferation with the combination of treatments (64.95 ± 8.2%, $p=0.46$). All experiments were n=3, paired t-test to compare 'both' versus 'PPP'.

5.4.5 VEGF neutralisation in combination with IGF1R inhibition results in decreased pAKT expression

When OAC cell lines were treated with VEGF neutralising antibody alone, there was no difference noted between phosphorylated Akt (pAkt) expression versus untreated cells. As noted previously, PPP treatment did not alter significantly the expression of pAkt at 24 or 48 hours (Table 5.2) and there was a non-significant increase in pAkt expression at 72 hours. Expression of pAkt was decreased at 48 and 72 hours (Figure 5.11) in both JH-Eso-Ad1 and OE33 cell lines following the combination of IGF1R inhibition with VEGF neutralisation. Although these results did not achieve statistical significance with n=3, likely related to the relative large standard deviations encountered using the Western blotting technique, there was a clear trend towards a decrease in protein expression (Figure 5.13).

Table 5.2 pAkt protein expression at 24, 48 and 72 hours following treatment with VEGF neutralising antibody, picropodophyllin and both in OAC cell lines

	Control	VEGF neut Ab	p-value (control v VEGF neut Ab)	PPP	Both	p-value (PPP v Both)
OE33						
24 hours	100 (1.0)	95.7 (2.7)	0.25	95.0 (5.3)	113.8 (6.4)	0.25
48 hours	100 (7.1)	101.4 (1.1)	1.0	109.1 (4.5)	140.8 (24.5)	0.25
72 hours	100 (9.5)	107.5 (9.0)	0.25	111.6 (2.3)	93.9 (5.4)	0.1
JH-Eso-Ad1						
24 hours	100 (5.8)	104.23 (6.03)	1.0	108.1 (4.2)	105.6 (3.4)	0.53
48 hours	100 (3.5)	107.1 (12.3)	0.23	132.6 (27.4)	120.5 (8.7)	0.75
72 hours	100 (15.5)	70.0 (22.5)	0.5	579.6 (165.1)	237.8 (55.8)	0.25

All data are mean percentage of control (SEM). t-test is Wilcoxon matched –pairs signed rank test for non-parametric data.

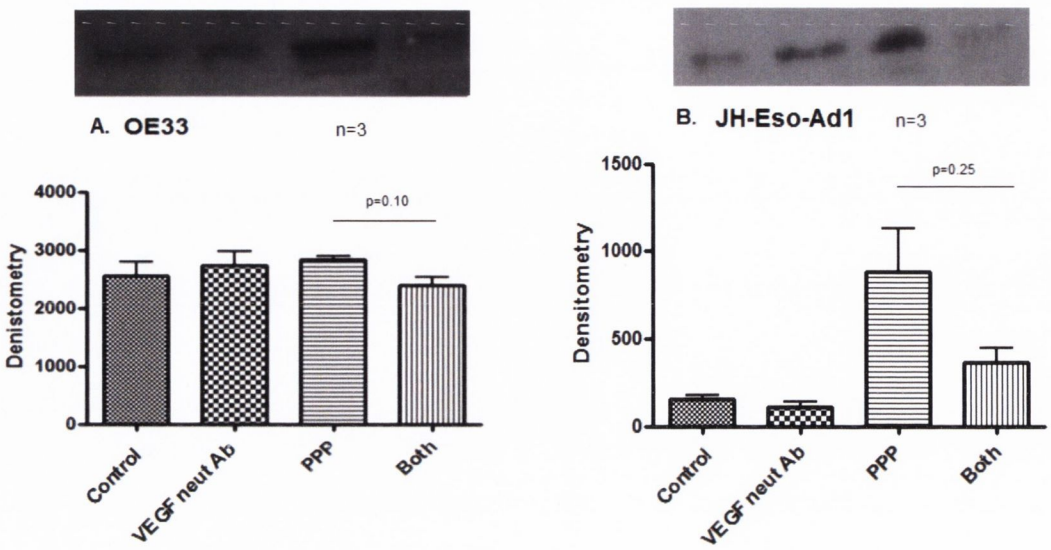


Figure 5.13 Treatment with PPP and VEGF neutralising antibody in combination leads to reduction in pAKT protein expression. Phosphorylated Akt (pAkt) was measured by Western blotting with the following treatments: control (untreated); VEGF neutralising antibody ND100 dose; PPP IC50 dose and combination of VEGF neutralisation + PPP, for 72 hours. Protein expression was corrected for loading variance by adjusting densitometry results relative to expression of the housekeeping protein β-actin. Representative Western blots from one set of experiments are included. Combination treatment with VEGF neutralisation and IGF1R inhibition lead to decreased pAkt expression in both (A). OE33 (p=0.10, paired t-test; n=3) and (B). JH-Eso-Ad1 cell lines (p=0.25, paired t –test; n=3).

5.4.6 Mechanism of upregulation of VEGF.

5.4.6.1 Treatment with PPP is associated with an increase in HIF-1α mRNA production

The OE33 cell line was treated with PPP for 24 hours and the changes in HIF-1α mRNA expression, the transcription factor mainly responsible for upregulation of VEGF was assessed at 6, 12 and 24 hours. There was a significant upregulation of HIF-1α mRNA at both 12 and 24 hours following PPP treatment (Fold change relative to control: 0.67 at 6 hours; 1.38 at 12 hours; 1.97 at 24 hours, p=0.017, ANOVA, Friedman correction for paired data) (Figure 5.14).

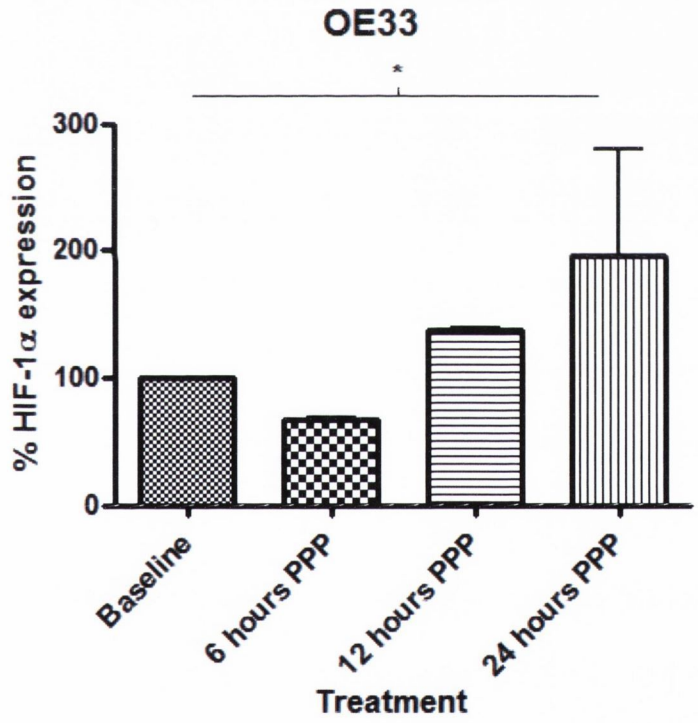


Figure 5.14 PPP treatment is associated with an increase in HIF-1α mRNA. There is an increase in HIF-1α mRNA expression following PPP treatment at both 12 and 24 hours following PPP treatment in the OE33 cell line. Fold change relative to control: 0.67 at 6 hours; 1.38 at 12 hours; 1.97 at 24 hours, p=0.017, ANOVA, Friedman correction for paired data.

5.4.6.2 OAC cell lines are relatively resistant to mTOR inhibition

In order to test the hypothesis that signalling via the mTOR pathway is responsible for the autocrine production of VEGF after PPP treatment, two mTOR inhibitors, rapamycin and temsirolimus were tested in both cell lines. In order to determine the relevant dose to use, dose response experiments using a MTT assay (as described in section 2.11.1) were performed. The "standard" IC_{50} dose of both temsirolimus and rapamycin as calculated in cell line screening experiments investigating its effect on proliferation was obtained from the literature. In the 60 tumour cell lines screened as part of the COMPARE programme at the National Cancer Institute (Bethesda, MN, USA) the average IC_{50} obtained for rapamycin over all cell lines was 8.2 nM when the highest concentrations tested was 1000 nM and 1800 nM, when the highest concentration tested is 10^6 nM (Vignot et al., 2005). The median reported IC_{50} of temsirolimus from 191 cell lines is 842nM with a range of 2.4 nM-20μM (Greshock et al., 2010).

The IC_{50} doses calculated for rapamycin in the OE33 cell line and JH-Eso-Ad1 were 32.96 μM and 19.04 μM respectively – which are ~4000–fold and ~2000–fold higher than the standard IC_{50}. Similarly the IC_{50} doses for temsirolimus in both OE33 (132.7 μM) and JH-Eso-Ad1 (49.19 μM) are ~150–fold and ~58–fold higher. This indicates that OAC cell lines are relatively resistant to mTOR inhibition, a finding not previously reported in the literature. Some of the difference in IC_{50} doses may be due to the different end points inspected in the different studies – most report the IC_{50} for decreasing proliferation, whereas these experiments calculated the effect on cell viability. Inhibition of mTOR in OAC cell lines does not induce significant apoptosis at

low doses and it may be necessary to co-target other pathways alongside mTOR inhibition to ensure cell death.

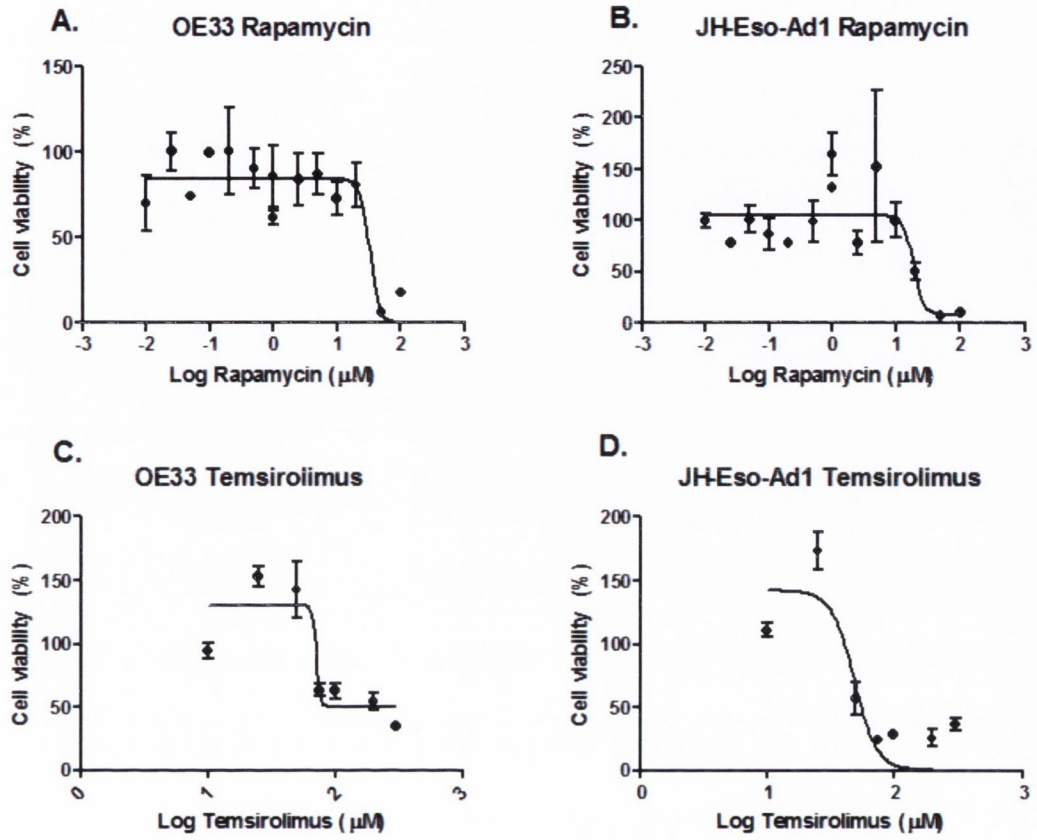


Figure 5.15 Dose response curves of OAC cell lines in response to mTOR inhibitors (temsirolimus and rapamycin). OE33 (A & C) and JH-Eso-Ad1 (B & D) cell lines were treated with a range of doses of mTOR inhibitors rapamycin (A & B) and temsirolimus (C & D). The IC_{50} dose was calculated by non-linear logistic regression analysis, which requires log transformation of drug concentration and which fits a line to the data. The dose response curves have a flat portion at lower doses indicating the relative resistance of these cell lines to treatment using mTOR inhibitors.

5.4.6.3 Autocrine VEGF production is inhibited by treatment with mTOR inhibitors

Following treatment with the published IC_{50} doses of temsirolimus (1.4μM) and rapamycin (100nM) there were no significant alterations in the production of VEGF by treated cells at 24 hours. Treatment with the IC_{50} doses of temsirolimus and rapamycin as calculated in section 5.4.6.2, lead to a significant decrease in autocrine VEGF production. These results may indicate that the failure of PPP to decrease mTOR signalling may be responsible for the upregulation of VEGF production in treated cell lines.

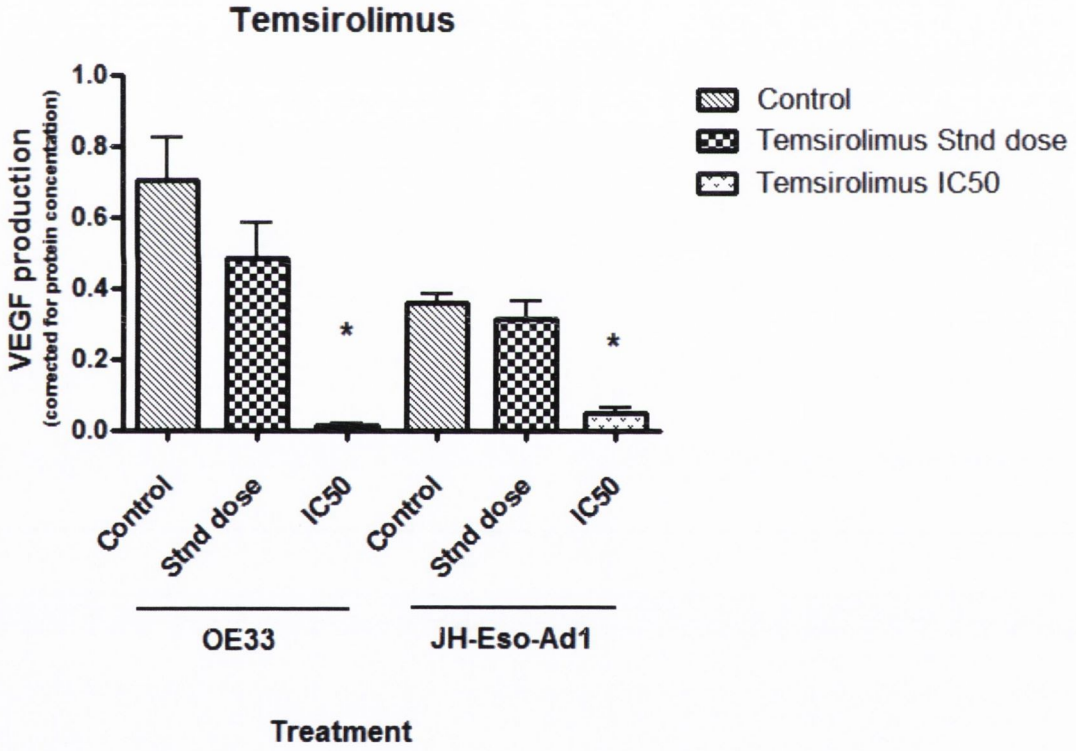


Figure 5.16 VEGF production in response to treatment of OAC cell lines with Temsirolimus. OE33 and JH-Eso-Ad1 cell lines were treated with both the calculated IC_{50} dose of temsirolimus (from section 5.4.6.2) and the standard IC_{50} from the published literature (1.4μM). The standard dose had no effect on VEGF production. There was a significant decrease in the production of VEGF (corrected for protein concentration) by the remaining viable cells in both cell lines when treated with the calculated IC_{50}. Data are mean ± SEM, comparison using Wilcoxon matched paired signed rank test.

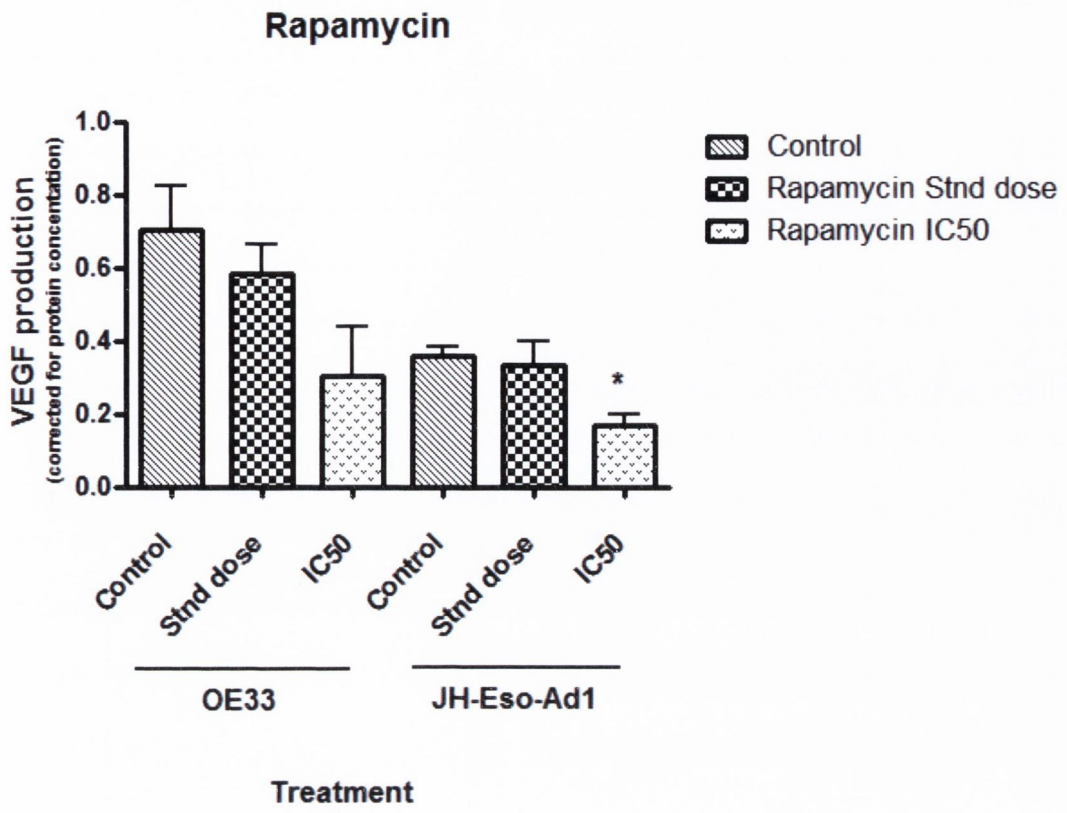


Figure 5.17 VEGF production in response to treatment of OAC cell lines with Rapamycin. (A). OE33 and (B). JH-Eso-Ad1 cell lines were treated with both the calculated IC_{50} dose of rapamycin (from section 5.4.6.2) and the standard IC_{50} (100nM) from the published literature. The standard dose had no effect on VEGF production. There was a significant decrease in the production of VEGF (corrected for protein concentration) by the remaining viable cells in both cell lines when treated with the calculated IC_{50}. Data are mean ± SEM, comparison using Wilcoxon matched paired signed rank test.

5.4.7 VEGF and IGF1R co-expression in oesophageal adenocarcinoma tumour samples

In order to investigate whether co-targeting VEGF and IGF1R may be a relevant strategy for the treatment of oesophageal adenocarcinoma, the protein expression of VEGF and IGF1R was determined by immunohistochemistry (section 2.8) in a set of 100 oesophageal adenocarcinoma samples arrayed in a tissue microarray (population characteristics detailed in section 2.3). The hypothesis was that if these two proteins are relevant targets in the treatment of oesophageal adenocarcinoma, the subgroup of patients with the lowest expression of these two proteins (i.e. tumour samples negative for IGF1R and with less than the median level of VEGF) would have a more favourable prognosis than samples with higher co-expression of the two proteins. That is, that this subgroup of patients would not derive benefit from targeted use of these agents and patients with higher levels of expression of these proteins may have a poorer prognosis and thus derive benefit from co-targeting. For these analyses patients were divided into four groups: <median VEGF and negative IGF1R; <median VEGF and positive IGF1R; >median VEGF and negative IGF1R and >median VEGF and positive IGF1R.

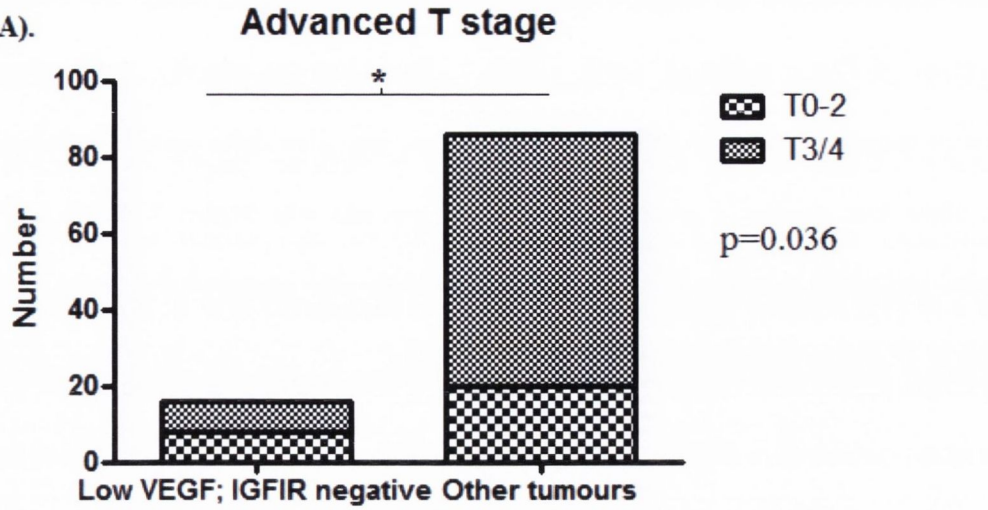
5.4.7.1 Clinicopathological variables

Patients with the lowest expression of VEGF and negative IGF1R expression (hereafter denoted the "low expression group") had favourable clinicopathological tumour features compared to patients with other levels of expression. With respect to T stage, there was a smaller proportion of patients with locally advanced (T3/4) tumours (defined in Table 1.1) amongst the low expression group versus other tumours (50% versus 76.7%, $p=0.036$ (X^2 test); Figure 5.18a). Similarly, there was a greater proportion of patients with well or moderately differentiated cancers versus poorly or undifferentiated tumours

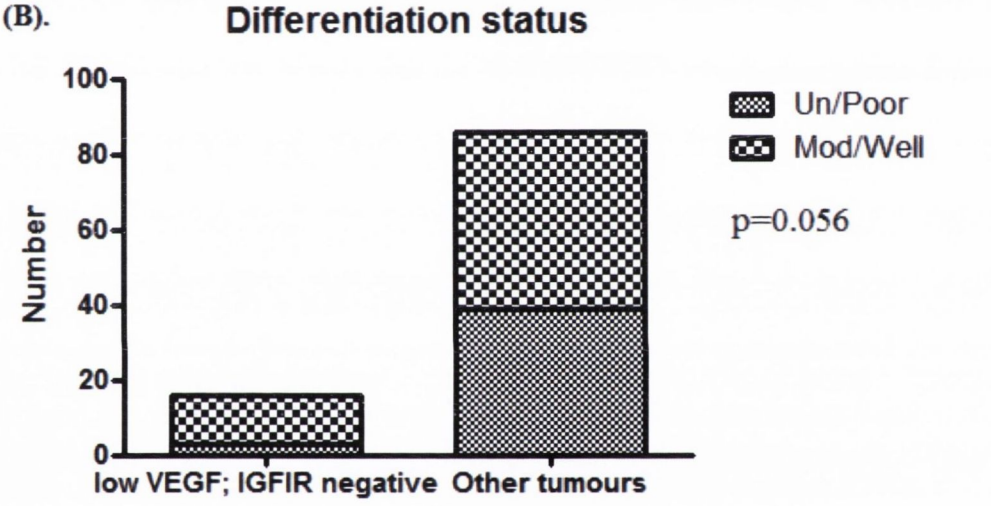
in the low expression group (81.3% well or moderately differentiated in low expression group versus 54.7%; p=0.056 (X^2 test); Figure 5.18b). Most importantly, the greatest predictor of tumour recurrence in OAC, nodal status, was also differentially involved between these two groups: a greater proportion of patients with higher VEGF/IGF1R expression had node positive tumours than those with low expression (low: 43.8% versus high: 75.6%, p=0.016 (X^2 test); Figure 5.18c).

None of these factors were significantly different when patients were grouped according to VEGF or IGF1R expression only – there was only evidence of different clinicopathological factors when patients were grouped according to both VEGF and IGF1R expression status.

(A).


Advanced T stage
*
100
80
60
40
20
0
Number
Low VEGF; IGFIR negative
Other tumours
T0-2
T3/4
p=0.036

(B).


Differentiation status
100
80
60
40
20
0
Number
low VEGF; IGFIR negative
Other tumours
Un/Poor
Mod/Well
p=0.056

(C).

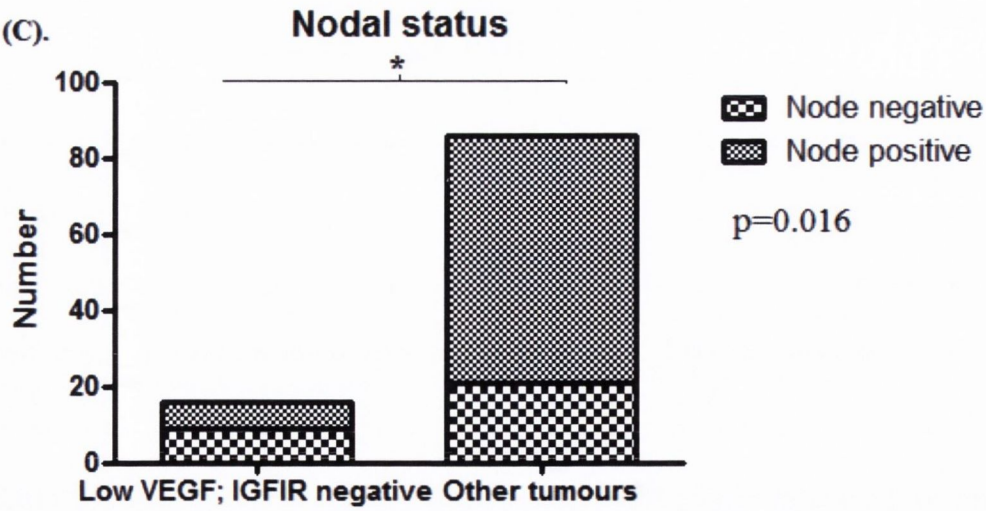
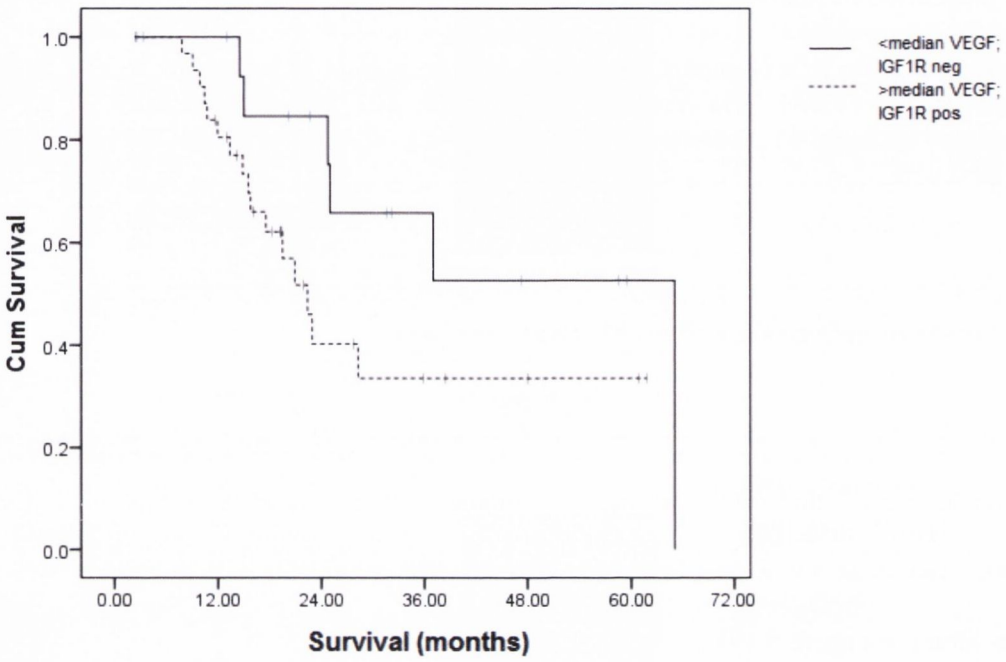

Nodal status
*
100
80
60
40
20
0
Number
Low VEGF; IGFIR negative
Other tumours
Node negative
Node positive
p=0.016

Figure 5.18 Oesophageal adenocarcinoma tumours with low VEGF and negative IGF1R expression have favourable clinicopathological features. Patients with oesophageal adenocarcinoma were divided into two groups: patients with less than median VEGF protein expression by immunohisotchemistry and negative IGF1R versus patients ("low expression group") with higher VEGF and IGF1R expression. Clinicopathological variables associated with favourable prognosis were investigated and the proportion of patients with these favourable prognostic indicators was compared between the two groups (X^2 test). Comparing the proportion of patients in the "low expression group" to other patients, the following observations were made: (A). Locally advanced tumours (T3/4): 50% versus 76.7%, p=0.036 (B). Well or moderately differentiated tumours: 81.3 versus 54.7%; p=0.056 (C). Node positive: 43.8% versus 75.6%, p=0.016.

5.4.7.2 Survival: univariate and multivariate analysis

When the survival of patients with the lowest co-expression of VEGF and IGF1R was compared to that of those with the highest co-expression (>median VEGF and IGF1R positive), median survival was longer at 65.1 months versus 22.4 months, p=0.089. (Kaplan-Meier in Figure 5.19).

When VEGF and IGF1R co-expression was analysed using cox regression multivariate analysis, co-expression was not an independent prognostic factor for disease-specific survival. Nodal status and T stage were independently prognostic (Table 5.1). This implies that the significantly different clinicopathological values reported above mediate the effect of VEGF/IGF1R expression.



Protein expression	Median survival	5 year survival	p-value
<median VEGF/ IGF1R negative	65.1 months	46%	0.089
>median VEGF/IGFIR positive	22.4 months	33%	

Figure 5.19 Oesophageal adenocarcinoma tumours with low VEGF and negative IGF1R expression have improved survival. Disease-specific survival was increased in patients with lowest VEGF and IGF1R expression versus patients with the highest co-expression of VEGF and IGF1R with median survival rates of 65.1 months versus 22.4 months (Log-rank test, p=0.089).

Table 5.3 Cox regression multivariate analysis: VEGF/IGF1R co-expression

Factor	Univariate analysis		Multivariate analysis	
	Hazard ratio (95%CI)	p-value	Hazard ratio (95%CI)	p-value
VEGF <median /IGF1R negative	0.697 (0.315-1.542)	0.234	X^2=1.197	0.274
Node negative versus node positive	0.309 (0.146-0.654)	0.002	0.398 (0.126-0.882)	**0.041**
Early cancer (T1-T2)	0.257 (0.114-0.578)	0.001	0.334 (0.165-0.961)	0.027

Multivariate analysis of disease-specific survival carried out using cox regression analysis of 100 patients with OAC. Co-variates included were VEGF/IGF1R expression (coded as <median VEGF/IGF1R negative versus others); nodal status (node negative versus positive) and T stage (Early cancer T1-2 versus T3-4). Nodal status and T stage were independent predictors of prognosis. There was no association between VEGF/IGF1R expression and prediction of disease-specific survival.

5.5 Discussion

A common theme arising from the use of targeted agents in malignancies is that with time, resistance inevitably develops (Ellis and Hicklin, 2009). Furthermore, only subgroups of patients are sensitive to the use of these agents and there is a clear need to understand the molecular responses that develop following targeted agent treatment in order to use these agents with any reliable ability to predict clinical successes.

From recent clinical trials, we know that responses to IGF1R inhibition are poor in unselected groups of patients (Pollak, 2012b). Work to identify biomarkers predicting response and resistance to IGF1R resistance are in the early stages of development (Gualberto et al., 2008a).

Models of acquired resistance to another tyrosine kinase receptor, EGFR, following tyrosine kinase inhibition (TKI) use have demonstrated that there is continued signalling along the PI3K pathway despite TKI treatment. This occurs via diverse acquired mechanisms of resistance (Engelman et al., 2007, Bianco et al., 2003, Yamasaki et al., 2007, Ogino et al., 2007). In a mouse sarcoma model IGF1R inhibition treatment failure was noted to correlate with a failure to downregulate IGF1R and to a failure to suppress pAKT at 24 hours following treatment (Kurmasheva et al., 2009). A similar result was found in this work, whereby despite functional activity from IGF1R inhibition, downstream PI3K activity was not decreased.

An early morphoproteomic profiling study of two patients with Ewing's sarcoma who initially responded to IGF1R inhibition demonstrated that resistance was associated with upregulation of pAkt and p-mTOR in these patient's tumours and that second line treatment with mTOR inhibition lead to further clinical responses (Subbiah et al., 2011). Therefore, this mechanism of treatment resistance – failure to adequately downregulate

PI3K pathway activity- may explain both primary (intrinsic) treatment resistance and secondary (acquired) resistance to IGF1R inhibition.

Preclinical data from this lab (section 5.2) has demonstrated that VEGF production is of relevance to visceral adiposity and may mediate some of the pro-tumourigenic effects of visceral adipose tissue (Lysaght et al., 2011). This study found increased autocrine VEGF production by cell lines following IGF1R inhibition with PPP. VEGF and IGF1R have been found to be co-regulated in other disease models (Economou et al., 2008, Kurmasheva et al., 2009, Bid et al., 2012, Slomiany and Rosenzweig, 2004). VEGF is primarily thought to be regulated via induction of hypoxia-inducible factor 1α (HIF1α) which leads to increase VEGF mRNA and subsequent protein expression (Ferrara et al., 2003).

The effects of constitutively active Akt on HIF-1α activity are cell-type specific. High levels of Akt signalling can modestly increase HIF-1α protein but this does not affect HIF-1α target gene expression (Arsham et al., 2002). In Akt-expressing, HIF-deficient cells, there is an increase in VEGF production by the tumour with Akt overexpression (Arsham et al., 2004), indicating that there are non-HIF-1α mediated roles for regulation of VEGF by Akt activity. IGF1R activity has been proposed as one of these mechanisms.

In a panel of sarcoma (Ewing's and osteo-) cell lines and xenografts, some cancer cells were responsive to the synergistic combination of IGF1R inhibition and rapamycin (Kurmasheva et al., 2009). The authors reported that this was due to the ability of IGF1R inhibition to decrease VEGF production by the tumour cells, as well as the ability of rapamcyin to decrease vascular endothelial VEGF production. Not all tumours displayed equal clinical responses to combination treatment; nor did they have

homogenous changes in VEGF production with either agent alone or in combination. In some instances rapamycin alone lead to increased VEGF production whereas in others it was decreased. This indicates that whilst there appears to be co-regulation of VEGF production by the IGF1R-PI3K and mTOR pathways, this is not well characterised and the results of inhibition of one or other arm of the pathway may lead to different results in different tumour subtypes.

Data from this study show that in OAC cell lines, neutralisation of VEGF leads to further decreases in proliferation when combined with IGF1R inhibition versus IGF1R inhibition alone. Furthermore, this combination of treatments can successfully decrease pAkt expression, indicating that this treatment combination may be of relevance to prevent treatment resistance. Looking at patient tumours samples, low VEGF and IGF1R expression is associated with an improved prognosis and more favourable clinicopathological variables including local invasion (T stage), differentiation and Nodal status.

To investigate whether it was persistent activation of the mTOR pathway which mediated the increase in VEGF production, two mTOR inhibitors; rapamycin and temsirolimus, were investigated in OAC cell lines. Both of the cell lines were highly resistant to both inhibitors- as measured by the large IC_{50} doses required to decrease cell viability in these cell lines. Treatment of the cell lines with mTOR inhibitors lead to a significant decrease in VEGF production. This provides circumstantial evidence that there may be persistent activation of the mTOR pathway following IGF1R inhibitor. These findings have not previously been investigated in OAC and warrants further in-depth study.

The mechanism of resistance identified in this chapter may only arise as a result of PPP treatment – treatment of cells with a monoclonal antibody IGF1R inhibitor R1507 did not results in a significant alteration in VEGF production. Whether other IGF1R tyrosine kinase inhibitors produce this effect remains to be investigated. Nevertheless, the functional consequences of PPP treatment are of clinical relevance as another cyclolignan, a pharmacological analogue of PPP is under clinical trial at present. This mechanism of resistance may be limited to oesophageal adenocarcinoma cell lines only. Furthermore, this may be only one of many other resistance mechanisms in the armamentarium of cells treated with IGF1R inhibition and may not enhance prevention of resistance.

Individualised tailored therapy with anti-IGF1R therapies will be aided by increased understanding of the interactions of the IGF axis and other growth factors as well as developments in the ability to characterise receptor expression and activation and alterations in signalling molecules. Identification of relevant biomarkers will facilitate selection of patients likely to benefit from treatment. A greater understanding of the intra-cellular responses to treatment will lead to rational selection of targeted agents. Prophylactic combination of targeted agents which are known to have complementary activity may help to prevent or delay resistance from developing. Furthermore, a greater understanding of the alterations that provide pro-survival advantages to oesophageal adenocarcinoma tumours, using whole genome and proteome molecular profiling, will allow targeted agents to be utilised in the setting of molecularly defined cancer populations.

5.6 Clinical relevance

What is already known: Treatment with targeted agents has been inevitably associated with treatment resistance – either primary intrinsic resistance or secondary acquired resistance. Resistance mechanisms associated with IGF1R inhibition in oesophageal adenocarcinoma have not been examined previously.

What's new: Failure to adequately downregulate PI3K pathway activity- may explain resistance to IGF1R inhibition. Upregulation of VEGF production leading to co-activation of the mTOR pathway maybe responsible for ongoing pAKT activation in the setting of IGF1R inhibition.

Potential clinical implications: Understanding the mechanisms of treatment resistance associated with specific targeted agents may allow the rational combination of targeted agents in order to improve clinical response rates. Co-inhibition of VEGF and IGF1R may be necessary in oesophageal adenocarcinoma for adequate clinical response rates to be achieved.

Chapter 6

STMN1 as a proxy marker of PI3K activity

6.1 Introduction

6.1.1 Obesity and pathway addiction

Eukaryotic cells coordinate cell growth in line with the availability of nutrients in their environment (Shaw and Cantley, 2006). Obesity as a condition of both systemic insulin resistance and nutrient excess may lead to activation of intra-cellular pathways that promote tumour growth and progression. The systemic alterations associated with obesity include a change in inflammatory, sex hormone, insulin and adipokine secretion which may directly influence the tumour microenvironment (Donohoe et al., 2010). Cancers which arise in this environment may go on to develop progressive mutations and epigenetic alterations which are influenced by this obese milieu.

The concept of oncogene addiction describes the apparent dependency of some tumours on one or a few genes to maintain the malignant phenotype (Weinstein, 2002). Clinical evidence of this is cited as the ability of therapies targeting specific genes or pathways to inhibit cancer cell growth or improve survival rates (Weinstein and Joe, 2006). Whole genome arrays have demonstrated that breast cancer may be subdivided into a number of types based on the genes over-expressed in certain subtypes and that targeting these predominant pathways provides avenues for chemotherapy treatment for each subtype (Perou et al., 2000, Sørlie et al., 2003, Sawyers, 2004). Molecular characterisation of tumours has been exploited to develop assays to predict subgroups of patients with poorer prognosis who may benefit from adjuvant therapy (Paik et al., 2004).

Cancers which develop within an obese environment may become selectively altered to signal via specific pathways. For examples, in patients with non-small cell lung cancer, only a subset (approximately 10-20%) respond to the EGFR targeted therapy gefitinib –

and these patients often have an activating mutation of EGFR (Lynch et al., 2004). Patients with activating mutations are more likely to have adenocarcinomas, to be female, non-smokers and Japanese (Taron et al., 2005). Similarly, obesity-related cancers may have a specific set of targets (malfunctioning molecules or pathways) which may be exploitable in clinical practice. Certainly a number of the putatively dysregulated adipokines and growth factors in obesity signal via the same intracellular signalling pathways.

Candidate pathways include the phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (STAT3) pathways. Activation of these pathways lead to multiple downstream effects which underpin cancer progression and metastasis (Huang et al., 2010, Yu et al., 2009, Aggarwal et al., 2009a). Importantly, inhibitors of these pathways are under development at present in order to provide new therapeutic avenues (Sebolt-Leopold and Herrera, 2004, Liu et al., 2009, Jing and Tweardy, 2005). Resistance to targeted therapies is an ongoing issue in translational oncology (Ellis and Hicklin, 2009) and the mechanisms underpinning resistance to targeted therapies will require detailed insights into the molecular signalling events associated with their use. Bypassing tyrosine kinases and instead focusing on inhibiting pathways downstream from them, may be the next logical step in developing new therapeutic targets. For this strategy to be successful, biomarkers of pathway activity will need to be developed.

6.1.2 Obesity and the PI3K pathway

One of the hypothesised pathways which is strongly implicated in mediating the effect of the obese environment is the PI3K pathway. Many of the factors upregulated in the obese state, and explored in more detail in chapter 1 (section 1.3.2), signal via the PI3K

pathway. These include leptin, IL-6, insulin, tumour necrosis factor and IGF-1 (Figure 6.1).

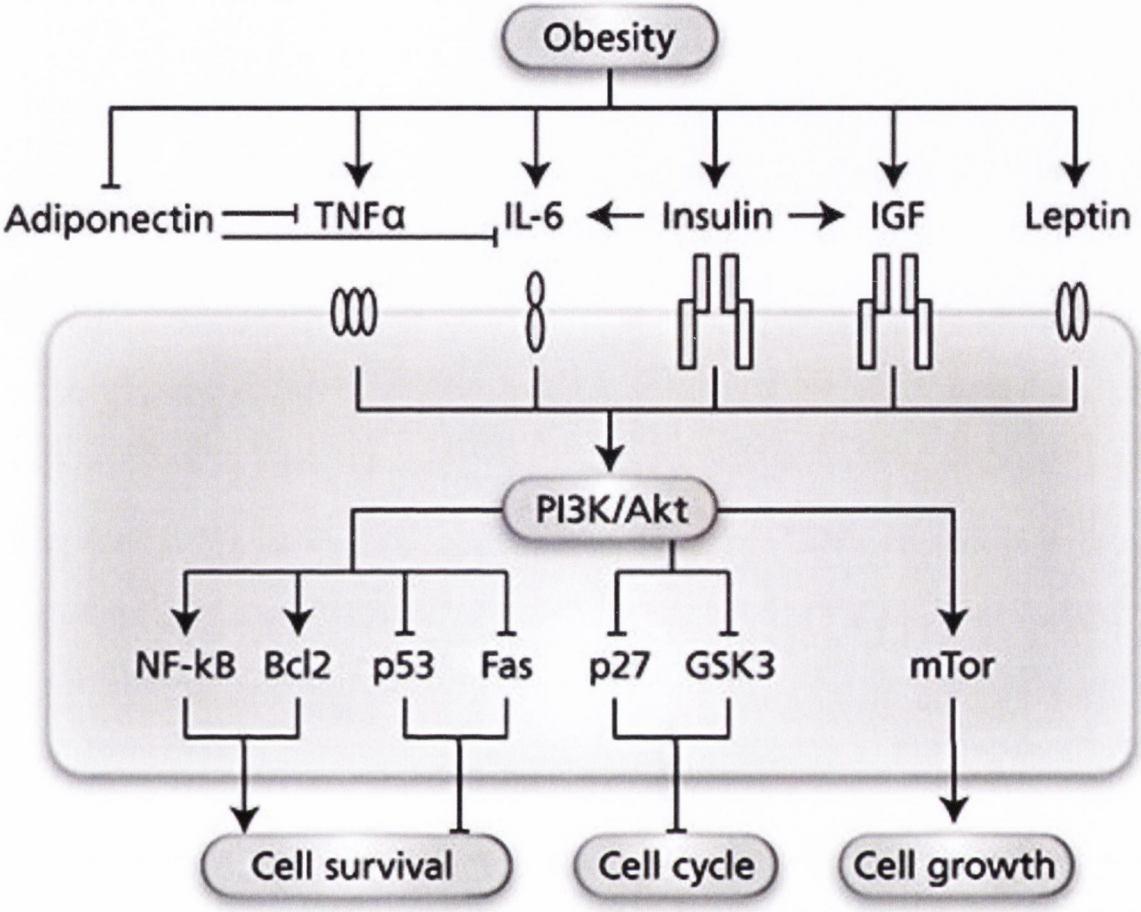


Figure 6.1 Adipokines signal via the PI3K pathway. A number of the pro-tumourigenic adipokines (discussed in detail in Chapter 1, section 1.4.2) including tumour necrosis factor (TNF), IL-6, insulin, leptin and IGF-1 all mediate their downstream activities primarily through the PI3K pathway. The downstream functional effects of PI3K pathway activity include increased cell survival, cell cycle progression and cell growth.

Is there any evidence that cancers which develop within the obese milieu are "addicted to" or preferentially signal via these pathways? Whole genome analysis of breast cancer tumours divided according to body mass index demonstrate that an obesity-associated gene signature pattern is associated with a shorter time to metastasis and is associated

with IGF signalling signature in multiple publically available breast cancer genome arrays (Creighton et al., 2011).

Most data to support this hypothesis are derived from mouse models. PI3K activity (measured by pAkt and mTOR protein levels) is increased in diet induced obesity in mice and is associated with an increased level of circulating IGF-1 compared to controls (Moore et al., 2008). Mice fed a high energy diet have twice the volume of tumours 17 days after colon cancer cell injection versus controls. PI3K pathway activity was demonstrated by increased phosphorylated Akt protein levels. The tumour growth effect was abrogated by metformin treatment, which led to decreased pAkt levels (Algire et al., 2010). In a mouse model of obesity-related skin cancer, obese mice had higher PI3K activity after UV exposure than lean mice (Sharma and Katiyar, 2010). Activity of MAPK phosphorylation and NF-kB signalling were also higher following UVB irradiation in the leptin-deficient mice (Katiyar and Meeran, 2007). In an obesity associated hepatoma model IL-6 and TNF-alpha induce the development of cancer via activation of STAT3 pathway (Park et al., 2010).

Excess energy balance associated with the obese state may influence tumour growth. Mouse tumour xenografts have decreased incidence and slower growth in mice who are fed a calorie restricted diet. Tumours which are resistant to dietary restriction have constitutive activation of the PI3K pathway (Kalaany and Sabatini, 2009).

6.1.3 Indicators of IGF-1 pathway activity

With other targeted agents, gene amplification leading to increased receptor numbers and ligand independent activity occurs, leading to a readily identifiable test predicting clinical activity of targeting agents (e.g. HER2-neu) (Slamon et al., 2001). No such biomarkers have been identified to predict IGF1R inhibition responses to date.

Evidence from some phase III studies indicate that patients with higher free IGF-1 levels may experience less toxicity and increased efficacy, hypothesised to be due the development of tumours in these patients in an IGF-1 rich milieu (Gualberto et al., 2008a) (Gualberto et al., 2011). Other candidate biomarkers are explored in more detail in Chapter 1 (section 1.4.4.4).

IGF1R expression in itself may be a useful initial biomarker. Variability in expression is noted across clinical tumour samples and in chapter 3, IGF1R expression was a predictor of disease-specific survival in OAC tumours. In rhabdomyosarcoma, receptor number seems to predict sensitivity to anti-IGF1R antibody (Cao et al., 2008) Immuno-SPECT imaging using a radiolabelled IGF1R monoclonal antibody has been used in a proof of principal setting involving mice with three different types of human bone sarcoma xenografts: those with high, moderate and low responses to the targeting MAB. Tumours with a high response to the drug had homogenous uptake versus heterogenous and nonspecific uptake in the moderate and low response tumours respectively (Fleuren et al., 2011). The efficacy of cixutumumab in mesothelioma was highly correlated with the number of IGF1R sites per cell (Kalra et al., 2012).

However, IGF1R expression does not necessarily equate with activity via IGF1R and the search for alternative biomarkers of both IGF-1 and PI3K pathway activity are likely

to be of relevance to the selection of patient populations who may derive benefit from IGF1R targeting agents.

6.1.4 Stathmin1 (STMN1) as a proxy indicator of PI3K activity

A 2007 genomic study established a gene expression signature for immunohistochemistry-detectable PTEN loss in breast cancer (Saal et al., 2007). In independent data sets of breast, prostate and bladder carcinoma samples, expression of the gene signature was significantly correlated with poor patient outcomes. Stathmin protein was an accurate IHC marker of the signature and was demonstrated to have prognostic significance in breast cancer and was therefore hypothesised to be a PI3K pathway activity indicator, with potential clinical applications. Evidence in support of this hypothesis comes from a study of endometrial cancer, whereby STMN1 expression had independent prognostic value in endometrial cancer and its expression correlated with PI3KCA amplification and overexpression (Salvesen et al., 2009).

In colorectal cancer, obesity was associated with high cancer-specific mortality in STMN1-positive patients but not in normal weight patients, implying that there may be a differential effect on PI3K activity in the obese state (Ogino et al., 2009).

Stathmin (also called STMN1 or oncoprotein-18) destabilises microtubules and reorganises the cytoskeleton and functions in cell cycle progression and cell migration. STMN1 is negatively regulated by Siva1. STMN1 increases microtubule formation, focal adhesion assembly, cell migration and epithelial-mesenchymal transition. Knockdown of Siva1, in mouse models promotes cancer dissemination (Li et al., 2011).

6.2 Specific aims

The overall hypothesis for this chapter is that STMN1 protein expression is associated with PI3K pathway activity and may be a useful biomarker *in vitro* and *in vivo* of IGF1R pathway activity. This divides into two specific aims:

- Firstly, that STMN1 protein expression in vitro is an indicator of PI3K pathway activity in the OE33 cell line
- Secondly, that STMN1 protein expression in oesophageal adenocarcinoma tumours is a biomarker of IGF1R pathway activity

6.3 Results

6.3.1 Human cancer pathway profiler: no alterations in genes following IGF-1 treatment

The human cancer profiler array (SABiosciences, Frederick, MD, USA) was used in an attempt to identify gene changes 24 hours following treatment in the OE33 cell line following stimulation with a saturating dose of recombinant IGF-1 (one that induced maximal proliferation in the cells at 24 hours using a BrdU proliferation assay: 1000pg/ml). The human cancer profiler array is a 96 well PCR array that contains 84 gene targets and endogenous and negative controls (section 2.7.6). None of the genes were significantly altered when treated cells were compared to controls. Appendix II contains a list of the genes assayed.

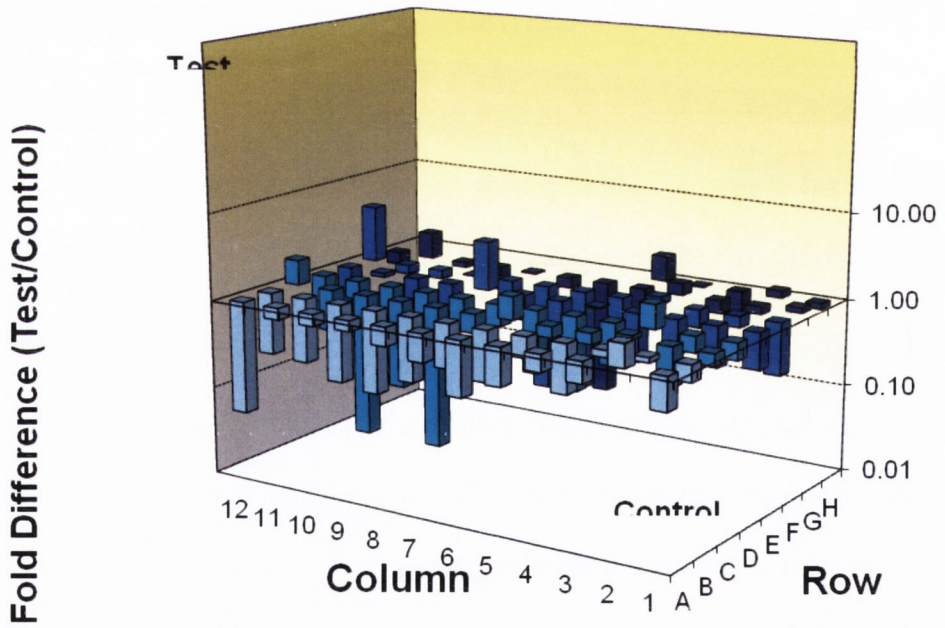


Figure 6.2 No genes included in the human cancer pathway profiler were significantly altered in the OE33 cell line 24 hours following treatment with recombinant IGF-1 (n=3). Fold changes in gene expression were calculated using the CT method (section 2.7.7)

6.3.2 *In vitro* STMN1 expression

Since using a sample of the most commonly differentially regulated genes in cancer cells did not yield any significantly alterations in gene expression, a potential biomarker was identified in the literature – STMN1 (section 6.1.4).

In order to investigate whether STMN1 was a marker of PI3K pathway activity in vitro, cells were treated with both activators and inhibitors of PI3K pathway activity and levels of STMN1 protein expression were assessed.

6.3.2.1 IGF-1 treatment leads to upregulation of STMN1 protein expression

The OE33 cell line was treated with a dose of recombinant IGF-1 (1000pg/ml; section 2.2) which led to maximal stimulation of proliferation by BrdU assay. Protein was harvested and STMN1 expression was investigated by Western blotting (section 2.17). There was an increase in STMN1 protein production by 48.4 ± 7.4%, p=0.09 (Figure 6.3).

6.3.2.2 PI3K inhibition leads to decreased STMN1 protein levels

OE33 cells were treated with the IC_{50} dose of the general PI3K inhibitor LY294002. Confirmation of inhibition of PI3K pathway activity was tested by probing for pAKT. There was a reduction in pAKT activity (Figure 6.4). Following treatment with LY294002 there was a significant decrease in the expression of the STMN1 protein at 24 hours (100 ± 7.6 vs 79.9 ± 9.7%, p=0.001) (Figure 6.5)

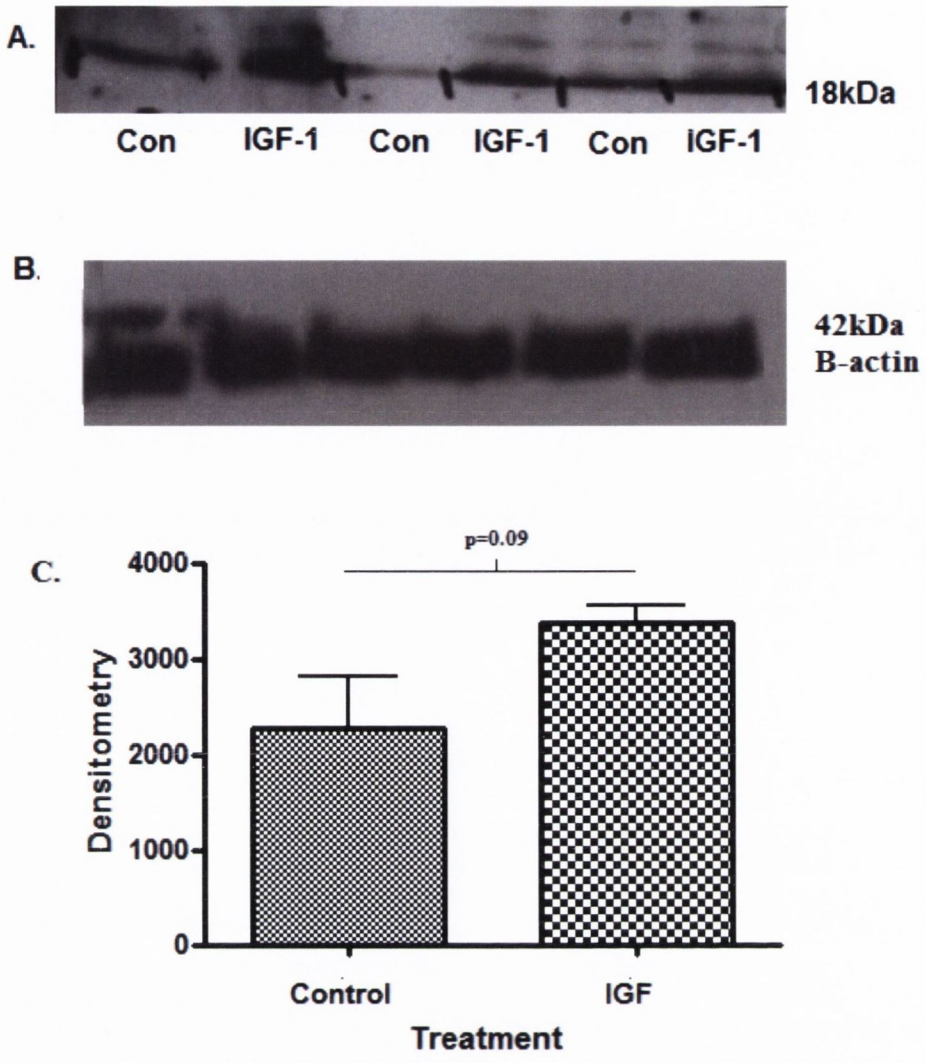


Figure 6.3 IGF-1 treatment leads to upregulation of STMN1 protein expression.

Treatment of the OE33 cell line with a stimulating dose of IGF-1 (1000pg/ml) led to an increase in STMN1 protein expression (A). Representative STMN1 Western blotting, n=3 (B).Loading control using B-actin to correct for total protein loaded onto the gel (C). Densitometry results show that the mean STMN1 protein expression was increased following IGF-1 stimulation at 24 hours (Control vs treated: 100 ± 23.1 vs 148.4 ± 7.3%, p=0.09); Wilcoxon paired-signed rank test;n=3, Data are mean ± SEM.

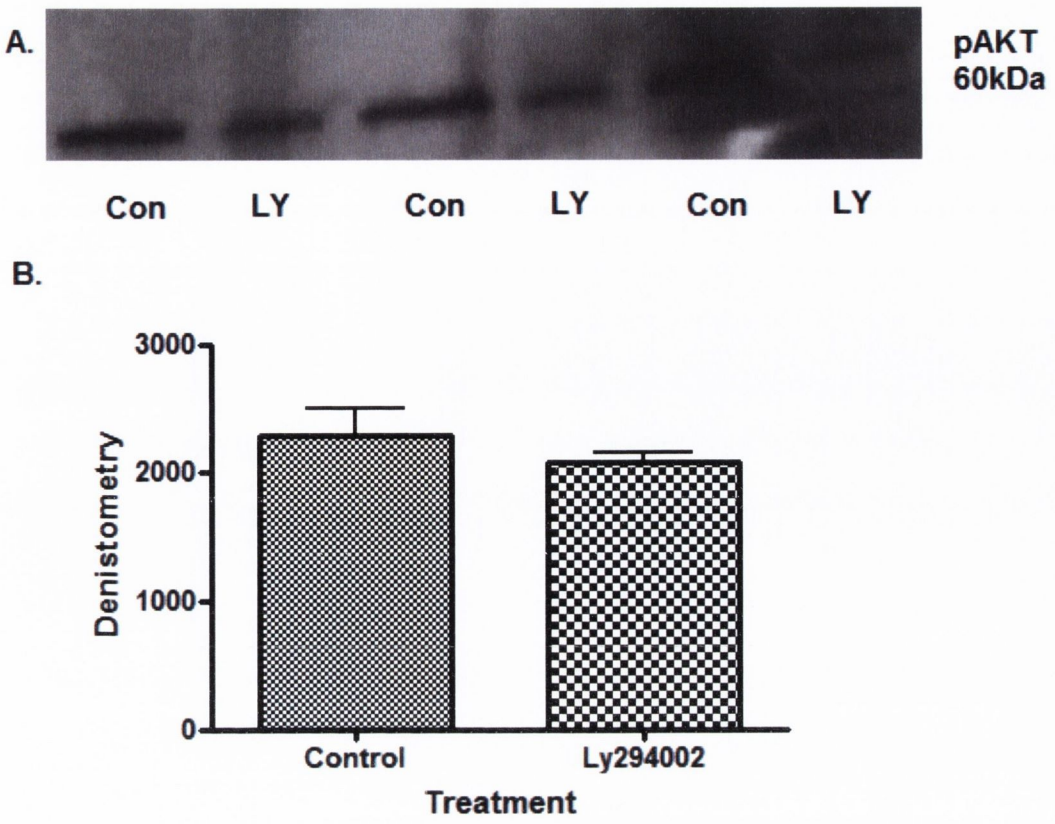


Figure 6.4 Treatment of OE33 with LY294002 leads to decreased pAKT expression at 24 hours. Following treatment of the OE33 cell line with PI3K inhibitor, LY294002, there was a decrease in pAKT protein (Control vs treated: 100.0± 9.03% vs 90.7 ± 3.74%, p=0.21). Data are mean ± SEM, n=3, Wilcoxon matched pairs signed rank test.

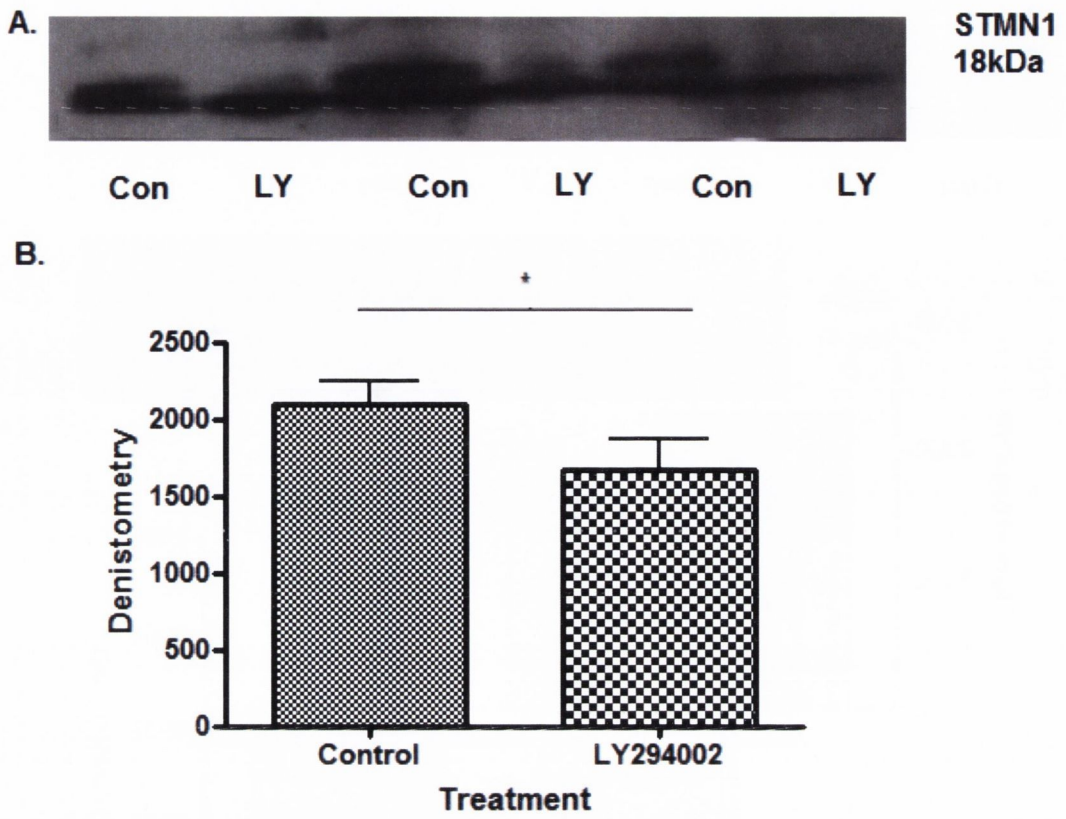


Figure 6.5 PI3K inhibition leads to decreased STMN1 protein levels. Treatment of the OE33 cell line with the IC_{50} dose of the PI3K inhibitor LY294002 leads to a significant decrease in the expression of STMN1 protein at 24 hours (Control vs treated: 100 ± 7.6 vs 79.9 ± 9.7%, p=0.001). N=3, Data are mean ± SEM, Wilcoxon matched pairs signed rank test.

6.3.3 STMN1 protein expression in patient tumour samples

In order to address the hypothesis that STMN1 is a biomarker of PI3K pathway activity, 148 oesophageal adenocarcinoma tumour samples, arrayed in a tissue microarray, were stained for STMN1 using immunohistochemisty (section 2.) Demographics of the patients included are displayed in table 6.1

Table 6.1 Characteristics of patients with oesophageal adenocarcinoma stained for STMN1

N=148		N (%)
Age (mean, SD)		64.6 (11.7)
Male: female		100:48 (67.6:32.4)
T stage	T1	18 (12.2)
	T2	36 (24.3)
	T3	89 (60.2)
	T4	5 (3.4)
N stage	N0	52 (35.1)
	N1	79 (53.4)
	N2	11 (7.4)
	N3	6 (4.1)
Neoadjuvant treatment		41 (27.7)

STMN1 staining was graded as described in section 2. Examples of grading are displayed in figure 6.5. The median grade was 100. Only 4 tumours (2.7%) were negative for STMN1 expression.

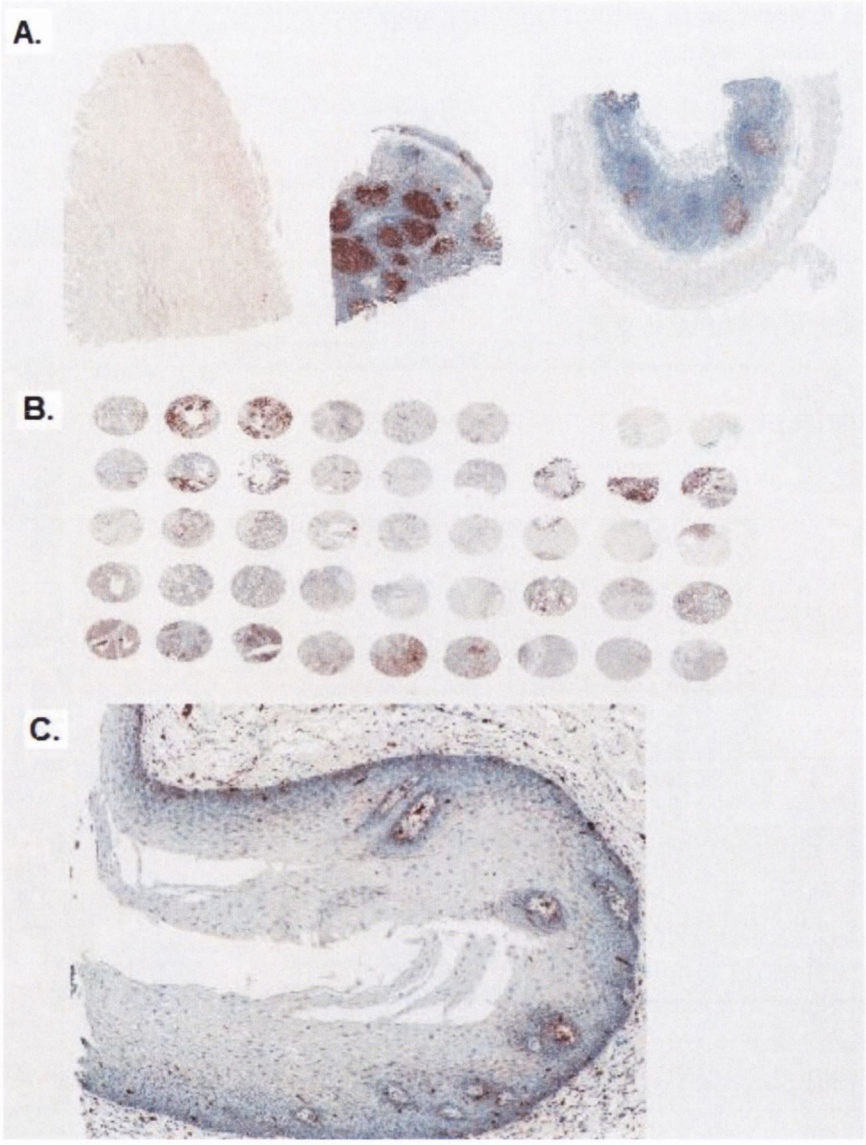


Figure 6.6 Examples of tissue microarray staining for STMN1: Paraffin-embedded tissue microarrays of oesophageal adenocarcinoma were stained using immunohistochemistry for STMN1 expression (1:3000) (section 2.8). (A). positive control, TAC tissue was stained which is a composite block of tonsil, lymph node and tumour tissue (left to right). Brown staining indicates positive cytoplasmic staining for STMN1. (B). Tissue microarray; three cores from negative controls (mouse prostate) are included in the top two cores at the top right-hand corner of the array. (C). Negative staining in normal squamous epithelium (5X magnificiation)

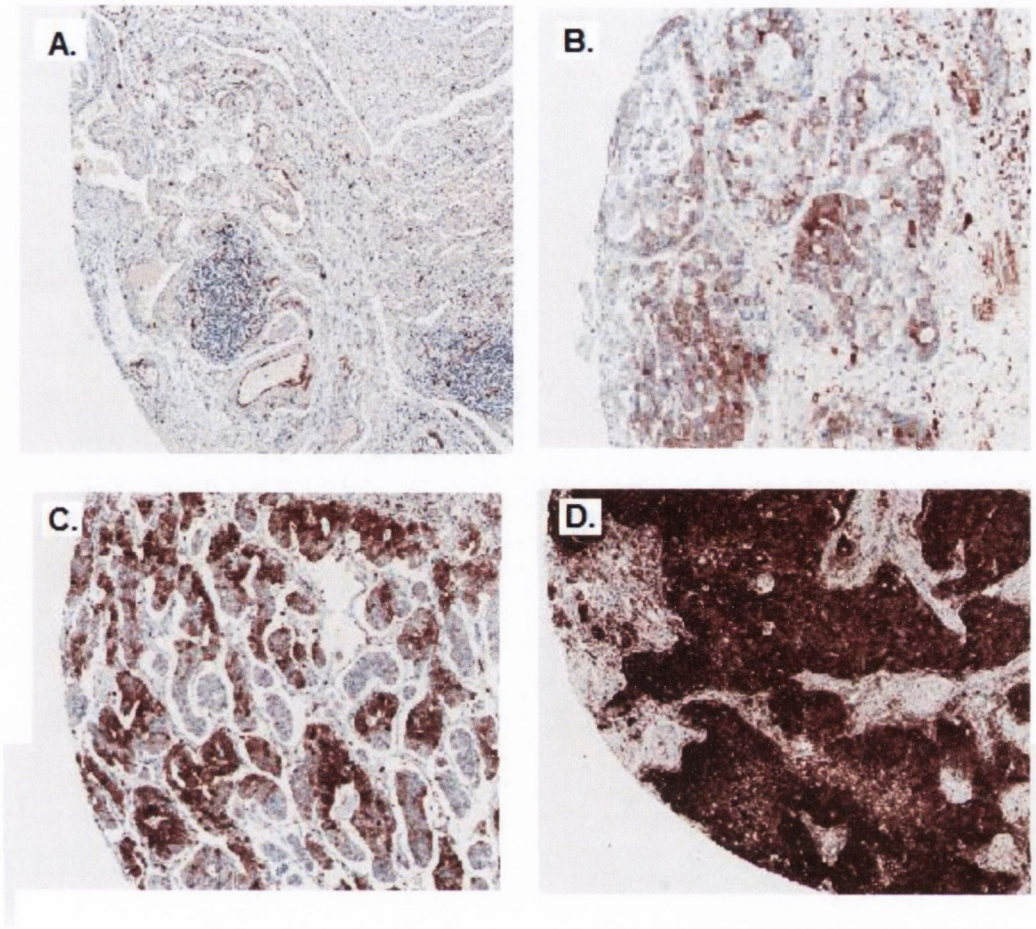


Figure. 6.7 Grading of STMN1 expression in TMAs. All figures are 5X magnification. The intensity of STMN1 staining of tumour-bearing areas on the tissue microarray cores was scored between 0-3, where zero was negative (A). one was weak (B)., two was moderate (C) and three represented strong staining (D). In addition, the quantity of positive staining within the tissue microarray core was scored between 0 and 100%. The intensity score and percentage positivity were multiplied giving an overall score for each core of 0-300.

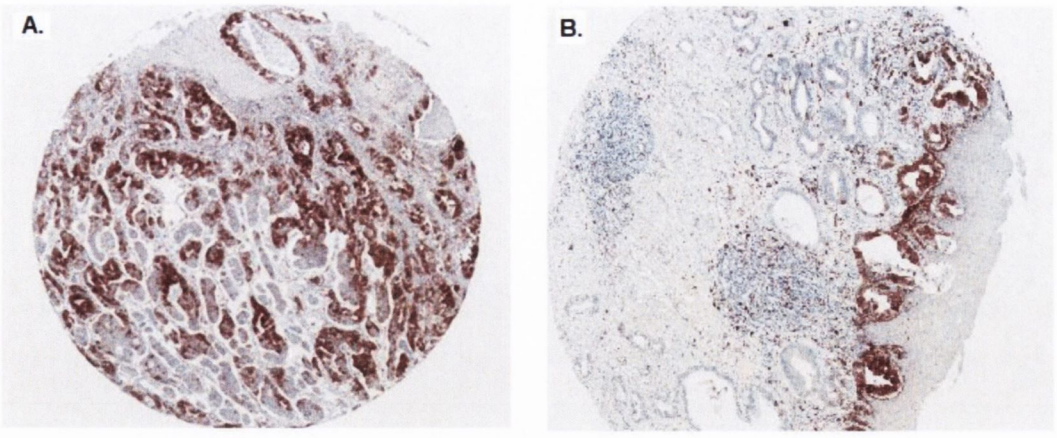


Figure 6.8 Variability in STMN1 expression. In most sections there was variability in the staining across sections – this finding may have contributed to the lack of association between overall grade and clinicopathological variables. Certainly, this presents challenges in the reproducible grading of STMN1 by IHC. (A). 50-75% grade 3 staining (B). 50-75% grade 2 staining

6.3.3.1 Association with clinicopathological variables

In order to investigate whether there was an association between clinicopathological variables and STMN1 expression, tumours were divided into those with less than the median STMN1 expression (low expression) and greater than the median (high expression) and the distribution of clinicopathlogical variables, such as T stage and nodal status, was compared between the two groups.

There was no difference in the proportion of advanced T stage tumours (T3 or 4) amongst patients with stronger STMN1 staining versus weaker STMN1 staining (43/67 (64.2%) vs 49/79 (62.0%), p=0.864).There was a similar proportion of patients with node positive disease in those with greater than the median STMN1 expression as tumours with lower staining (41/67 (61.2%) versus 53/79 (67.1%), p=0.491).

Table 6.2 Clinicopathological variables and STMN1 expression

	<median STMN1 expression	> median STMN1 expression	p-value
Lymphatic invasion	48/79 (60.8)	35/66 (53.0)	0.401
Venous invasion	36/78 (46.2)	27/65 (41.5)	0.615
Perineural invasion	30/79 (38.0)	24/65 (36.9)	1.00
Poor or undifferentiated	48/80 (60.0)	39/66 (59.1)	1.00

Data are n (%) p value calculated using X^2 test (Fisher's exact test).

STMN1 and IGF1R protein levels in tumours were not correlated (Spearman's ρ=0.044, p=0.604) (Figure 6.9).

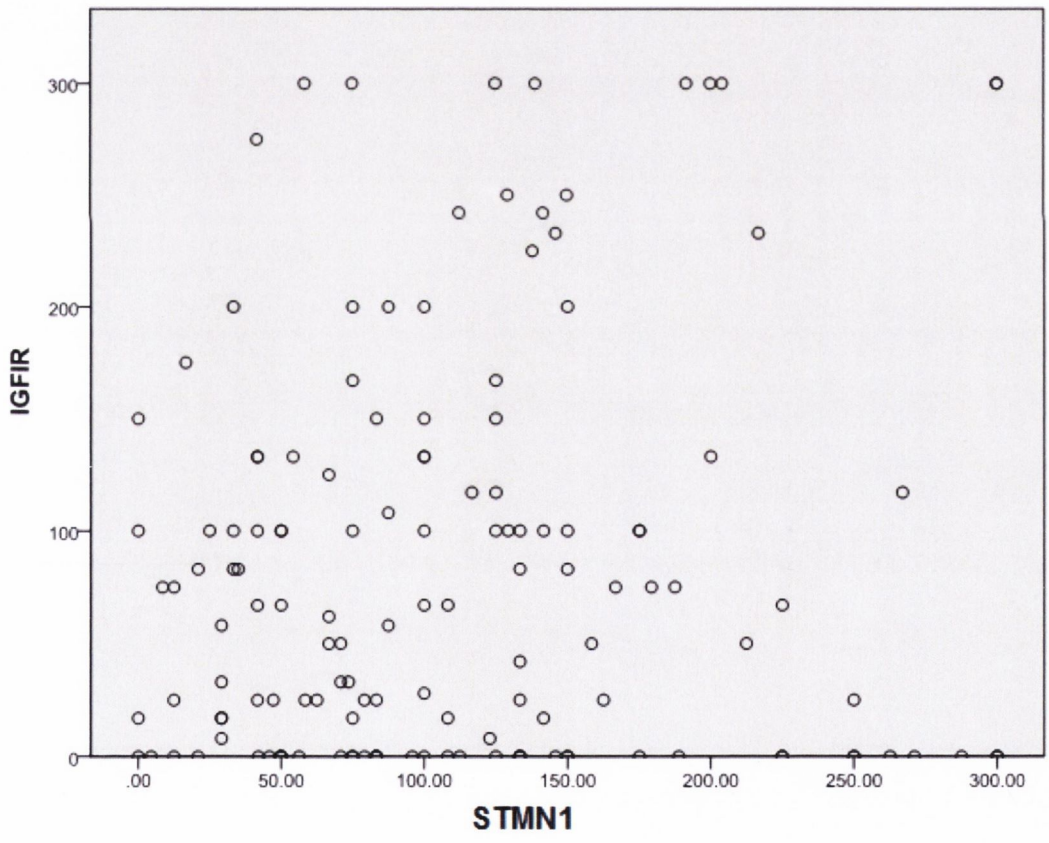
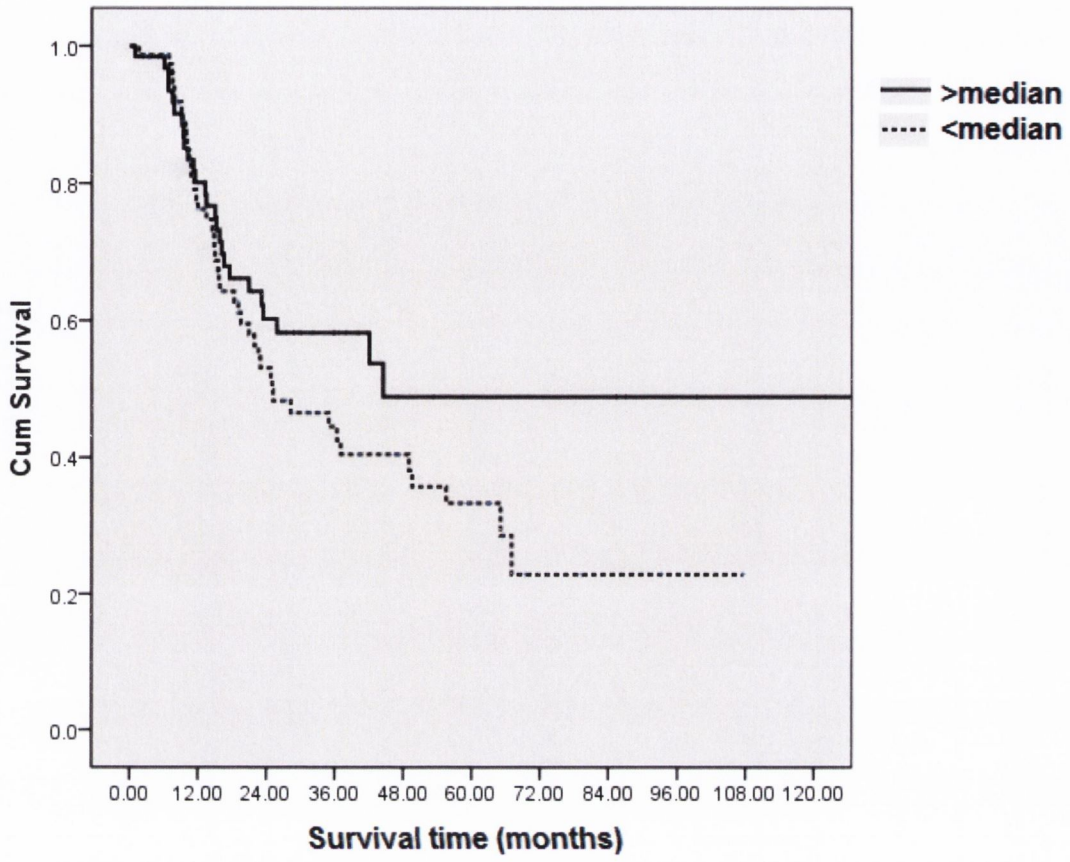


Figure 6.9 STMN1 and IGF1R protein expression in oesophageal adenocarcinoma tumours were not correlated. Spearman's ρ=0.044, p=0.604, n=100.

6.3.3.2 STMN1 and disease-specific survival

STMN1 grade was divided into greater and less than the median value (100) and disease-specific survival was investigated using Kaplan-Meier survival analysis (Figure 6.10). There was no statistically significant difference in survival between patients with the lowest and highest levels of STMN1 expression, although there was a longer median survival period in patients with higher STMN1 expression (44.5 months versus 22.0 months, p=0.198).



	Median survival (months; std error)	5 year survival	10 year survival	p-value (log rank test)
<median	25.0 (6.7)	40%	24%	0.198
>median	44.49 (9.2)	51%	25%	

Figure 6.10 Kaplan-Meier survival curve of STMN1 expression in oesophageal adenocarcinoma patients. There was no statistically significant difference in survival between patients with less than and greater than the median STMN1 expression.

6.4 Discussion

Activation of the PI3K kinase pathway appears to be a common event in obesity associated cancers – most of the adipokines upregulated in the obese state and the IGF-1 axis mediate their downstream signalling effects through this pathway (Donohoe et al., 2011a, Donohoe et al., 2010). In mice models of both diet-induced and genetically-acquired obesity, there is activation of the PI3K pathway within their tumours (Algire et al., 2010) (Sharma and Katiyar, 2010) (Moore et al., 2008). Activating mutations of the PI3K pathway have been relatively rare in small studies published to date (6% in 50 oesophageal adenocarcinoma samples) (Phillips et al., 2006) and thus the activation of the PI3K pathway reported in studies of Barrett's oesophagus and oesophageal cancer (Beales et al., 2007) is likely due to activation of activating receptors proximal to the signalling pathway .

Activation of PI3K pathway signalling leads to a number of functionally relevant pro-survival effects including growth, proliferation, differentiation, motility, survival and intracellular trafficking (Engelman, 2009). Attempts to identify altered genes using a PCR profiler array following treatment with IGF-1 did not yield any significant alterations in the genes included on the array. A whole genome array which included over 22,000 genes found only approximately 800 differentially regulated genes following treatment of the MCF-7 cell line with IGF-1, thus this finding is not wholly unexpected (Creighton et al., 2009).

Findings from a number of other studies as reported in 6.1.4 provided encouraging evidence that expression of STMN1 may be a proxy indicator of PI3K pathway activation and thus by extension, IGF1R activity (Saal et al., 2007, Ogino et al., 2009).

STMN1 itself may also be pro-oncogenic – it increases microtubule formation, focal adhesion assembly, cell migration and epithelial-mesenchymal transition (Li et al., 2011). Activation of IGF-1 axis can induce neo-expression of mesenchymal markers and E-cadherin downregulation (Julien-Grille, 2005). In a number of clinical trials, patients with epithelial-mesenchymal transition tumours (defined by E-cadherin and high IRS-1 expression) have a higher response rate (Karp et al., 2009a, Gualberto et al., 2008a). This finding has also been reported *in vitro* (Buck et al., 2008), whereby epithelial but not mesenchymal tumour cells are sensitive to IGF1R inhibition. fIGF-1 levels correlate with the mesenchymal marker vimentin and inversely with E-cadherin in pre-treatment tumour samples in a clinical trial of patients treated with figitiumumab which targets IGF1R (Gualberto et al., 2011).

Despite encouraging findings from the *in vitro* arm of this study - with OAC cell lines displaying an increase in STMN1 expression following treatment with IGF-1 and decreases following PI3K inhibitor treatment - there appeared to be no association between clinicopathological variables or survival and STMN1 protein expression. Furthermore, there was no correlation between IGF1R protein expression and STMN1. Of note, there was considerable variability in the IHC protein expression across individual tumours which would limit the ability of IHC to accurately assess protein expression in diagnostic biopsies. Furthermore, analysis of grade according to either maximal staining (data not shown) or a median of overall staining grade (as described) had no significant impact on the utility of STMN1 as a prognostic indicator.

The first step in mining the data from whole genome studies has been to distil the information in order to sub-type cancers into prognostic groups (Paik et al., 2004, Gray et al., 2011) and predict response to treatment (Paik et al., 2006). The next step will involve pathway analysis in order to understand how the genetic alterations in cancer

cells enable them to have a survival advantage. Initial experience with this has proven difficult (Wang et al., 2010). The difficulty with the application of our greater understanding of molecular oncology is the amount and complexity of the data generated. Both mathematical modelling and automated network analysis methodologies will need to improve if the real benefits of all of the data generated are to be harvested. Using single proxy markers, as was attempted in this study, fails to reflect the complexity of pathway interactions.

Furthermore, the application of findings from other tumour types does not necessarily translate. The next logical step in the exploration of new therapeutic avenues in oesophageal cancer is the molecular profiling of patient tumours. The Oesophageal cancer clinical and molecular stratification (OCCAMS) incorporating International Cancer genome Consortium (ICGC) multicentre study (Stratton et al., 2009) is currently underway. It is planned that 500 patient's tumours (including samples from lymph node metastases and both prior to and after neoadjuvant therapy) will have whole genome sequencing and transcriptomics performed. This will lead to a biorepository of molecular biology information which is linked to clinicopathological information and will allow validation of previously identified and putative targets in oesophageal cancer. The potential difficulty will be in the understanding the complex and dynamic interplay of altered gene and transcription factor levels and the implications of these findings for treatment strategies.

6.5 Clinical relevance

What is already known: Biomarkers of pathway activity may be relevant to the identification of valid targets for therapeutic activity. Activation of the PI3K kinase pathway appears to be a common event in obesity associated cancers and if a valid biomarker of activity of this pathway could be identified, tumours which may respond to pathway inhibition could be readily identified. STMN1, a protein which increases microtubule formation, focal adhesion assembly, cell migration and epithelial-mesenchymal transition, as been previously identified as a potential biomarker of PI3K pathway activity

What's new: Although there may be an association between STMN1 expression and PI3K pathway activity in oesophageal adenocarcinoma cell lines, there was no associated found between STMN1 expression and patient's outcomes in clinical samples.

Potential clinical application: STMN1 is not a useful biomarker in oesophageal adenocarcinoma. The utility of single protein biomarkers for pathway activity is limited. and further research using genomic or proteomic analysis of patient tumour samples may be necessary in order to identify clinically relevant biomarkers.

Chapter 7

General Discussion

7.1 Oesophageal adenocarcinoma: the need for new therapeutic avenues

Modern benchmarks in oesophageal cancer management include a cure rate of approximately 40% in patients treated with curative intent, and low postoperative mortality rates. A recent study of 1067 patients treated over 15 years with curative intent in this academic department highlights patterns of care underpinning improved outcomes, in particular an increase in early diagnoses, higher nodal yields, and an increase in non-operative approaches (Reynolds et al., 2012). Despite these improvements in outcome, the fact remains that fewer than 20% of patients diagnosed with oesophageal cancer remain alive five years post-diagnosis (Enzinger and Mayer, 2003, Reynolds et al., 2012). Clearly, there is a need to identify new treatment options for oesophageal cancer.

Targeted therapies hold the promise of providing selective and specific personalised cancer treatment. These are agents are designed utilising detailed knowledge of molecular biology in order to exploit pro-survival mechanisms of the tumour and its microenvironment (Keefe and Bateman, 2011, Sawyers, 2004).

Oncogene addiction is the hypothesis underlying the principle of utilising targeted agents for the treatment of cancer (Weinstein and Joe, 2006). It is thought that cancer cells preferentially utilise the function of certain oncoproteins in order to gain a survival advantage and the targeting these oncoproteins may be a method of intelligently designing new cancer treatments (Sawyers, 2004).

7.2 Oesophageal adenocarcinoma: an exemplar model of an obesity associated cancer

The incidence of oesophageal cancer has increased by 27% in Ireland over the last 15 years (National Cancer Registry, 2011). This is accounted for by a 50% increase in the rate of oesophageal adenocarcinoma (OAC) whilst the rate of oesophageal squamous cell carcinoma had fallen by 4% over the same period (National Cancer Registry, 2011). These changes have been paralleled internationally in Western societies, whereby the incidence of OAC is now greater than that of squamous tumours (Brown et al., 1995, Horner MJ, Wayman et al., 2001).

Obesity is now recognised as one of the foremost modifiable cancer risk factors. Numerous, large-scale epidemiological studies have demonstrated that there is a clear association between the risk of cancer development at a number of sites and raised BMI (Renehan et al., 2008b, Calle et al., 2003, Fund, 2007). The relative increased risk for the development of oesophageal cancer in patients with a raised BMI is higher than for most other cancers. Although this may be partly due to the inclusion of smaller studies in the meta-analysis, the finding is consistent across studies and populations (Renehan et al., 2008b, Calle et al., 2003, Fund, 2007). Few large studies have been performed to investigate the association between visceral adiposity and cancer incidence, although it is thought increased abdominal obesity is of more biologic relevance to cancer development than subcutaneous fat.

The increasing prevalence of obesity in the general population over the past decades is thought to be causative factor in the alteration in the epidemiology of OAC. A known precursor of OAC, Barrett's oesophagus is similarly increased in patients with increased waist circumference (Corley et al., 2007b, Edelstein et al., 2007, Cook et al., 2008, Akiyama et al., 2009) and investigations into OAC incidence and increased waist circumference have revealed a positive association (Corley et al., 2008, MacInnis et al., 2006).

Thus, OAC may be thought of as an exemplar model of an obesity-associated cancer in which to investigate pro-carcinogenic mechanisms associated with the obese state and, by increased understanding of the molecular biology of these factors, to uncover new therapeutic strategies.

7.3 Obesity and OAC: mechanisms of carcinogenesis

Although not the ideal measure of body adiposity (Donohoe et al., 2010), the finding of a link between raised BMI and cancer incidence is consistent and has biological plausibility. Adipocytes from obese subjects exhibit an altered endocrine function and secretory profile leading to an increased release of pro-inflammatory molecules resulting in a chronic low-grade inflammatory state that has been linked to the development of chronic diseases, including cancer (Schroeder et al., 2005). Despite an expanding body of epidemiological evidence in support of the link between obesity and cancer, the underlying molecular mechanisms responsible are poorly characterised.

Excess adipose tissue results in elevated levels of pro-inflammatory adipokines, resulting in an imbalance between increased inflammatory stimuli and decreased anti-inflammatory mechanism leading to persistent low-grade inflammation (Esposito and Giugliano, 2004, Wajchenberg, 2000a, Das, 2001). The level of adipokine production from adipose tissue is strongly influenced by immune cell populations present in adipose tissue (Kershaw and Flier, 2004, Schaffler et al., 2006, Xu et al., 2003, Weisberg et al., 2003). Adipose tissue in obese people is infiltrated with macrophages and the number of macrophages correlates with the degree of adiposity (Neels and Olefsky, 2006). Altered adipokine production by adipose tissue, and in particular inflamed visceral adipose tissue, may influence the tumour microenvironment.

7.4 The role of the insulin-like growth factor-1 family in OAC

A study previously carried out in this academic unit demonstrated an increase in $CD68^+$ cells, a cell surface macrophage marker, in the stromal tissue surrounding the invasive edge of oesophageal tumours (Figure 1.15) (Doyle et al., 2011). Furthermore, there was increased expression of the insulin-like growth factor receptor in oesophageal adenocarcinoma tumour's invasive edge (Figure 1.16), which may indicate that there is a paracrine mechanism involving interplay between tumour-associated macrophages-derived IGF-1 and tumour cells which express IGF1R.

The insulin-like growth factor family is on oft cited mechanism which mediates the effect of obesity on tumourigenesis (Roberts et al., 2010, Renehan et al., 2008a, LeRoith and Roberts, 2003). The overall aim of this thesis was to investigate the systemic role of the IGF-1 axis in oesophageal adenocarcinoma.

The main biological processes that the IGF axis is involved with can be summarised as: control of normal growth (Ohlsson et al., 2009, Pollak, 2008) (and perhaps lifespan (Holzenberger et al., 2003)); maintenance of tissue homeostasis (Sutherland et al., 2008) and a differentiated phenotype (Belfiore et al., 2009); alteration in the balance of proliferation and apoptosis (LeRoith and Roberts, 2003); angiogenesis, cell adhesion, migration and wound healing (LeRoith and Roberts, 2003).

In chapter 3 of this thesis, an association between visceral obesity and oesophageal adenocarincoma was reported. In patients with an increased waist circumference, there were significant increases in circulating free and total IGF-1. Whilst there was an increase in the disease-free survival in patients with less than median expression of free IGF-1 at 23.9 months versus 15.6 months in patients, this result did not achieve statistical significance ($p=0.104$). However, this study included only 100 patients and

doing a power calculation, with assumptions of an 80% probability of detecting a 20% difference (HR 1.2) in survival at a two sided 5.0% significance level, resulted in a requirement of 976 patients in order for the study to be adequately powered. Therefore, the finding that there appeared to be some difference in outcomes between patients with low and high circulating free IGF-1 levels lends some support to the hypothesis investigated in other studies that free IGF-1 is a biomarker of IGF1R activity.

Population based studies have provided evidence that relate circulating ligand levels as well as polymorphic variation of relevant genes to cancer risk and prognosis (Ma et al., 1999, Giovannucci et al., 2000, Palmqvist et al., 2002, Chan et al., 1998, Chan et al., 2002, Harman et al., 2000, Stattin et al., 2000). An important question with respect to the role of circulating IGF-1 levels is whether they may be used as a biomarker of response to IGF1R targeted therapies in patients with cancer. In a phase I expansion cohort study of figitumumab in 31 patients with relapsed sarcoma, which suggested that pre-treatment plasma levels of IGF-1>110ng/ml conferred a significant treatment advantage compared with lower levels (10.5 v 4.5 months overall survival, $p<0.001$) (Olmos et al., 2010).

In the ADVIGO trial of figitumumab (a monoclonal IGF1R inhibitor) in combination with paclitaxel and carboplatin for treatment of advanced NSCLC, there were objective response rates in patients with higher IGF1R expression (correlating with the squamous subtype) and improved progression free survival (PFS) in patients with high pre-treatment free IGF-1 levels compared to low levels (PFS 6 v 3 months, $p=0.007$) (Jassem J, 2010). Low IGF-1 levels have previously been associated with prolonged survival in NSCLC (Han et al., 2006). However, in this trial elevated baseline fIGF-1 was found in female patients with adenocarcinoma, which may indicate this, rather than predicted therapeutic efficacy, influences survival and fIGF-1 is merely a prognostic

biomarker. In comparison, in the control chemotherapy only treatment arm, fIGF-1 was not a prognostic biomarker, which may indicate that it is of relevance in predicting response to figitumumab (Gualberto et al., 2011). In addition, fIGF-1 levels correlated with tumour vimentin expression – a mesenchymal marker and inversely with E-cadherin expression – indicating that fIGF-1 may play a role in EMT (Gualberto et al., 2011).

IGF1R expression was associated with changes in disease-specific survival in chapter 3. The patient cohort included is restricted to those undergoing surgical treatment, which is exclusive to patients undergoing treatment with curative intent, representing approximately 40% of patients referred to this centre (Reynolds et al., 2012). Notwithstanding the lack of association with standard clinicopathological variables, the IGF-1 axis was associated with prognosis, with significantly ($p < 0.05$) reduced survival associated with IGF1R expression and a trend ($p=0.1$) towards longer disease-free survival in patients with lower circulating IGF-1 levels.

Thus, IGF1R expression has been found to be a prognostic factor in oesophageal adenocarcinoma in this (Donohoe et al., 2012) and other studies (Kalinina et al., 2010). The implications of the data presented in this chapter are that IGF1R expression is not universal in oesophageal adenocarcinoma patients and only a subset of patients may be suitable for treatment with anti-IGF1R targeting agents. Furthermore, IGF1R protein expression on tumour tissue may be a prognostic biomarker. Variability of IGF1R expression across different cell lines representing the same histological tumour type and across clinical tumour samples may be an indicator of variability of response. For example, in rhabdomyosarcoma receptor number seems to predict sensitivity to anti-IGF1R antibody (Cao et al., 2008). IHC has a number of important limitations both

technical and interpretative, which may impact on its reproducibility and accuracy (La Thangue and Kerr, 2011)

7.5 IGF1R inhibition is a therapeutic target in oesophageal adenocarcinoma

The association between IGF1R expression and survival prompted us to investigate whether IGF1R might be a valid therapeutic target using *in vitro* OAC cell lines and a novel tyrosine kinase inhibitor, picropodophyllin (PPP). Picropodophyllin (PPP) is a cyclolignan tyrosine kinase inhibitor specific to the insulin-like growth factor receptor with little effect on insulin (IR), fibroblast growth factor (FGFR), platelet derived growth factor (PDGFR) or epidermal growth factor receptors (EGFR) (Girnita et al., 2004). It may be a potentially attractive targeted agent as it has shown activity in both *in vitro* and *in vivo* settings (Menu et al., 2006, Economou et al., 2008) and a current clinical trial is underway at present examining the clinical efficacy of an oral analogue (AXL-1717) with a good safety profile reported and tumour necrosis in some of the NSCLC patients who received the drug (Ekman et al., 2010a).

In this study, despite induction of increased autocrine production of IGF-1 in response to PPP treatment, there was no corresponding increase in cell viability and IGF-1 stimulated proliferation was significantly inhibited with PPP treatment. Attempts to artificially knockdown IGF1R using siRNA were unsuccessful due to large amounts of toxicity observed with relevant concentrations of siRNA. Numerous reports in the literature have reported difficulty with stable and transient knockdown of IGF1R or the expression of inactive IGF1R mutants –as it results in the induction of apoptosis (Economou et al., 2008). This may indicate that IGF1R is a critical agent in the maintenance of cellular viability in oesophageal adenocarcinoma.

There were important functional consequences of IGF1R inhibition using PPP demonstrated in this study. PPP is a strong inducer of apoptosis and induction of apoptosis is now thought to be a critical component of effective chemotherapeutic agents (Pommier et al., 2004). PPP has been previously demonstrated to induce apoptosis by decreasing phosphorylation of Bad which leads to its dissociation from Bcl-2, thus activating cytochrome c release from the mitochondria (Economou et al., 2008). Consistent with previous studies, there was a substantial increase in the fraction of cells in the G2/M phase, since signalling via IGF1R is necessary for late cell cycle phases (Adesanya et al., 1999, Sell et al., 1994).

Previous studies have demonstrated that PPP downregulates IGF1R and that this is mediated via MDM2 E3 ligase, which ubiquinates and causes degradation of IGF1R. The bridging molecule β-arrestin 1 links MDM2 and IGF1R and is necessary for the downregulation of IGF1R (Vasilcanu et al., 2007). β-arrestin 1 has also been shown to mediate HIF-1α induced VEGF expression in hypoxic conditions (Shenoy et al., 2012). Other IGF1R inhibiting antibodies lead to downregulation of the IGF1R receptor (Sachdev et al., 2006, Huang et al., 2009, Kurmasheva et al., 2009) and this response to treatment may indicate clinical efficacy (Kurmasheva et al., 2009). In fact downregulation of IGF1R expression may be a critical step in the promotion of apoptosis, whereas inhibition of IGF1R phosphorylation only decreases proliferation (Hashemi et al., 2011). Re-biopsy following induction IGF1R inhibition for assessment of IGF1R expression may be a potential clinical application of this finding.

7.6 Resistance to IGF1R inhibition

As experience has accrued in the clinical setting with the use of targeted agents the problem of resistance has emerged (Ellis and Hicklin, 2009). Even in the mutationally homogenous haematological malignancies, continued use of tyrosine kinase inhibitors has lead to resistance developing to the agents (Weisberg et al., 2007). Understanding the mechanisms behind intrinsic (primary) resistance will allow us to identify predictive biomarkers of response and understanding the mechanisms behind acquired resistance will allow the rational combination of synergistic agents to mitigate against resistance.

Chapter 5 examined factors which may underpin the lack of inhibition of pAKT activity following PPP treatment and examined the potential role of autocrine VEGF expression as a factor which promotes resistance to IGF1R inhibition. The phenomenon of compensation for targeted agent activity by enhanced activity in reciprocal RTKs has been described in a number of tumour types involving a number of RTKs (Bean et al., 2007, Engelman et al., 2007, Chaft et al., 2011). While the lack of decreased pAKT activity beyond 24 hours may be specific to the two cell lines under investigation, differential effects of PPP on pAKT levels in cell lines from the same cancer subtype have also previously been observed (Karasic et al., 2010) and this finding has biological precedents in other tumour types.

VEGF is a critical growth factor for angiogenesis with a number of other proposed pro-tumourigenic mechanisms (Ellis and Hicklin, 2008). Results reported from this academic department have demonstrated a potential role for VEGF in mediating the link between obesity and carcinogenesis. Utilising the KEGG database and pathway analysis techniques, the VEGF signalling pathway was found to be significantly upregulated in a whole genome array of OAC cell lines after co-culture with human adipose tissue,

performed by this academic department (Allott, 2010). VEGF production by adipose tissue was greatest in viscerally obese patients and neutralisation of VEGF lead to significantly decreased tumour cell proliferation of co-cultured OAC cell lines (Lysaght et al., 2011).

In other disease models, VEGF and IGF1R may be co-regulated with IGF1R activation leading to upregulation of VEGF production (Reinmuth et al., 2002, Akagi et al., 1998). Thus with inhibition of IGF1R activity, decreased VEGF production would be anticipated to occur, however, both increases and decreases of VEGF have been reported in the literature following IGF1R inhibition. Increased VEGF production following treatment with an anti-IGF1R monoclonal antibody was attributed to weak agonistic effects of the MAB in one study (Warren et al., 1996). However, there is a complex and poorly understood regulation of VEGF production, especially with respect to how IGF1R may act to regulate VEGF. This interaction may potentially involve the mTOR pathway.

Data from this study show that in OAC cell lines, following PPP treatment there is persistent signalling through the PI3K pathway. This coincides with an increase in autocrine VEGF production by OAC cell lines. Neutralisation of VEGF leads to further decreases in proliferation when combined with IGF1R inhibition versus IGF1R inhibition alone. Furthermore, this combination of treatments can successfully decrease pAkt expression, indicating that this treatment combination may be of relevance to prevent treatment resistance. Looking at patient tumours samples, low VEGF and IGF1R expression is associated with an improved prognosis and more favourable clinicopathological variables including local invasion (T stage), differentiation and Nodal status.

To investigate whether it was persistent activation of the mTOR pathway which mediated the increase in VEGF production, two mTOR inhibitors, rapamycin and temsirolimus, were investigated in OAC cell lines. Both of the cell lines were highly resistant to both inhibitors- as measured by the large IC_{50} doses required to decrease cell viability in these cell lines. Treatment of the cell lines with mTOR inhibitors lead to a significant decrease in VEGF production. This provides circumstantial evidence that there may be persistent activation of the mTOR pathway following IGF1R inhibitor. These findings have not previously been investigated in OAC and warrants further in-depth study.

It must be acknowledged that there may be some limitations to the findings presented in this thesis. The mechanism of resistance identified in this chapter may only arise as a result of PPP treatment – treatment of cells with a monoclonal antibody IGF1R inhibitor R1507 did not results in a significant alteration in VEGF production. Whether other IGF1R tyrosine kinase inhibitors produce this effect remains to be investigated. Nevertheless, the functional consequences of PPP treatment are of clinical relevance as another cyclolignan, a pharmacological analogue of PPP is under clinical trial at present (Ekman et al., 2010b). This mechanism of resistance may be limited to oesophageal adenocarcinoma cell lines only. Furthermore, this may be only one of many other resistance mechanisms in the armamentarium of cells treated with IGF1R inhibition and may not enhance prevention of resistance.

Chapter 5 illustrates the potential for resistance to develop in cancer cells when there is aberrant control of complementary pathways. The resistance which develops in this situation could present in the clinical setting as either primary or secondary resistance to IGF1R inhibition and thus elucidating these mechanisms *in vitro* is of relevance to the rational combination of targeted agents in clinical practice and for future clinical trials.

7.7 Proxy indicators of PI3K activity

As previously discussed, an important aspect of utilising targeted agents is to identify the patient populations in which successful use of the targeted agent is maximised (Ellis and Hicklin, 2009). The disappointing results from clinical trials of IGF1R inhibition to date may be as a result of their use in broad unselected cancer populations (Pollak, 2012a, Pollak, 2012b). All successful targeted agents in current clinical practice are used in patients in whom there is a biomarker of reliable clinical responsiveness (Ellis and Hicklin, 2009). These biomarkers are usually related to the biology of the pathway targeted, for example HER2 amplification leads to increased signalling via this pathway (Slamon et al., 2001) or with anti-EGFR agents whereby a mutation in the EGFR receptor leads to inherent resistance to targeting agents (Lynch et al., 2004).

Activation of the PI3K kinase pathway, as reported in chapter 6, appears to be a common event in obesity associated cancers – most of the adipokines upregulated in the obese state and the IGF-1 axis mediate their downstream signalling effects through this pathway (Donohoe et al., 2011a, Donohoe et al., 2010). In mice models of both diet-induced and genetically-acquired obesity, there is activation of the PI3K pathway within their tumours (Algire et al., 2010) (Sharma and Katiyar, 2010) (Moore et al., 2008).

A 2007 genomic study established a gene expression signature for immunohistochemistry-detectable PTEN loss in breast cancer (Saal et al., 2007). In independent data sets of breast, prostate and bladder carcinoma samples, expression of the gene signature was significantly correlated with poor patient outcomes. Stathmin protein (STMN1) was an accurate IHC marker of the signature and was demonstrated to have prognostic significance in breast cancer and was, therefore, hypothesised to be a PI3K pathway activity indicator, with potential clinical applications. Studies in other

cancer types also demonstrated that STMN1 was also prognostic in colorectal (Ogino et al., 2009) and endometrial cancers (Salvesen et al., 2009).

STMN1 itself may also be pro-oncogenic – it increases microtubule formation, focal adhesion assembly, cell migration and epithelial-mesenchymal transition (Li et al., 2011). Activation of IGF-1 axis can induce neo-expression of mesenchymal markers and E-cadherin downregulation (Julien-Grille, 2005). In a number of clinical trials, patients with epithelial-mesenchymal transition tumours (defined by E-cadherin and high IRS-1 expression) have a higher response rate (Karp et al., 2009a, Gualberto et al., 2008a). This finding has also been reported *in vitro*,(Buck et al., 2008) whereby epithelial but not mesenchymal tumour cells are sensitive to IGF1R inhibition. fIGF-1 levels correlate with the mesenchymal marker vimentin and inversely with E-cadherin in pre-treatment tumour samples of patients in a clinical trial of patients treated with figitiumumab (Gualberto et al., 2011).

Thus, identification of STMN1 as a proxy marker of both IGF1R and PI3K pathway activation in OAC could be used to identify patients with tumours responsive to targeted agents. In the *in vitro* model, OAC cell lines displayed an increase in STMN1 expression following treatment with IGF-1 and decreases following PI3K inhibitor treatment. However, STMN1 was not a useful clinical biomarker in oesophageal adenocarcinoma tumours – there was no difference in STMN1 staining in tumours with different clinicopathological variables including T-stage, nodal status, differentiation, lymphovascular or perineural invasion. Patients with high STMN1 (>median) expression had a longer disease-specific survival (25.0 months) than patients with low expression (44.49 months) but this difference was not statistically significant.

These findings indicate that efforts to reduce the complexity of microarray data by using proxy indicators of activity are not universally of clinical utility. They do not reflect the complexity of the intra-cellular signalling pathways in solid tumours.

7.8 Future directions

A major recent advance in cancer biology has been the development of high through-put technologies which have enabled the sub-typing of cancers with similar histomorphological appearances (Sorlie et al., 2001). Prognostication has long been a clinical challenge in surgical oncology whereby, despite pathological staging and refinements with some known prognostic markers, the ability to predict which patients are likely to have a poor prognosis remains limited. This is because within cancer stage groups for tumours of certain histological types, there are heterogenous tumour types with different biological behaviour, which until now, were not distinguishable. The ability to sub-classify tumours according to their molecular profile, which can be used in a meaningful way to predict prognosis, should enable advances in cancer therapeutics. We may finally uncover the molecular changes in tumours with poor prognosis.

The difficulty with the application of our greater understanding of molecular oncology is the amount and complexity of the data generated(Martini et al., 2011). Both mathematical modelling and automated network analysis methodologies will need to improve if the real benefits of all of the data generated are to be harvested.

Another consideration which must be borne in mind in future studies is the cost effectiveness of targeted therapies. As delineated in this thesis, targeted agents are not universally effective and outcome improvements associated with their use are modest. The recent increase in progression free and overall survival following the addition of

transtuzumab to standard therapy to advanced oesophago-gastric tumours (Bang et al., 2010) was only found to have a borderline incremental cost effectiveness ratio (ICER) of between £45,000 and £50,000 for patients with strong staining of the HER2 receptor using immunohistochemistry ((NICE), 2010). It was only approved for use in this setting in the National Health Service in the United Kingdom by applying special criteria which took into account the lack of alternative options in an end of life setting and a likely increase in survival of greater than 3 months.

There has been a 14% annual increase in spending on cancer drugs in the US (Jackson and Sood, 2011), and 90% of the anticancer agents approved by the FDA over the past 4 years have cost more than $20,000 for a 3 month course (Bach, 2009). Typically use of agents such as bevacizumab costs $100,000 per year of treatment with modest clinical benefit achieved (Hayes, 2011). In socialised health care systems difficult choices are encountered with the prospective large spend to extend the lives of small numbers of patients. A major challenge to be faced is how to achieve maximal clinical efficacy without substantial costs accruing during the drug discovery and testing phases and critical to this will be the understanding of the molecular implications of targeted agents at a subcellular level in order to accurately predict response and resistance.

The next logical step in the exploration of new therapeutic avenues in oesophageal cancer is the molecular profiling of patient tumours. The Oesophageal cancer clinical and molecular stratification (OCCAMS) incorporating International Cancer genome Consortium (ICGC) multicentre study (Stratton et al., 2009) is currently underway. It is planned that 500 patient's tumours (including samples from lymph node metastases and both prior to and after neoadjuvant therapy) will have whole genome sequencing and transcriptomics performed. This will lead to a biorepository of molecular biology information which is linked to clinicopathological information and will allow validation

of previously identified and putative targets in oesophageal cancer. The potential difficulty will be in the understanding the complex and dynamic interplay of altered gene and transcription factor levels and the implications of these findings for treatment strategies.

As demonstrated in this study, inhibition of the IGF1R may be a promising target in the treatment of oesophageal adenocarcinoma, however, in order to delay or prevent inevitable treatment resistance developing we must first understand the complex interactions of the IGF-1 pathway in oesophageal cancer. Identification of response biomarkers in patients and combination of IGF1R inhibition with other agents to circumvent resistance mechanisms is necessary for clinical success. Once we have a greater understanding of the complex interacting molecular alterations within cancers, we can rationally and accurately tailor treatments to the biology of individual patient's tumours and the era of personalised medicine will have truly arrived.

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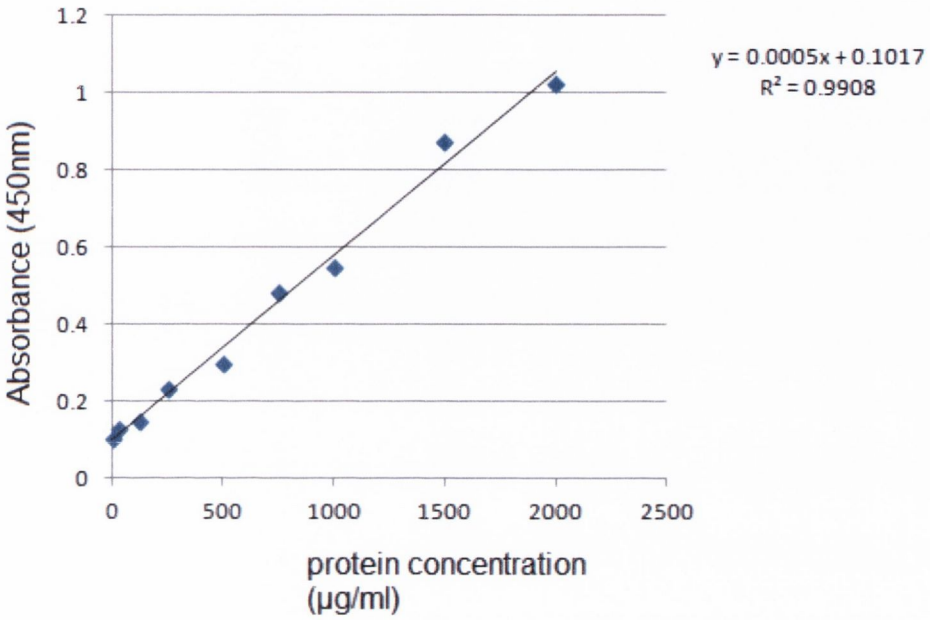
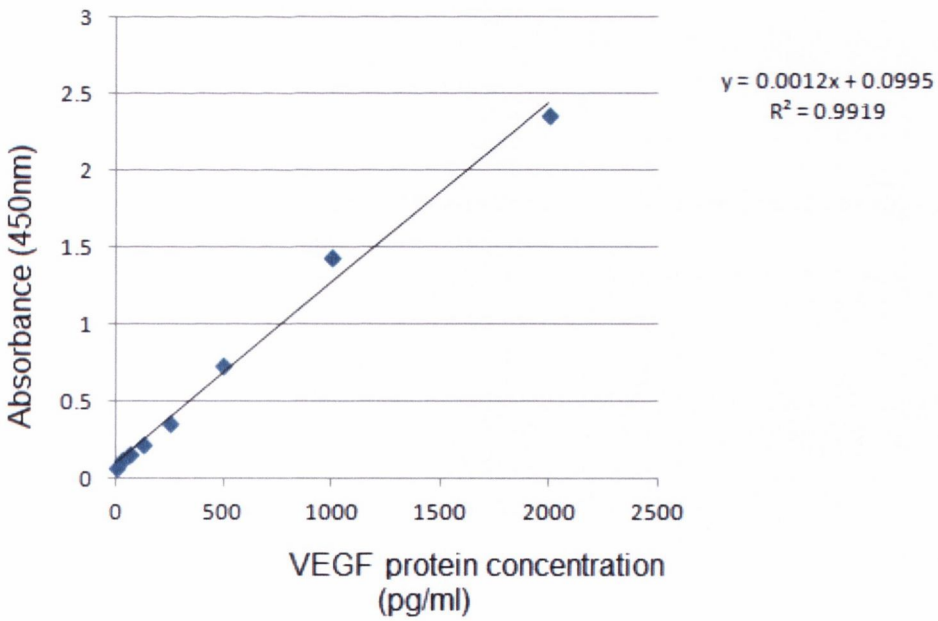
Appendices

Appendix I: Standard Curves

BCA assay (section 2.17.3)



VEGF ELISA (section 2.6)



Appendix II: Genes on Human Cancer pathway profiler

UniGene	RefSeq	Symbol	Description	Gene Name
Hs.525622	NM_005163	AKT1	V-Akt murine thymoma viral oncogene homolog 1	PKB/PRKBA
Hs.369675	NM_001146	ANGPT1	Angiopoietin 1	AGP1/AGPT
Hs.553484	NM_001147	ANGPT2	Angiopoietin 2	AGPT2/ANG2
Hs.552567	NM_001160	APAF1	Apoptotic peptidase activating factor	CED4
Hs.435561	NM_000051	ATM	Ataxia telangiectasia mutated (includes complementation groups A, C and D)	AT1/ATA
Hs.370254	NM_004322	BAD	BCL2-antagonist of cell death	BBC2/BCL2L8
Hs.159428	NM_004324	BAX	BCL2-associated X protein	Bax zeta
Hs.150749	NM_000633	BCL2	B-cell CLL/lymphoma 2	Bcl-2
Hs.516966	NM_138578	BCL2L1	BCL2-like 1	BCL-XL/S
Hs.194143	NM_007294	BRCA1	Breast cancer 1, early onset	BRCAI/BRCC1
Hs.369736	NM_001228	CASP8	Caspase 8, apoptosis-related cysteine peptidase	FLICE/MACH
Hs.244723	NM_001238	CCNE1	Cyclin E1	CCNE
Hs.1634	NM_001789	CDC25A	Cell division cycle 25A	CDC25A2
Hs.19192	NM_001798	CDK2	Cyclin-dependent kinase 2	p33(CDK2)
Hs.95577	NM_000075	CDK4	Cyclin-dependent kinase 4	CMM3/PSK-J3
Hs.370771	NM_000389	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CAP20/CDKN1
Hs.512599	NM_000077	CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	ARF/CDK4I
Hs.390736	NM_003879	CFLAR	CASP8 and FADD-like apoptosis regulator	CASH/CASP8AP1
Hs.291363	NM_007194	CHEK2	CHK2 checkpoint homolog (S. pombe)	CDS1/CHK2
Hs.517356	NM_030582	COL18A1	Collagen, type XVIII, alpha 1	KNO
Hs.96055	NM_005225	E2F1	E2F transcription factor 1	E2F-1/RBBP3
Hs.446352	NM_004448	ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	HER-2/HER-2
Hs.517296	NM_005239	ETS2	V-Ets erythroblastosis virus E26 oncogene homolog 2 (avian)	c-Ets2
Hs.244139	NM_000043	FAS	Fas (TNF receptor superfamily, member 6)	ALPS1A/APO-1
Hs.533683	NM_000141	FGFR2	Fibroblast growth factor receptor 2	BEK/BFR-1
Hs.25647	NM_005252	FOS	V-fos FBJ murine	c-fos

			osteosarcoma viral oncogene homolog	
Hs.90708	NM_006144	GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	CTLA3/HFSP
Hs.90753	NM_006410	HTATIP2	HIV-1 Tat interactive protein 2, 30kDa	CC3/TIP30
Hs.37026	NM_024013	IFNA1	Interferon, alpha 1	IFL/IFN
Hs.93177	NM_002176	IFNB1	Interferon, beta 1, fibroblast	IFB/IFF
Hs.160562	NM_000618	IGF-1	Insulin-like growth factor 1 (somatomedin C)	IGFI
Hs.624	NM_000584	IL8	Interleukin 8	3-10C/AMCF-I
Hs.519304	NM_181501	ITGA1	Integrin, alpha 1	CD49a/VLA1
Hs.482077	NM_002203	ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	BR/CD49B
Hs.265829	NM_002204	ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	CD49C/GAP-B3
Hs.553495	NM_000885	ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	CD49D/IA4
Hs.436873	NM_002210	ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	CD51/MSK8
Hs.429052	NM_002211	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	CD29/FNRB
Hs.218040	NM_000212	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	CD61/GP3A
Hs.13155	NM_002213	ITGB5	Integrin, beta 5	Integrin b5
Hs.525704	NM_002228	JUN	V-jun sarcoma virus 17 oncogene homolog (avian)	AP1
Hs.145442	NM_002755	MAP2K1	Mitogen-activated protein kinase kinase 1	MAPKK1/MEK1
Hs.511397	NM_006500	MCAM	Melanoma cell adhesion molecule	CD146/MUC18
Hs.567303	NM_002392	MDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	hdm2
Hs.132966	NM_000245	MET	Met proto-oncogene (hepatocyte growth factor receptor)	HGFR/RCCP2
Hs.83169	NM_002421	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	CLG/CLGN
Hs.513617	NM_004530	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	CLG4/CLG4A
Hs.297413	NM_004994	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa	CLG4B/GELB

			gelatinase, 92kDa type IV collagenase)	
Hs.525629	NM_004689	MTA1	Metastasis associated 1	Mta-1
Hs.173043	NM_004739	MTA2	Metastasis associated 1 family, member 2	DKFZp686F2281/MTA1L1
Hs.336994	NM_014751	MTSS1	Metastasis suppressor 1	MIM/MIMA
Hs.202453	NM_002467	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	c-Myc
Hs.431926	NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	DKFZp686C01211/EBP-1
Hs.81328	NM_020529	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	IKBA/MAD-3
Hs.118638	NM_000269	NME1	Non-metastatic cells 1, protein (NM23A) expressed in	AWD/GAAD
Hs.9235	NM_005009	NME4	Non-metastatic cells 4, protein expressed in	NM23H4/nm23-H4
Hs.376032	NM_002607	PDGFA	Platelet-derived growth factor alpha polypeptide	PDGF-A/PDGF1
Hs.1976	NM_002608	PDGFB	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	PDGF2/SIS
Hs.132225	NM_181504	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	GRB1/p85-ALPHA
Hs.77274	NM_002658	PLAU	Plasminogen activator, urokinase	ATF/UPA
Hs.466871	NM_002659	PLAUR	Plasminogen activator, urokinase receptor	CD87/UPAR
Hs.409965	NM_002687	PNN	Pinin, desmosome associated protein	DRS/SDK3
Hs.159130	NM_002880	RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	CRAF/Raf-1
Hs.408528	NM_000321	RB1	Retinoblastoma 1 (including osteosarcoma)	OSRC/RB
Hs.81256	NM_002961	S100A4	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	18A2/42A
Hs.55279	NM_002639	SERPINB5	Serpin peptidase inhibitor, clade B (ovalbumin), member 5	PI5/maspin
Hs.414795	NM_000602	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	PAI/PAI-1
Hs.349470	NM_003087	SNCG	Synuclein, gamma (breast cancer-specific protein 1)	BCSG1/SR
Hs.371720	NM_003177	SYK	Spleen tyrosine kinase	Syk
Hs.89640	NM_000459	TEK	TEK tyrosine kinase,	CD202B/TIE-2

			endothelial (venous malformations, multiple cutaneous and mucosal)	
Hs.492203	NM_003219	TERT	Telomerase reverse transcriptase	EST2/TCS1
Hs.1103	NM_000660	TGFB1	Transforming growth factor, beta 1 (Camurati-Engelmann disease)	CED/DPD1
Hs.494622	NM_004612	TGFBR1	Transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kDa)	ACVRLK4/ALK-5
Hs.164226	NM_003246	THBS1	Thrombospondin 1	THBS/TSP
Hs.522632	NM_003254	TIMP1	TIMP metallopeptidase inhibitor 1	CLGI/EPA
Hs.297324	NM_000362	TIMP3	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	HSMRK222/K222
Hs.241570	NM_000594	TNF	Tumor necrosis factor (TNF superfamily, member 2)	DIF/TNF-alpha
Hs.521456	NM_003842	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	DR5/KILLER
Hs.279594	NM_001065	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	CD120a/FPF
Hs.462529	NM_003790	TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	APO-3/DDR3
Hs.408312	NM_000546	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	LFS1/TRP53
Hs.66744	NM_000474	TWIST1	Twist homolog 1 (acrocephalosyndactyly 3; Saethre-Chotzen syndrome) (Drosophila)	ACS3/BPES2
Hs.416007	NM_017549	EPDR1	Ependymin related protein 1 (zebrafish)	MERP-1/MERP1
Hs.73793	NM_003376	VEGF	Vascular endothelial growth factor	VEGFA/VPF
Hs.534255	NM_004048	B2M	Beta-2-microglobulin	B2M
Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	HGPRT/HPRT
Hs.546356	NM_012423	RPL13A	Ribosomal protein L13a	RPL13A
Hs.544577	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD
Hs.520640	NM_001101	ACTB	Actin, beta	b-Actin

Appendix III: Related publications by author

First Author publications:

Total: 17

Original clinical research

	Title	Authors	Journal, year	PMID
1	Long-term Health-related Quality of Life in Disease-free Esophageal Cancer Patients.	**Donohoe CL**, McGillycuddy E, Reynolds JV	*World Journal of Surgery*, 2011	21553202
2	The Impact of Young Age on Outcomes in Esophageal and Junctional Cancer	**Donohoe CL**, MacGillycuddy E, Reynolds JV	*Diseases of the Esophagus*, 2011	21385286
3	Eversion endarterectomy for external iliac occlusive disease	**Donohoe CL**, Dowdall JF, McDonnell CO, O'Malley MK, O'Donohoe MK	*Vascular and Endovascular surgery*, 2010	20810406
4	Palliative endoscopic trans-anal resection of advanced rectosigmoid carcinoma	**Donohoe CL**, Brannigan AE, O'Connell PR	*Irish Journal of Medical Science*, 2010	20953977
5	Evidence-based selective application of transhiatal oesophagectomy in a high-volume oesophageal centre	**Donohoe CL**, O'Farrell NJ, Ravi N, Reynolds JV	*World Journal of Surgery*, 2011	21979584

Clinical literature reviews and commentary

	Title	Authors	Journal, year	PMID
6	Defining a successful esophagectomy	**Donohoe CL**, Reynolds JV	*Ann Surg*, 2012	22580934

7	Perioperative evaluation of the obese patient	**Donohoe CL**, Feeney C, Carey M, Reynolds JV	*J Clin Anesthesia*, 2011	22050805
8	Cancer cachexia: mechanisms and clinical implications	**Donohoe CL**, Ryan AM, Reynolds JV	*Gastroenterol Res Pract*, 2011	21760776
9	Short Bowel Syndrome	**Donohoe CL**, Reynolds JV	*The Surgeon*, 2010	20709285
10	Fast-track surgery protocols in colorectal surgery	**Donohoe CL**, Nguyen M, Cook J, Murray SG, Chen N, Zaki F, Mehigan BJ, McCormick PH, Reynolds JV	*The Surgeon*, 2011	21342674
11	European Working Time Directive: Implications for surgical training	**Donohoe CL**, Sayana MK, Kennedy MT, Niall DM	*Irish Medical Journal*, 2010	20666060
12	Allowances for Training	**Donohoe CL**, Sayana MK	*Irish Medical Journal*, 2009	19405325
13	Aspirin as thromboprophylaxis for lower limb arthroplasty: review of international guidelines	**Donohoe CL**, Sayana MK, Niall DS	*Irish Journal of Medical Science*, 2010	21286842
14	Challenges in the treatment of gastroesophageal cancer	**Donohoe CL**, Reynolds JV	*World J Surgery*, 2011	21409608

Publications related to present thesis

	Title	Authors	Journal, year	PMID
15	Obesity and gastrointestinal malignancy	**Donohoe CL**, Lysaght J, Pidgeon GP, Reynolds JV	*British Journal of Surgery*. 2010	20306531
16	Role of Insulin-like growth factor 1 axis with visceral adiposity in oesophageal adenocarcinoma	**Donohoe CL**, Doyle SL, Lysaght J, Cathcart MC, Pidgeon GP,	*British Journal of Surgery*. 2011	22241325

		Reynolds JV		
17	Visceral adiposity, insulin and cancer risk	**Donohoe CL**, Doyle SL, Reynolds JV	*Diabetology & Metabolic Syndrome*, 2011	21696633

Co-Author Peer reviewed Publications:

Total published: 11

Original scientific research

	Title	Authors	Journal, year	PMID
1	T-lymphocyte activation in visceral adipose tissue of patients with oesophageal adenocarcinoma	Lysaght J, Allot EH, ***Donohoe CL***, Howard JM, Pidgeon GP, Reynolds JV	British Journal of Surgery, 2011	21520028
2	Pro-inflammatory and tumour proliferative properties of excess visceral adipose tissue	Lysaght J, van der Stok EP, Allott EH, Casey R, ***Donohoe CL***, Howard JM, McGarrigle SA, Ravi N, Reynolds JV, Pidgeon GP	Cancer Letters, 2011	21890265
3	IGF-1 and Its Receptor in Esophageal Cancer: Association with Adenocarcinoma and Visceral Obesity	Doyle SL, ***Donohoe CL***, Finn SP, Howard JM, Lithander FE, Reynolds JV, Pidgeon GP, Lysaght J	American Journal of Gastroentero logy, 2011	22146489
4	MMP9 expression in oesophageal adenocarcinoma is up regulated with visceral obesity and correlates with poor tumour differentiation	Allott EH, Lysaght J, Cathcart MC, ***Donohoe CL***, Cummins R, McGarrigle S, Ravi N, Reynolds JV, Pidgeon GP	Molecular carcinogenes is, 2011	22121096

Original clinical research

	Title	Authors	Journal, year	PMID
1	Evolving Improvement in Oncologic and Operative Outcomes for Esophageal and Junctional Cancer: Lessons from the experience of a High-Volume Center	Reynolds JV, **Donohoe CL** et al	Journal of Thoracic and Cardiovascular Surgery, 2011	22244551
2	Modern Oncologic Outcomes in a High volume Oesophageal Unit	Reynolds JV, McGillycuddy E, Ravi N, **Donohoe CL**, O'Toole D, O'Byrne K, Hollywood D	Irish Medical Journal, 2011	22125876
3	Acute intestinal failure in surgical patients: an audit of incidence, management and outcomes in an Irish hospital and compliance with ASGBI guidelines	Geoghegan AR, **Donohoe CL**, Reynolds JV	Irish Journal of Medical Science, 2012	22231901
4	^{18}F-FDG PET –detected Synchronous neoplasms in the Staging of Esophageal Cancer: Incidence , Cost and Impact on Management	Malik V, Johnston C, **Donohoe CL**, Claxton Z, Ravi N, Reynolds JV	Clinical Nuclear Medicine, 2012	Awaited
5	Establishing CT-defined visceral fat area thresholds associated with metabolic syndrome in patients with gastrointestinal cancer.	Doyle S, Bennett AM, **Donohoe CL**, Howard J, Lithander F, Pidgeon GP, Reynolds JV, Lysaght J.	Submitted	

Literature reviews and commentary

	Title	Authors	Journal, year	PMID
1	Visceral obesity, the metabolic syndrome, insulin resistance and cancer	Doyle SL, **Donohoe CL**, Lysaght J, Reynolds JV	Proceedings of the Nutrition Society, 2011	22051112
2	Diet, Obesity and Cancer	Reynolds JV, **Donohoe CL**, Doyle SL	*Irish Journal of Medical Science.* 2010	21174166