Molecular Mechanisms of Advanced Prostate Cancer

A thesis submitted to the University of Dublin, Trinity College for the degree of

Doctor of Philosophy (Ph.D.)

by

Lauren Brady

SUPERVISOR:

Prof Stephen Finn,

Department of Histopathology

University of Dublin

Trinity College

April 2018

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the Library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

_____

Lauren Brady

Summary

Globally, prostate cancer is the most common cancer for males. In the era of PSA screening, survival rates for primary localised prostate cancer are high, with the majority of cases classed as clinically insignificant. However, in advanced or metastatic prostate cancer, five year survival rates decrease drastically. Improved understanding of the biological mechanisms involved in disease progression and metastasis is paramount. The overall aim of this study was to elucidate the relationship between circulating tumour cells, inflammation, obesity and exercise in patients with metastatic prostate cancer.

Characterisation of molecularly distinct subtypes of prostate cancer may aid the development of targeted therapies for patients with this disease. Three sets of patients, grouped according to progression status, were screened for the presence of SPOP mutations and for genomic alterations, ERG and PTEN. No SPOP mutations were observed within each group, however decreased ERG expression was demonstrated as significantly associated with immediate progression after surgery in this cohort. ERG status remained significant within this cohort of patients, with the addition of age, Gleason grade and PTEN status to the model. This finding highlights the possible role of distinct genomic alterations in prostate cancer disease progression.

The ExPeCT trial was conceived to further understanding on the biology of metastasis in prostate cancer. ExPeCT aimed to examine the effects of exercise on circulating tumour cells, obesity and inflammation in patients with metastatic prostate cancer. Participants were randomised into exercise or control groups, and the exercise group undertook six months of aerobic exercise. The impact of exercise was determined in relation to circulating tumour cell number and the hypothesised platelet cloaking of tumour cells. Significant changes in circulating tumour cell number were observed within each group over time, in addition to the presence of platelet cloaking and circulating tumour cell clusters. Significant positive correlations were also observed between circulating tumour cells and clinical variables, which may hold potential as an

aggressive disease signature, as well as a screening tool for patients with high risk of metastatic spread.

In addition to circulating tumour cells, the role of inflammation and obesity in metastatic prostate cancer was examined. Obesity is associated with aggressive disease and the onset of a pro-inflammatory environment. Understanding the role played by inflammation in metastatic disease, may provide insight into disease progression. Significant differences in adipokine and inflammatory mediator expression were observed within group, in addition to significant correlations with clinical variables. The relationship between obesity, inflammation, circulating tumour cells and platelet cloaking was also assessed. Significant correlations were observed, suggestive of an association between these biological processes in promoting metastatic disease.

Diagnostic needle core biopsies from ExPeCT participants were assessed for expression of a lethality associated gene signature. Significant correlations between gene expression and inflammatory mediators, IL4 and IL-13 were observed, suggesting a relationship in early stage disease. In conjunction with the clinical trial, an *in vitro* cell line model was constructed to study the impact of obesity in prostate cancer. Significant differences in expression between malignant and normal prostate cells were observed, as well as differences between cell lines cultured in adipocyte conditioned media. These data highlight the need for further research in this area.

In conclusion, this research contributed significantly to the understanding of the relationship between circulating tumour cells, obesity and inflammation in metastatic prostate cancer and aimed to improve the lives of patients living with this disease.

Acknowledgements

Firstly, I would like to extend my deepest gratitude to my supervisor, Prof Stephen Finn. Your guidance, good humour and encouragement over the last four years has been invaluable.

Thank you to Prof Orla Sheils for the mentorship, kind words and advice at every stage throughout my PhD. Your support has been immeasurable.

Thank you to Dr Brian Hayes, Dr Tatjana Vlajnic, Dr Bryan Stanfill and Dr Jessica Carlsson, all of whom made significant contributions to this research.

Thank you to all of the clinical trials staff, site principal investigators and the ExPeCT research team in London. It has been a pleasure to work with you and I thank you for your assistance and hard work throughout the ExPeCT trial.

Thank you to Grainne and Anne-Marie, this experience would not have been the same without either of you. Grainne –thank you for all of the teamwork, laughs and memories, and for making sure we got through everything together. Anne-Marie – thank you for being a constant source of advice, kindness and reassurance, and for remaining unfazed by whatever comes your way. Your strength is endless and I am forever grateful for you taking me under you wing. Special mention to John H, for the IT support and for introducing Jim into our lab lives!

To all of the lab members, both past and present, John G, Marvin, David, John A, Louise, Mark, Gordon, and Chris, thank you for making so many hours spent in the lab bearable and filled with laughter and support. Thank you to Paul, for always humouring my long list of questions with endless patience and guidance. Thank you to Orla C, for your positivity and encouragement. Thank you to Emma, for your advice and ideas throughout this project. Thank you to Martin for your kindness and good humour throughout. Thank you to Steven G for the technical advice and ideas.

To my parents, John and Marguerite, thank you for your infinite love and support. Thank you for always looking out for me and for re-watching *As Time Goes By* a never ending number of times. I would be half of the person I am today without you both. To my brother, Aidan, champion of longwinded storytelling, thank you for always cheering me on in anything I do. A special mention must go to Niamh, for somehow managing to put up with you. To my grandparents, Bridge, Vic and Frank, thank you for the care packages, lifts and general fussing over me (in spite of my age). To my second family the Smiths, Esther, David, Steve and Maeve, I am very fortunate to have your support and backing in anything I choose to do. To my favourite little people, Ruan, Finn and Jersey, the chubby faces, phonecalls and video messages have been the best pick me up I could hope for.

To my wonderful group of friends, who I am so fortunate to have in my arsenal, thank you for putting up with my absences and moaning, and for always being a source of dancing, belly laughs and no-nonsense advice.

To Smithy, I am grateful to have your love and kindness in my life. Thank you for always being there for me, for humouring my desire to see every corner of the world and for the inevitable adventures that ensue.

Lastly, I would like to thank all of the patients who took part in ExPeCT. It was a privilege to meet such an inspiring and positive group of people.

This research was funded by the World Cancer Research Fund.

Everything will be alright in the end, and if it is not, it is not yet the end.

The Best Exotic Marigold Hotel

Dedication

This thesis is dedicated to my parents, John and Marguerite, for everything.

It is also dedicated to all of the patients who participated in the ExPeCT trial.

Table of Contents

List of Tables

List of Figures

Abbreviations

ACK – acetate kinase

ACM – adipocyte conditioned media

AdipoR1 – adiponectin receptor 1

AdipoR2 – adiponectin receptor 2

ADT – androgen deprivation therapy

AKT – protein kinase

AMNCH – Adelaide and Meath National Children's Hospital

ANOVA – analysis of variance

AR – androgen receptor

BCL-2 – B-cell lymphoma 2

BCR – biochemical recurrence

BET – bromodomain and extraterminal

BMI – body mass index

BPH – benign prostatic hyperplasia

BTB – bric-brac, tramtrack and broad complex

cAMP – cyclic adenosine monophosphate

CAP1 – andenylyl cyclase-associated protein 1

cdDNA – circulating free DNA

cFLIP – cellular FLICE like inhibitory protein

CRP – C-reactive protein

CRPC – castrate resistant prostate cancer

CTC – circulating tumour cell

CTM – circulating tumour microemboli

CVD – cardiovascular disease

CXCL – chemokine ligand

DHT – dihydrotestosterone

DMEM – Dulbecco’s modified eagle’s medium

EGF – epidermal growth factor

EMT – epithelial mesenchymal transition

EpCAM – epithelial cell adhesion molecule

ERG – ETS-related gene

ERK – extracellular receptor kinase

ETS – E26 transformation specific

ETV1 – ETS variant 1

ExPeCT – exercise, prostate cancer and circulating tumour cells

EZH2 – enhancer of zeste homolog 2

FBS – fetal bovine serum

FFPE – formalin fixed paraffin embedded

H&E – Haematoxylin and Eosin

Hb - haemoglobin

HPFS – Health Professionals Follow Up Study

HSP – heat shock protein

IAP – inhibitor of apoptosis

ICAM -1 – intracellular adhesion molecule 1

IMS – industrial methylated spirits

IFN - interferon

IGF-1 – insulin growth factor

IHC – immunohistochemistry/Immunohistochemical

IL – interleukin

JAK1 – janus kinase 1

LCM – laser capture microdissection

LepR – leptin receptor

MAPK – mitogen-activated protein kinase

MCP – monocyte chemoattractant protein 1

MIP-2 – macrophage inflammatory protein

MEK – mitogen activated kinase

MEM – minimum essential medium eagle

MetS – metabolic syndrome

MGG – May Grunwald Giemsa

MMP – matrix metalloproteinase

mTOR – mammalian target of rapamycin

MYC – myeloctomatosis oncogene cellular homolog

NCB – needle core biopsy

NK – natural killer cell

NKX3 – homeobox protein NKx-3

NFκB – nuclear factor kappa B

NLS – nuclear localisation sequence

PA – physical activity

PAI2 – plasminogen activator inhibitor 2

PMBC – peripheral blood mononuclear layer

PBS – phosphate buffered saline

PBST – PBS tween

PCR – polymerase chain reaction

PCRC – Prostate Cancer Research Consortium

PDK1 – phosphoinositide-dependent kinase 1

PI3K – phosphoinositide 3-kinase

PIP3 – phosphatidylinositol (3,4,5)-trisphosphate

PIN – prostatic intraepithelial neoplasia

PKA – protein kinase A

PrCa – prostate cancer

PSA – prostate specific antigen

PTEN – phosphatase and tensin homologue

QoL – quality of life

OS – overall survival

RA – rheumatoid arthritis

RANTES – regulated upon activation normal T-cell expressed and secreted

RT – room temperature

RTK – receptor tyrosine kinase

SFM – serum free media

SGBS – Simpson Golabi Behmel Syndrome

SMI – seminal vesicle invasion

SPOP – speckle-type POZ protein

SRC – steroid receptor co-activator

STAT3 – signal transducer and activator of transcription 3

T – testosterone

T0 – baseline

T3 – three months

T6 – six months

TBS – tris-buffered saline

TE – Tris-EDTA

TMPRSS2-ERG – transmembrane protease serine ETS-related gene fusion

TMA – tissue microarray

TNF – tissue necrosis factor

TRAIL – TNF-related apoptosis inducing ligand

VCAM – vascular cell adhesion molecule

VEGF – vascular endothelial growth factor

WCC – white cell count

WHO – World Health Organisation

WT - wildtype

Units

bp	base pairs
°C	degrees Celsius
g	grams
h	hour(s)
µg	microgram
µL	microlitre
µM	micromolar
kB	kilobases
kg	kilograms
L	litre(s)
M	molar
mg	milligram
min	minutes
mL	millilitre
mM	millimolar
mm	millimetre
n	number (sample size)
pg	picogram
rpm	revolutions per minute
s	second(s)
U	unit(s)
v/v	volume per volume

Publications and presentations

Publications

ExPeCT (Examining Exercise, Prostate Cancer and Circulating Tumour Cells): A Randomised Control Trial. Sheill G*, Brady L***** et al. (*Joint first authors) 2017. Trials. 18(1):456

Exercise and Prostate Cancer. Brady L, Sheill G *et al*. 2016. *Cancer Professional.* Summer Issue

Exercise and prostate cancer: evidence and proposed mechanisms for disease modification. Hayes BD, Brady L, Pollak M, Finn SP. 2016. *Cancer Epidemiology Biomarkers and Prevention*. 25(9), 1281-1288

Oral presentations

'The Importance of Research in Prostate Cancer' Prostate Cancer: A Patient Information Evening about Research and Exercise (Nov 2017)

'ExPeCT Trial Update' Guy's and St Thomas' Hospital, London UK (Sept 2017)

'Exercise and Patients with Advanced Prostate Cancer' Gathering Around Cancer Conference, Dublin, Ireland (Oct 2015)

'ExPeCT Trial Update: Circulating Tumour Cells and Platelet Cloaking' ToPCaP (Transdisciplinary Prostate Cancer Partnership) Meeting, Dublin, Ireland (Sept 2015)

Posters

'Examining the link between obesity, inflammation and exercise in patients with metastatic prostate cancer - An interim analysis from the ExPeCT trial' AACR Prostate Cancer: Advances in Basic, Translational and Clinical Research (Dec 2017)

‘Adipokine Status Post-Exercise in a Cohort of Patients with Metastatic Prostate Cancer’ Trinity College Dublin 10th International Cancer Conference (Oct 2016), Dublin, Ireland

‘The Impact of a Structured Exercise Programme on Adipokine Status in Metastatic Prostate Cancer Patients’ World Obesity and WCRF Hot Topic Conference: Obesity, Physical Activity and Cancer, London, UK (Sept 2016)

The ExPeCT Trial Exercise, Prostate Cancer and Circulating Tumour Cells’ MMI Public Information Day, Dublin, Ireland (Nov 2015)

Awards

Awarded a poster prize at the Trinity College Dublin 10th International Cancer Conference (Oct 2016)

Awarded Janssen (Ireland) Young Investigator Travel Bursary at ToPCaP meeting (Sept 2015)

Chapter 1:
General Introduction

1.0 Introduction

1.1 Prostate cancer

1.1.1 Incidence and mortality

Globally, prostate cancer (PrCa) is the second most common cancer type in men and accounted for 1.1 million new cancer cases in 2012 (Ferlay et al., 2015). The majority of reported cases occur in developed regions in comparison to developing countries (Figure 1.1), conceivably in part related to the widespread use of prostate specific antigen (PSA) testing and biopsy screening (Ferlay et al., 2015). In terms of European statistics, Western Europe has the highest levels of PrCa incidence, with Ireland placing as third highest for PrCa Incidence (EUCAN, 2018) (Figure 1.2). PrCa is the most common cancer for men in Ireland, with the National Cancer Registry Ireland estimating 3364 new diagnoses and 527 deaths per annum (NCRI, 2017). While five year survival rates for primary localised PrCa are extremely high at almost 100%, metastatic disease infers a much worse prognosis with five year survival rates reducing to 31% (Jin et al., 2011).

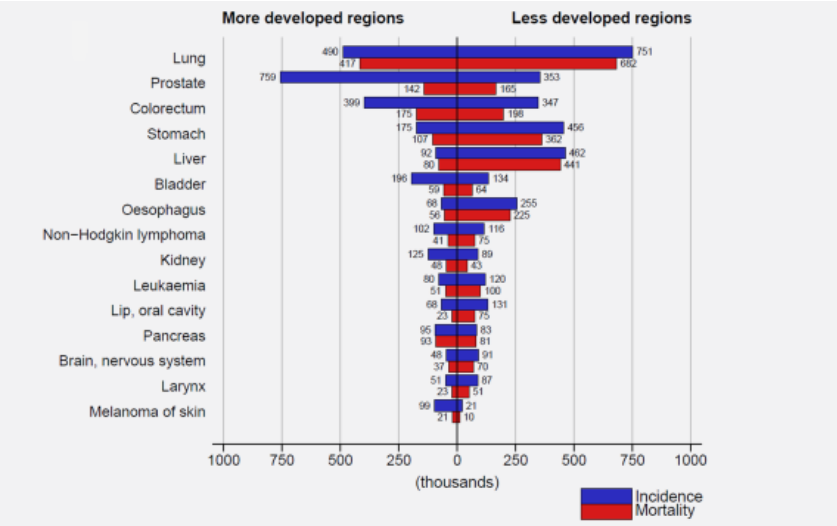
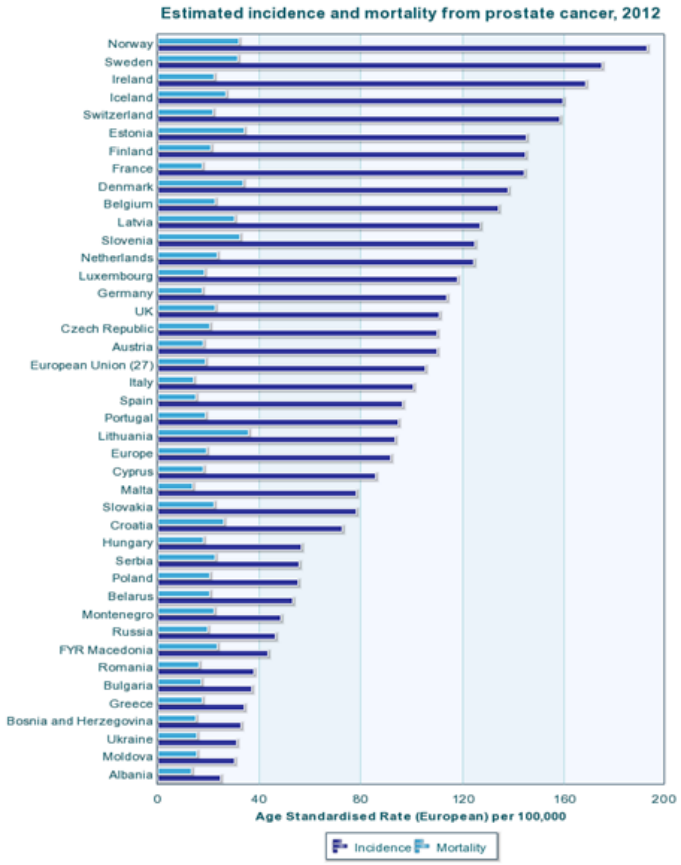
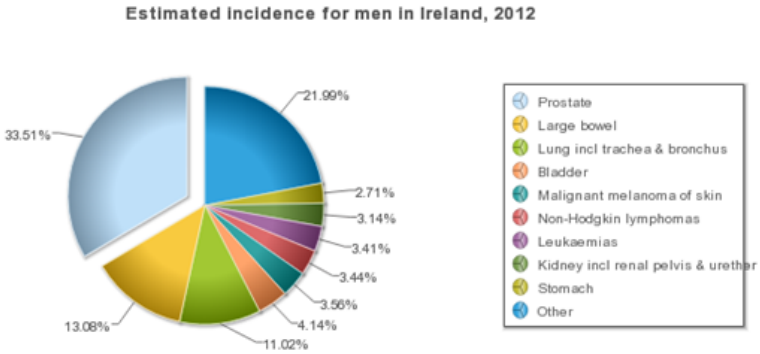


Figure 1.1: Outline of cancer incidence and mortality rates among men in developed *vs.* less developed regions.

Image adapted from (Ferlay et al., 2015).





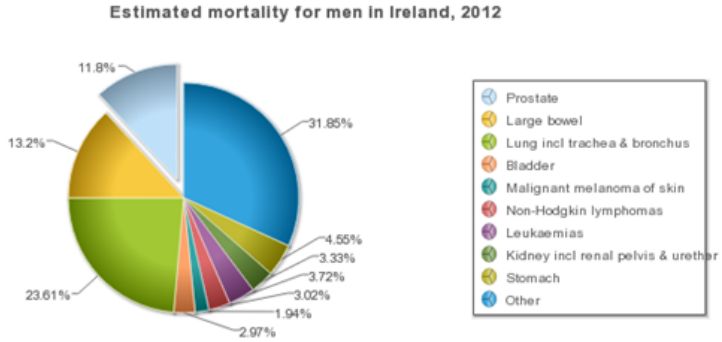


Figure 1.2: European prostate cancer statistics including incidence and mortality rates in Ireland. Images adapted from EUCAN (https://eco.iarc.fr/eucan).

1.1.2 Development of prostate cancer

The key role of the prostate is the addition of secretory proteins to seminal fluid (Abate-Shen and Shen, 2000). It is divided into four main sections (Figure 1.3) and consists of glands lined with basal and secretory epithelial cells; secretory epithelial cells rely on androgens and produce PSA. In addition to basal and epithelial cells, neuroendocrine cells are dispersed throughout the gland (Oh W.K., 2003).

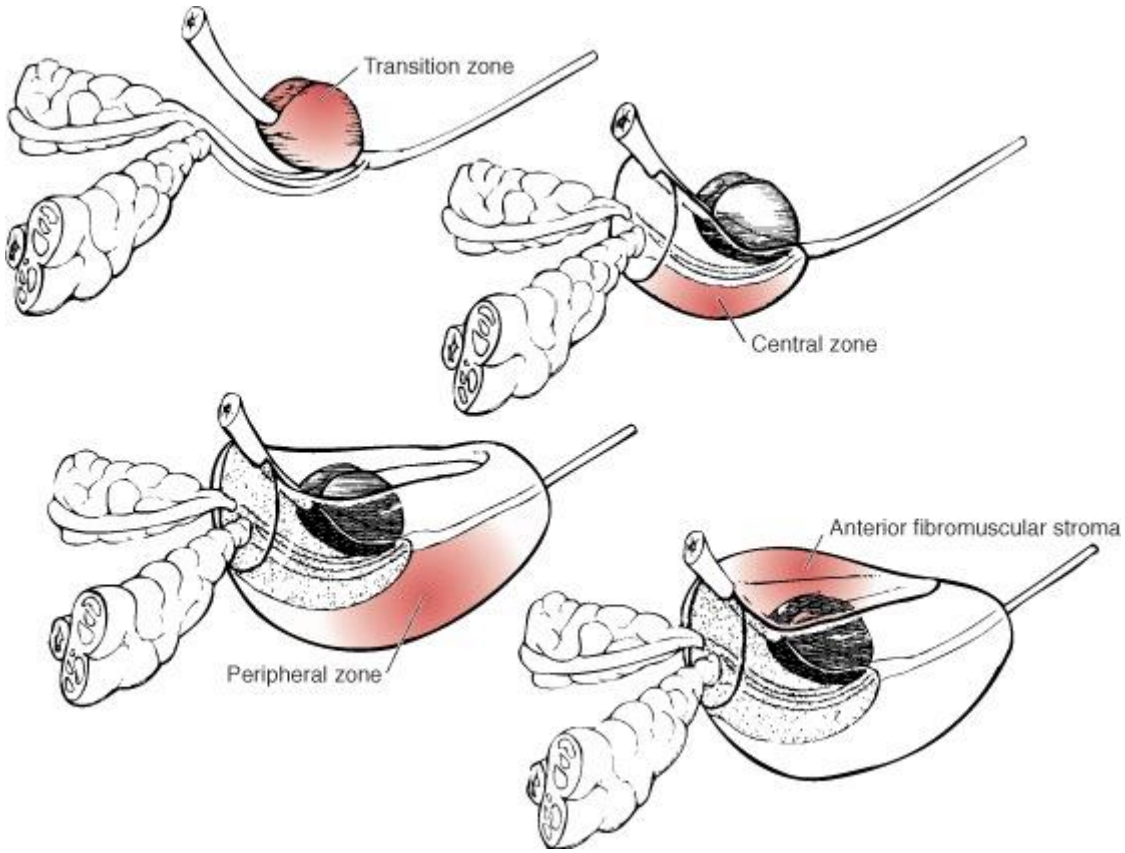


Figure 1.3: Prostate anatomy showing the four zones. Image from (Oh W.K., 2003)

Prostatic conditions are common, with risk of non-malignant conditions, such as benign prostatic hyperplasia (BPH), increasing with age (Roehrborn, 2005). The pre-malignant condition, prostatic intraepithelial neoplasia (PIN) involves aberrant, non-invasive proliferation and may be determined as a precursor to malignant disease (Bostwick and Qian, 2004). The development of PrCa is an event most commonly associated with older males (>60) and is viewed as a multi-focal, heterogeneous disease state (Shen and Abate-Shen, 2010). The development of PrCa (Figure 1.4) occurs through interplay between differentiated cells, loss of cell programmed death, oncogene activation and the cell environment (Taichman et al., 2007). Lifestyle factors (e.g. dietary elements), inflammation and somatic mutations, (e.g. phosphatase and tensin homologue (PTEN) loss), are all risk factors associated with PrCa (Nelson et al., 2003). DNA repair defects have been identified in 20-30% of advanced PrCa cases, suggesting a prominent role in metastatic disease and a potential use as a therapeutic target (Mateo et al., 2017). Multiple different types of PrCa have been determined based on the cells of origin, including transitional cell, squamous cell and neuroendocrine PrCa, with adenocarcinomas presenting as the most common type (CancerResearchUK, 2018). Due to different histologic patterns within PrCa tumours, a grading system called the Gleason grade works on the basis of adding the score of the two most common histologic tumour patterns, with a maximum score of Gleason grade 10 (Gordetsky and Epstein, 2016). To improve accurate assessment of the clinical significance of disease, amendments to the Gleason grading system were recently recommended. The new five tier system eliminates scoring of 2-5 Gleason grades, and assigns a grade 1 to previously scored Gleason grade 6. These recommendations have been incorporated into the European Association of Urology guidelines (De Nunzio et al., 2018).

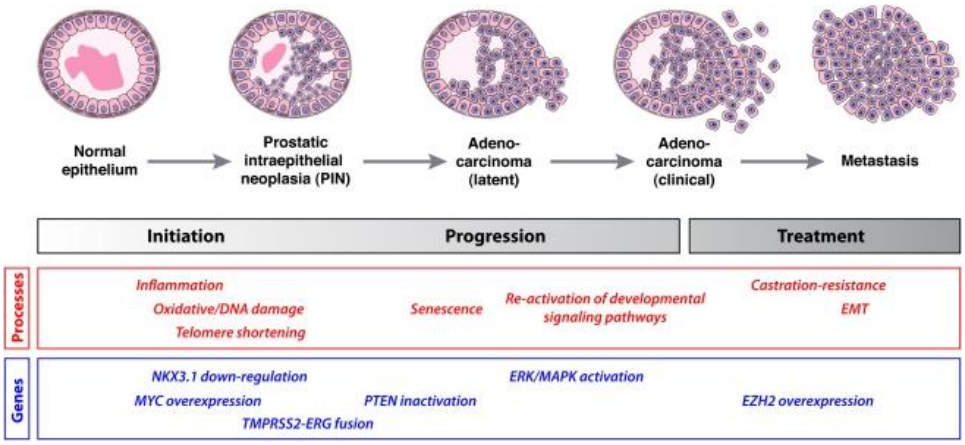


Figure 1.4: Stages of prostate cancer progression. Image taken from Shen and Abate Shen, 2010.

EMT: epithelial mesenchymal transition, NKX3: homeobox protein Nkx-3, PTEN: phosphatase and tensin homolog, ERK: extracellular receptor kinase, MAPK: mitogen-activated protein kinase, MYC: myeloctomatosis oncogene cellular homolog, EZH2: enhancer of zeste homolog 2, TMPRSS2-ERG: transmembrane protease serine 2 ETS-related gene fusion.

1.1.3 PSA and screening outcomes

Continuing research in the area of PrCa derives the need for a prostate specific biomarker suitable for the detection and monitoring of PrCa. PSA was approved in the early 1990s and subsequent screening programmes have witnessed an increased incidence with a concomitant decrease in patients presenting with metastatic disease (Lilja et al., 2008). PSA is a serine protease which, when present in the bloodstream, can exist in multiple forms. Factors such as inflammation within the prostate can cause a change from a normal state to a diseased state, allowing elevated levels of PSA to disseminate into the blood (Greene et al., 2013). Increased PSA levels in the blood are found in men with PrCa, but can also be attributed to other non-malignant prostate conditions such as BPH (Lilja et al., 2008). Patients with early stage PrCa can present as asymptomatic, thus PSA screening may allow for early prognosis and disease monitoring (Cabarkapa et al., 2016). PSA screening detects

many cases of clinically insignificant PrCa, posing the threat of over treatment for indolent disease (Chen et al., 2016). Separating clinically significant disease that may progress to advanced disease from indolent, poses a significant clinical challenge (Moschini et al., 2017). Active surveillance, consisting of regular PSA screening, biopsy and digital rectal exams for patients presenting with low risk PrCa (defined as Gleason score ≤6) may help to combat the overtreatment of indolent disease (Klotz, 2005).

1.1.4 Castrate-resistant/lethal prostate cancer

For primary, localised PrCa survival rates are high, with surgery for radical prostatectomy or radiation treatment the most common treatment choices (NCI, 2011). Androgen deprivation therapy (ADT), an anti-hormone therapy suppressing androgen synthesis, has become the mainstay of treatment for progressive PrCa. ADT encompasses anti-androgen drugs (e.g. Bicalutamide (Casodex)), luteinising hormone releasing hormone (LHRH) agonists that decrease levels of testosterone (e.g. Leuprolide) and LHRH antagonists (e.g. Degarelix) (ACS, 2018). Androgen receptor (AR) is a transcription factor associated with angiogenesis and proliferation in PrCa (Culig, 2014b), with PrCa cells relying on AR for prolonged survival and cancer dissemination (Lonergan and Tindall, 2011) (Figure 1.5). Testosterone, the most common androgen, is transformed into dihydrotestosterone, which is a more active hormone capable of increased AR binding (Feldman and Feldman, 2001). ADT inhibits androgen synthesis leading to decreased levels of serum testosterone (Yang et al., 2016). ADT is initially successful in the majority of patients, promoting cancer regression for a median of several months. However, almost all patients become resistant to ADT and are deemed castrate-resistant (Nakazawa et al., 2017, Watson et al., 2015). Castrate resistant prostate cancer (CRPC) is a lethal form of PrCa during which cancer cells can adapt to a low androgen status and continue proliferation. Disease progression can present as a biochemical recurrence (BCR) and/or the presence of distal metastases (Nakazawa et al., 2017). BCR is defined as a rise in PSA level post localised

treatment and may be viewed as a precursor to disease recurrence and progression (Paller and Antonarakis, 2013). Docetaxel was the leading treatment of CRPC until the emergence of new AR therapies such as abiraterone and enzalutamide (van Soest et al., 2015). These second-generation AR therapies target different pathways as well as preventing AR binding and AR translocation to the nucleus (Rodriguez-Vida et al., 2015). However, the eventual therapeutic resistance to these second-generation therapies (Nakazawa et al., 2017) demonstrates a need for continued research into treatment options for CRPC and metastatic PrCa.

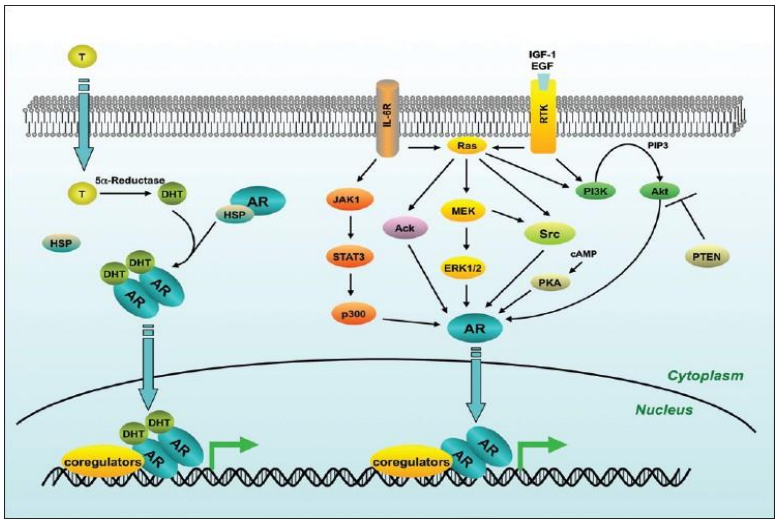


Figure 1.5: Overview of major AR signalling pathways in PrCa. AR binds to dihydrotestosterone and translocates to the nucleus. Upon translocation, AR binds to target genes and begins to regulate gene expression. Image taken from Lonergan and Tindall, 2011.

AR: androgen receptor, T: testosterone, DHT: dihydrotestosterone, HSP: heat shock protein, IL-6R: interleukin 6 receptor, IGF-1: insulin growth factor, EGF: epidermal growth factor, JAK1: janus kinase 1, STAT3: signal transducer and activator of transcription 3, p300: histone acetyltransferase p300, Ack: acetate kinase, MEK: mitogen activated kinase, ERK1/2: extracellular receptor kinase, RTK: receptor tyrosine kinase, Src: steroid receptor co-activator, PKA: protein kinase A, cAMP: cyclic adenosine monophosphate, PI3K: phophoinositide 3-kinase, PIP3: phosphatidylinositol (3,4,5)-trisphosphate, AKT- protein kinase b, PTEN: phosphatase and tensin homolog.

1.1.5 Biomarkers and molecular characterisation of prostate cancer

A biomarker can be defined as a measurement that when evaluated against a normal range may hold prognostic, predictive and diagnostic potential (Armstrong, 2012, Cary and Cooperberg, 2013). The most commonly used biomarker in PrCa, PSA, has been associated with a lack of specificity leading to over-treatment of clinically insignificant disease (Cary and Cooperberg, 2013). A PSA level of >0.4 ng/mL is generally accepted as an indicator for further investigation, however increased levels can be present in non-malignant conditions (Saini, 2016). Levels of overtreatment vary widely, with a systematic review highlighting the range of over diagnosis as being between 1-67% (Loeb et al., 2014). Based on these findings, PSA screening may be especially relevant for monitoring disease progression and recurrence (Saini, 2016). A key challenge in PrCa research is the development of novel PrCa specific biomarkers. In CRPC, biomarkers, such as epigenetic modifications and gene alterations, hold the potential to be used effectively to personalise targeted therapy in terms of course and duration of treatment (Armstrong, 2012).

1.1.5.1 Epigenetic

Epigenetic modifications are changes within the structure of DNA or chromatin, which do not alter the original DNA sequence (Chiam et al., 2014). Two main modifications, DNA methylation and histone modifications can alter gene expression, which may hold relevance for patients with metastatic CRPC. Post translational histone modifications can include methylation, phosphorylation and acetylation, with over 60 modification sites documented within histones (McAnena et al., 2017). In AR dependent LNCaP cells, histone acetylation and other histone modifications are shown to activate the PSA gene (Kang et al., 2004), while demethylases were shown to encourage AR dependent gene transcription (Wissmann et al., 2007). In tissue studies of patients with metastatic PrCa, a potentially predictive DNA methylation signature was identified for the first time (Friedlander et al., 2012). A hypermethylated epigenetic signature found in mPrCa was notably different to benign

tissue. Furthermore, mixed methylation signatures were determined in a subset of genes, such as genes linked with AR repression (Friedlander et al., 2012). These findings outline the potential role epigenetic markers could play in PrCa disease outcomes.

1.1.5.2 Gene alterations

Loss of PTEN has been extensively documented as one of the most frequent gene alterations in PrCa and is associated with poorer prognosis; with loss of expression present in >50% of metastatic PrCa cases (Murphy et al., 2016). The prevalence of PTEN loss increases with disease progression, making it a significant candidate as a PrCa biomarker (McCall et al., 2008). The phosphatidylinositide 3-kinase (PI3K) pathway (Figure 1.6) promotes phosphorylation of phosphatidylinositols (PIPs), which results in the recruitment of Akt. Akt mediates cell survival and promotes proliferation. PTEN can regulate the PI3K pathway, with PTEN loss resulting in over expression of Akt, causing a loss of apoptotic behaviour and increased proliferation (Phin et al., 2013, McCall et al., 2008). Thus, PTEN loss is closely linked with disease progression and metastasis (Noh et al., 2016).

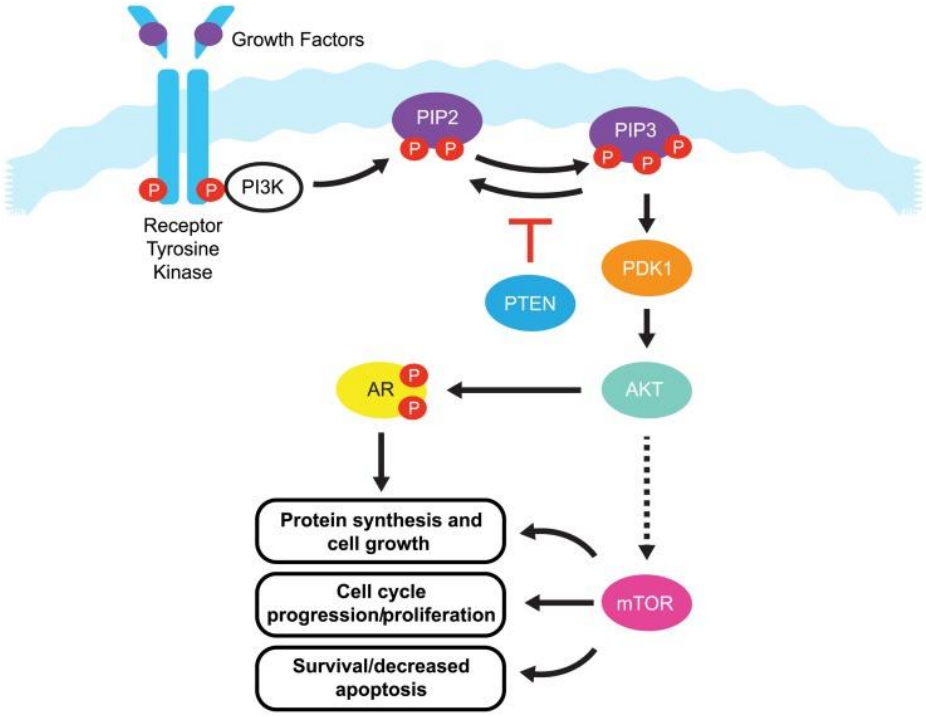


Figure 1.6: Overview of the PIK3/PTEN/Akt pathway. Growth factors bind to receptor tyrosine kinase and activates the receptor complex which in turns activates PI3K. PI3K converts PIP2 to PIP3 and mediates the phosphorylation of Akt. PTEN acts as a tumour suppressor that regulates the pathway, converting PIP3 back to PIP2. Image taken from (Phin et al., 2013).

PIP2: phosphatidylinositol 4,5-bisphosphate, PI3K: phosphoinositide 3-kinase, PTEN: phosphatase and tensin homolog, PDK1: phosphoinositide-dependent kinase 1, AR: androgen receptor, mTOR: mammalian target of rapamycin.

Recurrent gene fusions are one of the most common genetic alterations in cancer. The ETS family of transcription factors are associated with multiple biological processes including angiogenesis, metastasis and proliferation (Gasi Tandefelt et al., 2014). Members of the E26 transformation specific (ETS) family include ETS variant 1 (ETV1) and ETS-related Gene (ERG), both of which are altered in PrCa cases (Rubin, 2012). Rearrangements in ETV1 are expressed in 5-10% of PrCa and mutual exclusivity between ETS family rearrangments has been documented (Gasi Tandefelt et al., 2014). The TMPRSS2-ERG rearrangement is estimated to be present in >50% of all PrCa cases (Wang et al., 2017). TMPRSS2-ERG has also been linked to more aggressive, metastatic subtypes of PrCa (Kumar-Sinha et al., 2008). The use of TMPRSS2-ERG as a prognostic biomarker in PrCa has not been fully realised, however, presence of the gene fusion is strongly correlated with a more aggressive type of disease (Hägglöf et al., 2014).

Investigation into distinct molecular subtypes of PrCa may hold the potential to identify prognostic biomarkers. Heterozygous speckle-type POZ protein (SPOP) mutations have been identified in 6-13% of PrCa cases and have demonstrated mutual exclusivity with ERG fusions or ETS gene family rearrangements (Barbieri et al., 2012). The structure of the SPOP gene is comprised of several parts, of which an N-terminal MATH domain mediates substrate binding. SPOP binding can promote substrate degradation and is involved in significant biological processes (Mani, 2014). Investigation into the SPOP wildtype (WT) determined its tumour suppressor properties, which suppress AR activity (Geng et al., 2013). SPOP mutants lack this tumour suppressor role, thus postulating a relationship between SPOP mutants and a more aggressive disease outcome (Garcia-Flores et al., 2014). This evidence highlights the need for further research into the indentification of PrCa biomarkers and the classification of distinct molecular PrCa subtypes, to provide benefits to patients with PrCa.

1.2 Circulating tumour cells

Repeated biopsy of metastatic lesions is invasive and is not common practice in a clinical setting, preventing frequent genomic analysis of metastatic tumour biology. The term 'liquid biopsy' can include blood, saliva and urine and is a non-invasive, real-time approach that may reflect the molecular profile of the metastatic environment (Di Meo et al., 2017), which in turn, may hold particular relevance in terms of disease progression and clinical outcomes. Studies have shown that tumour cells possess the ability to shed off the primary tumour, enter the bloodstream and extravasate to distant sites, forming metastatic lesions (Maheswaran and Haber, 2010). These cells are known as circulating tumour cells (CTCs) (Figure 1.7) and may hold value in monitoring cancer progression.

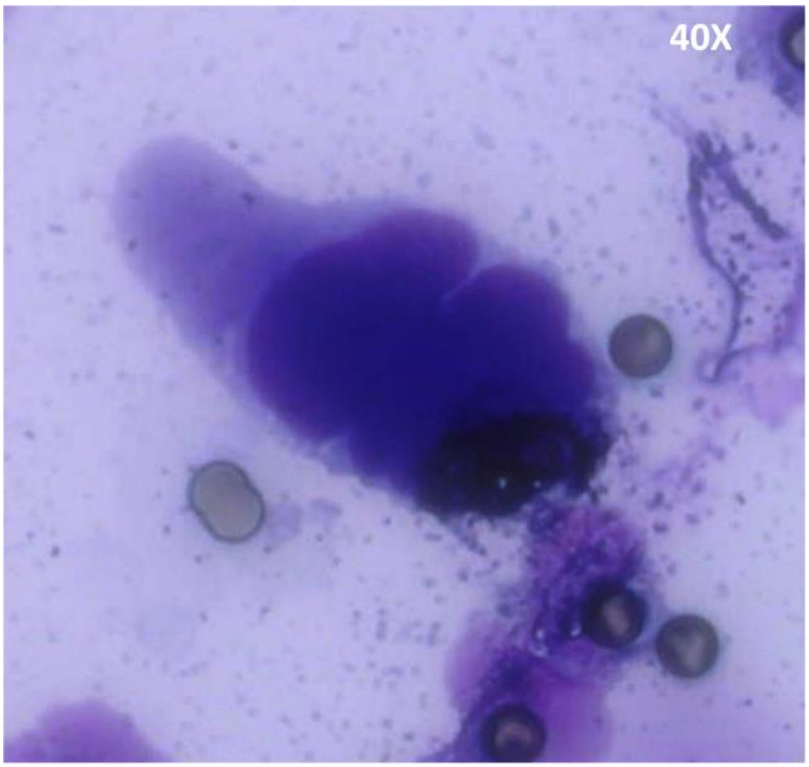


Figure 1.7: Representative image of a CTC (40X).

(Brady L et al., Manuscript in preparation)

The presence of CTCs has been extensively documented in multiple different cancer types, including breast, lung, ovarian and prostate (O'Shannessy et al., 2016, Chalfin et al., 2017). Metastasis is the most common cause of cancer related morbidity and CTCs may provide insight into tumour heterogeneity and risk of metastatic invasion (Gorges and Pantel, 2013). A majority of CTCs are detected as single cells; however, CTC clusters have been identified using cluster specific technology and are associated with poorer overall survival rate (Sarioglu et al., 2015). In addition to CTC clusters, circulating tumour microemboli (CTM), consisting of CTC aggregates which contain similar properties to CTCs have been identified circulating in the bloodstream of metastatic breast, prostate, pancreatic and non-small cell lung cancers (Cho et al., 2012). Circulating free DNA (cfDNA) has also been identified in patients with cancer. Molecular analysis of cfDNA *vs.* matched metastatic tumour tissue from patients with metastatic PrCa noted similar genomic characteristics and somatic attributes (Wyatt et al., 2017). Tumour heterogeneity may account for differences in therapy efficacy and disease recurrence (Hayes and Paoletti, 2013); hence evaluation of CTCs, CTM and cfDNA may provide insight into tumour heterogeneity and hold clinical relevance as a non-invasive method for profiling metastatic PrCa.

The method by which CTCs exit the bloodstream undetected is not well understood, with platelets postulated to play a role in aiding CTC extravasation (Figure 1.8) (Stegner et al., 2014). Platelets are small cell fragments found circulating in the peripheral blood and are primarily involved in the coagulation cascade (Stegner et al., 2014). Upon entering the bloodstream CTCs are subject to shear stress and immune surveillance and may partner with platelets to avoid detection (Massague and Obenauf, 2016). Using ovarian cancer cell lines, it has been established that platelets aggregate to CTCs and provide a protective pro-survival environment (Egan et al., 2011). P-selectin, an adhesion receptor involved in the immune response, is present in the granules of platelets. Once activated by tumour cells, P-selectin can mediate the binding of platelets to small cell lung cancer, neuroblastoma and

teratocarcinoma cells (Stone and Wagner, 1993). Natural Killer (NK) cells are members of the body's innate immune response and are integral to the detection and destruction of CTCs (Fregni et al., 2012). Platelet depleted murine models demonstrated the impact of NK cells on uncloaked tumour cells, decreasing the level of spontaneous metastases, inferring that the 'platelet cloaking' of CTCs provide a barrier to the body's immune response (Nieswandt et al., 1999). In addition to the cloaking effect of platelets on CTCs, platelets contain multiple growth factors within their alpha granules (Lubkowska et al., 2012), which may aid tumour formation. It has been demonstrated *in vivo* that transforming growth factor beta (TGFβ), when activated by platelets, significantly contributes to the extravasation process and onset of metastasis (Labelle et al., 2011). Murine models deficient in Gαq protein (essential for platelet activation) demonstrated a significant decrease in the levels of metastases, leading to the assumption that platelets aid the metastatic cascade (Palumbo et al., 2005). The role of platelets in metastatic PrCa is poorly understood; with platelets hypothesised to play a key role in both aiding CTCs evade immune surveillance and promoting metastatic spread.

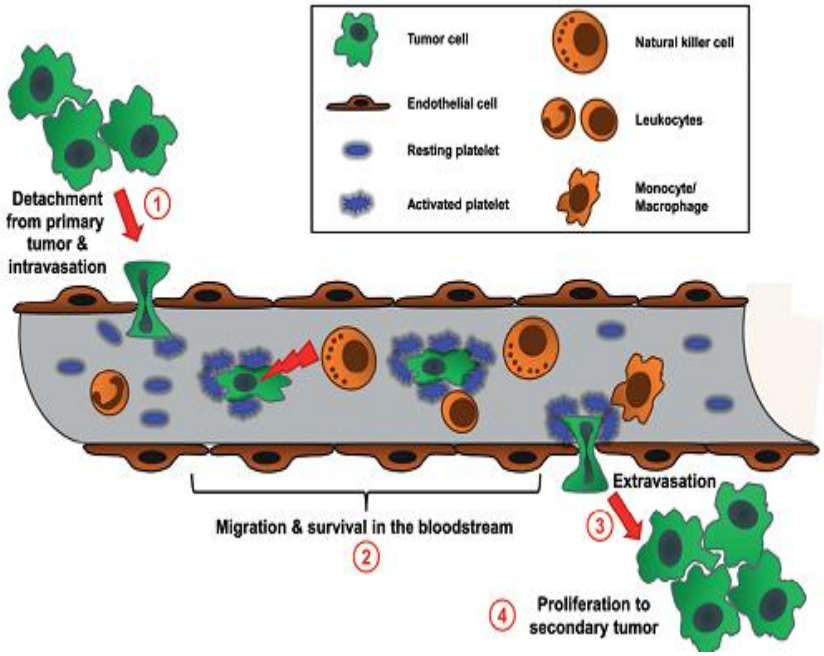


Figure 1.8: Impedance of immune surveillance by 'platelet cloaking' of CTCs.

Image taken from Stegner et al. 2014.

In spite of the proposed clinical relevance of CTCs, a 'gold standard' approach for detection of CTCs has proven problematic. Numerous platforms have been developed for the detection, isolation and enumeration of these rare cells. CellSearch® is currently the only FDA approved device for the isolation of CTCs. It centres on the use of immunomagnetic beads coated with epithelial cell adhesion molecule (EpCAM) to extract CTCs expressing this molecule from whole blood samples (Gorges and Pantel, 2013). EpCAM is a glycosylated membrane protein present on the majority of epithelial cells, operating as a cell-cell adhesion molecule (Munz et al., 2009). Previous studies with patients with malignant or metastatic PrCa disease, utilising the CellSearch® technology have demonstrated poor overall survival in those with ≥ 5 CTCs per 7.5 mL of whole blood, while < 5 CTCs per 7.5 mL, was classed as favourable (de Bono et al., 2008). Further studies using this platform in PrCa, have determined significantly higher counts in patients with metastatic disease (Thalgott et al., 2013). However, some CTCs may not

express EpCAM on their cell surface due to epithelial-mesenchymal transition (EMT) (Mikolajczyk et al., 2011) and it is therefore postulated that this methodology may not capture all CTCs (Desitter et al., 2011). Based on this evidence, size based filtration methods have been developed. ScreenCell® is a size based filtration method established on the assumption that CTCs are generally larger than other cells present in whole blood and will adhere to a polycarbonate filter membrane allowing for their segregation and enumeration (Desitter et al., 2011). While this methodology has many advantages, tumour cell diversity has been widely studied and considerable heterogeneity can exist between primary and metastatic tumours (Shah et al., 2009) resulting in a heterogeneous population of CTCs. The positive identification of CTCs after isolation, may be supported by the use of Immunohistochemical (IHC) markers such as CD45 (El-Heliebi et al., 2013). The current literature highlights the potential role of CTCs and platelet cloaking as reliable disease markers in metastatic PrCa.

1.3 Obesity

1.3.1 General overview

Obesity is a global healthcare burden, which is associated with lifestyle choices and is classified using body mass index (BMI), a height to weight ratio, with overweight characterised as a BMI ≥ 25 and obesity as a BMI ≥ 30 (WHO, 2016). BMI is considered a recognised clinical measurement for total body fat. Overweight and obesity are defined as an increased accumulation of fat, which may be detrimental to overall health. Furthermore, in 2014, the World Health Organisation (WHO) estimated that greater than 1.9 billion adults are classed as overweight globally (WHO, 2016). In Ireland in 2011, the Irish Universities Nutrition Alliance estimated that 37% of all adults were overweight, with a further 24% characterised as obese (IUNA, 2011). Using the UK Foresight Obesity Model it has been demonstrated that the rate of overweight and obese adults in Ireland will increase to 89% in males and 85% in females by 2030 (Figure 1.9) (Keaver et al., 2013). This obesity epidemic is further

underscored by overweight estimates in 9 year old Irish children, with 30% of girls and 22% of boys presenting as overweight (McCrory, 2011).

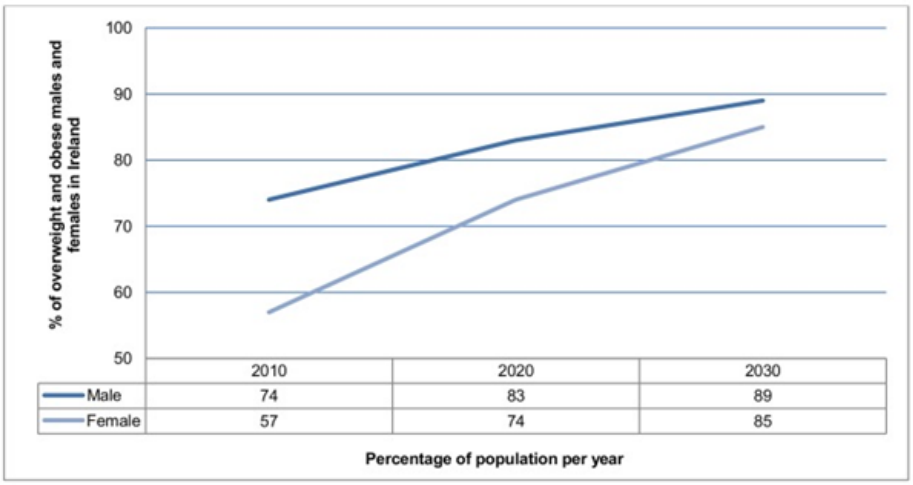


Figure 1.9: Graph outlining the projected increase in overweight and obese adults in Ireland in 2020 and 2030.
Image taken from (Keaver et al., 2013).

1.3.2 Risk factors associated with obesity

Diet and sedentary lifestyle behaviours greatly influence obesity risk (Hruby and Hu, 2015). Sedentary activities, such as sitting, greatly reduces energy expenditure (Matthews, 2008) and inactive leisure activities (e.g. prolonged television watching), greatly increase the probability of developing obesity and obesity related illnesses, when compared to light or moderate physical activity (Hu et al., 2003). Obesity is implicated as a risk factor for a number of significant health care concerns including cardiovascular disease (CVD), hypertension and diabetes (Poirier et al., 2006). CVD is considered the leading cause of mortality worldwide, with central adiposity and abnormal lipid levels classed as high risk factors for the development of CVD (Yusuf et al., 2004). Diets concentrated in saturated, monounsaturated and trans-fat heavy foods, which promote a pro-inflammatory response, may contribute to weight gain and the onset of obesity (Ramallal et al., 2017). Metabolic syndrome (MetS) is defined as low level systemic inflammation and a group of factors (Table 1.1), such as central adiposity and

hypertension, which together pose a greater risk for CVD (Emanuela et al., 2012). The risk of developing MetS is greatly increased in individuals who fall into the obese BMI range > 30 (Han et al., 2002).

Table 1.1: MetS factors

Risk Factor for MetS
Central adiposity
Hypertension
High level of triglycerides
Abnormal cholesterol
High blood sugar

Additionally, the relationship between obesity and inflammation has been extensively documented (Rodriguez-Hernandez et al., 2013). An enhanced inflammatory state has been suggested in obesity with increased levels of pro-inflammatory mediators (tissue necrosis factor (TNF)) witnessed in obese individuals (van Dielen et al., 2001). This is further strengthened by data demonstrating a reduction of TNF receptors 2 years post gastric restrictive surgery (Nijhuis et al., 2009). Increased C-reactive protein (CRP), a marker of systemic inflammation, has been associated with worse overall survival in PrCa (Xu et al., 2015). Elevated levels of CRP are correlated with a higher BMI, thus postulating that increased levels of CRP are more likely in obese individuals (Visser et al., 1999). Obesity decreases life expectancy and the likelihood of mortality from MetS associated co-morbidities (Kitahara et al., 2014).

1.3.3 Obesity and cancer

Increased body fat composition and obesity have been attributed to multiple different cancer types (AICR, 2007). In 2012, it was estimated

that 3-6% of all cancer diagnoses were associated with a high BMI, with Europe falling in the upper range of 2-9% of total cancer cases (Arnold et al., 2015). Evidence suggests that obesity, a higher BMI and waist circumference greatly increase the risk of developing certain cancer types including gastric cancer (Chen et al., 2013), liver cancer (Campbell et al., 2016) and endometrial cancer (Dougan et al., 2015).

In PrCa, while obesity and a high BMI alone may not influence overall PrCa risk, emerging research has shown a correlation between high BMI and advanced PrCa (Grotta et al., 2015). The Cancer Prevention Study II Nutrition Cohort outlined the increased possibility of obese men developing an aggressive disease subtype, while men who underwent lifestyle related weight loss decreased their probability of developing aggressive PrCa (Rodriguez et al., 2007). A meta-analysis further strengthened this hypothesis by identifying obesity as a significant risk factor for the development of high Gleason grade associated with advanced PrCa (Zhang et al., 2015). The probability of patients with MetS developing high grade tumours was 1.7 times more frequent than those men without MetS, while patients with MetS also displayed an increased susceptibility to BCR (Morote et al., 2013). This data may suggest an interplay between obesity, MetS and an aggressive PrCa disease state. The mechanisms of action associated with the obesity-cancer relationship may involve growth factors, adipokines and chronic inflammation (Ford et al., 2013).

1.3.4 Adipokines and prostate cancer

A range of adipokines, such as adiponectin, leptin and resistin, are secreted from adipose tissue, with elevated levels of some adipokines present in obese states (Figure 1.10) (Hauner, 2005). Adipose tissue can be widely dispersed around the body and the production of pro-inflammatory adipokines is a common occurrence (Nakamura et al., 2014). Adipokines play a leading role in obesity and are thought to contribute to processes such as inflammation, proliferation and apoptosis. Adiponectin, leptin and resistin are of particular interest in

PrCa as it is postulated that these adipokines play a role in the advancement of disease (Vansaun, 2013).

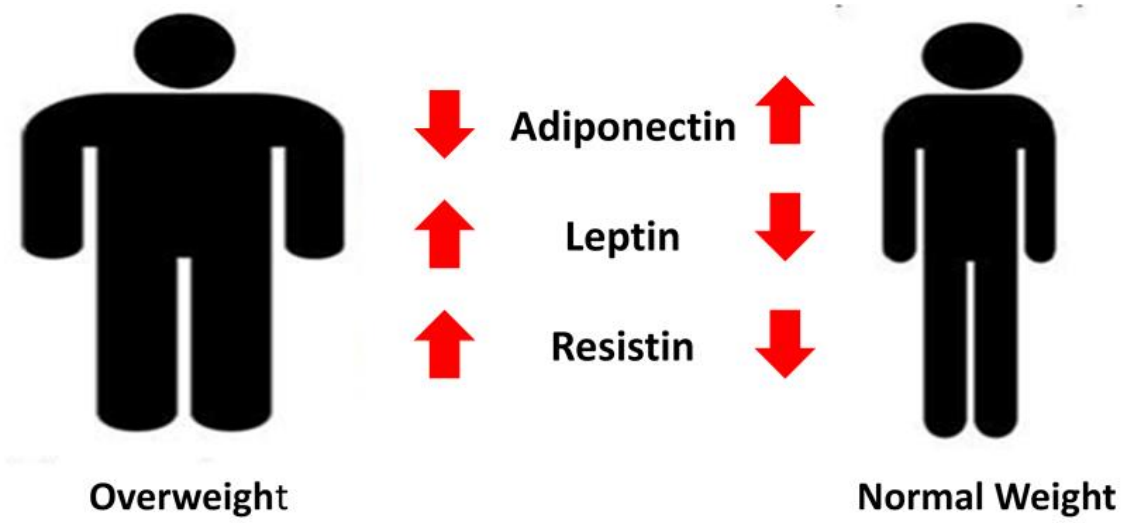


Figure 1.10: Image outlining levels of adiponectin, leptin and resistin expression in overweight and normal weight individuals.

1.3.4.1 Leptin

Leptin is an adipokine secreted by adipose tissue and was originally discovered in mouse models in 1994 as part of the obese gene mutation studies (Zhang et al., 1994). Leptin is synthesised in white adipose tissue and is a regulator of homeostasis. Leptin is also implicated in multiple cellular functions, including the regulation of food intake, energy expenditure, metabolism and immune function (Park and Ahima, 2014). Leptin is found in circulation in either its free, unbound active state or bound to the leptin receptor (LepR) (Sinha et al., 1996). Alternative splicing of the LepR gene produces a range of isoforms, however LepR acts as the predominant receptor for all cellular processes (Villanueva and Myers, 2008). Levels of leptin are linked to caloric intake, with higher levels present in obese individuals (Park and Ahima, 2014). In PrCa, a study comparing patients with BPH to patients with PrCa found that leptin expression was significantly higher in patients with PrCa (Hoon Kim et al., 2008). Furthermore, leptin may be associated with an aggressive

PrCa phenotype, with high levels significantly associated with greater Gleason grade (Di Sebastiano et al., 2017). Excess adipose tissue and levels of free leptin are further correlated with increased PrCa aggressiveness (Lopez Fontana et al., 2011). *In vitro* cell line experiments with androgen independent PrCa cell lines, PC-3 and Du145, demonstrated a dose dependent proliferative effect (Hoda et al., 2012), suggesting a relationship between high levels of leptin and advanced PrCa.

1.3.4.2 Adiponectin

Adiponectin is an adipokine found circulating in plasma in higher concentrations in non-obese individuals when compared to obese individuals (Arita et al., 1999). Adiponectin has two associated receptors with full binding affinity, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). Both receptors are known to play a role in fatty acid metabolism and can also regulate inflammatory induced NF-κB activation (Yamauchi et al., 2014). Epidemiology studies have noted reduced levels of adiponectin in CVD and diabetes, with low levels of circulating adiponectin thought to be predictive of the development of type 2 diabetes (Lihn et al., 2005). Treatment with a constructed adiponectin adenovirus in adiponectin knock-out mouse models exhibited improved fatty acid metabolism and alleviated insulin resistance (Maeda et al., 2002), demonstrating the role played by adiponectin in these pathways. Notably, adiponectin levels are approximately 40% lower in males when compared to females, based on an inhibitory effect of androgen on adiponectin (Lihn et al., 2005). An *in vitro* study using PrCa cell lines, DU145, PC-3 and LNCaP-FGC, demonstrated increased PrCa cell growth when cultivated in adipocyte conditioned medium (Bub et al., 2006). This effect was ameliorated by the introduction of adiponectin, suggesting an anti-proliferative effect of adiponectin in PrCa cells (Bub et al., 2006), which has been further demonstrated using *in vitro* PrCa cell lines models (Gao and Zheng, 2014). The US Physicians' Health Study found that men with higher levels of circulating adiponectin had a markedly reduced risk of

developing high grade PrCa when compared to those men with lower adiponectin levels (Li et al., 2010).

1.3.4.3 Resistin

Resistin is an adipokine that was originally discovered in mouse models (Steppan et al., 2001). Steppan et al. established a role for resistin in altering insulin action *in vivo*, providing an association between obesity, resistin levels and insulin resistance (Steppan et al., 2001). In mouse models, resistin was secreted from adipose tissue, however very low mRNA levels of resistin were detected in human adipose tissue (Patel et al., 2003). In humans, resistin is primarily expressed in peripheral blood mono-nuclear cells with levels increasing upon differentiation into macrophages (Park and Ahima, 2013). Investigation into resistin receptors has identified adenylyl cyclase-associated protein 1 (CAP1) as a potential candidate. CAP1 was found to bind directly to human resistin and to regulate the pro-inflammatory impact of human resistin both *in vitro* and *in vivo* (Lee et al*.,* 2014*)*. Serum levels of resistin were significantly elevated in obese individuals when compared to non-obese/lean individuals (Degawa-Yamauchi et al., 2003) and are implicated in CVD, atherosclerosis, inflammation and obesity (Filkova et al., 2009). In cancer, an association between high expression of resistin and obesity-related cancer has been established (Gong et al., 2016). The role of resistin in PrCa is not as well understood as other adipokines, however *in vitro* PrCa cell line experiments demonstrated a link between increased resistin expression and proliferation (Kim et al., 2011), postulating a role between resistin and PrCa progression.

1.4 Inflammation

1.4.1 The immune system and chronic inflammation

The body's immune system can enact a protective response, which reacts to foreign substances such as microbes, viruses and cancer cells (Warrington et al., 2011). The immune system can be divided into two main types, innate immunity and adaptive immunity. The main role of the innate immune system is to respond to infectious agents that pass

through the body's physiological barriers, such as the skin and mucous membranes. The innate immune response is comprised of multiple cell types, such as neutrophils and macrophages, who respond immediately to pathogens by igniting an inflammatory response (Turvey and Broide, 2010). Adaptive immunity is triggered by signalling received from the innate immune response, which alerts to pathogens within the host (Bonilla and Oettgen, 2010). Cells of the adaptive immune response, T-cells and B-cells, function by producing specific antigens which eradicate distinct pathogens, and by developing an immune memory to target and remove a repeat offender quickly in the event of a subsequent infection (Warrington et al., 2011). Acute inflammation is the initial response to infection such as influenza and usually resolves quickly. However, in some instances a chronic inflammatory state develops, where self-limiting ability is lost and irreparable damage can occur over time (Gabay, 2006). Examples of this include systemic autoimmune diseases such as rheumatoid arthritis (RA) (Straub and Schradin, 2016). Chronic inflammation and chronic inflammatory disorders (e.g. Crohn's disease, RA, psoriasis and ankylosing spondylitis) are linked to an increase in all-cause mortality (Dregan et al., 2017), as are elevated markers of systemic inflammation such as CRP, platelet counts and neutrophils (Proctor et al., 2015). Chronic inflammation has been linked to the development of multiple malignant disease states (Coussens and Werb, 2002).

1.4.2 Inflammation and cancer

Six traditional hallmarks of cancer have been defined as opposing apoptosis, evading growth suppressors, proliferative signalling, angiogenesis, replication and invasion and metastasis (Hanahan and Weinberg, 2011). A seventh hallmark, inflammation, has been proposed due to its ability to aid and incite multiple existing hallmarks (Figure 1.11) (Colotta et al., 2009). The concept of a relationship between inflammation and cancer was first proposed by Rodolph Virchow in 1863 (Balkwill and Mantovani, 2001), and since then it has been demonstrated in multiple cancer types, including lung cancer (Gomes et al., 2014), colorectal

cancer (Janakiram and Rao, 2014) and PrCa (Sfanos and De Marzo, 2012). The tumour microenvironment is a complex mix of inter-connected cells (tumour cells, stromal cells, immune cells) which can communicate an immune response (Mei et al., 2014). Cells of the tumour microenvironment can promote progression and aid the metastatic cascade by releasing growth factors and cytokines to stimulate invasion and proliferation (Eiró and Vizoso, 2012). Multiple mediators including cytokines, chemokines and proteases, work in harmony and may contribute to inflammation associated with cancer (Diakos et al., 2014).

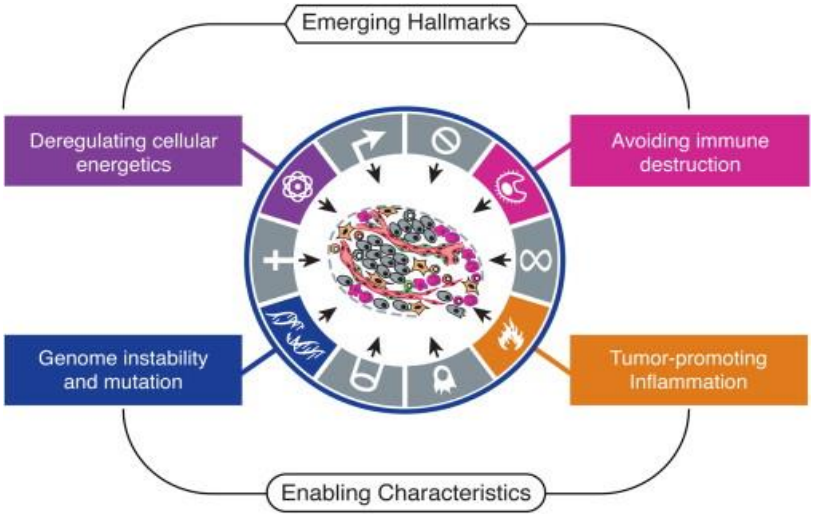


Figure 1.11: Inflammation as an emerging hallmark of cancer.

Image taken from Hanahan and Weinberg, 2011.

1.4.2.1 NFκB

Several transcription factors are thought to be involved in the tumour driven inflammatory process. One of the most extensively studied transcription factors is nuclear factor kappa B (NFκB). NFκB is a master regulator of inflammation and is comprised of trans-activator subunits Rel A and c-rel, and DNA binding subunits (Levine, 2013). NFκB can be activated by inflammatory cytokines, bacteria and viruses and can promote the production of angiogenic factors and growth signals which aid malignant growth (Karin, 2006). In addition, NFκB can promote anti-apoptotic behaviour, promote proliferation and aid tumour progression (Karin, 2009). The signal transducer and activator of transcription (STAT) family of proteins is interconnected with NFκB (Yu et al., 2009).

Both NFκB and STAT3 can operate as nuclear transcription factors which aid the regulation of inflammatory genes associated with cancer promotion and can regulate the expression of oncogenic genes (Yu et al., 2009). Transcription factors are an integral part of the inflammation and cancer relationship, both of which can be activated by inflammatory cytokines (Liu et al., 2017).

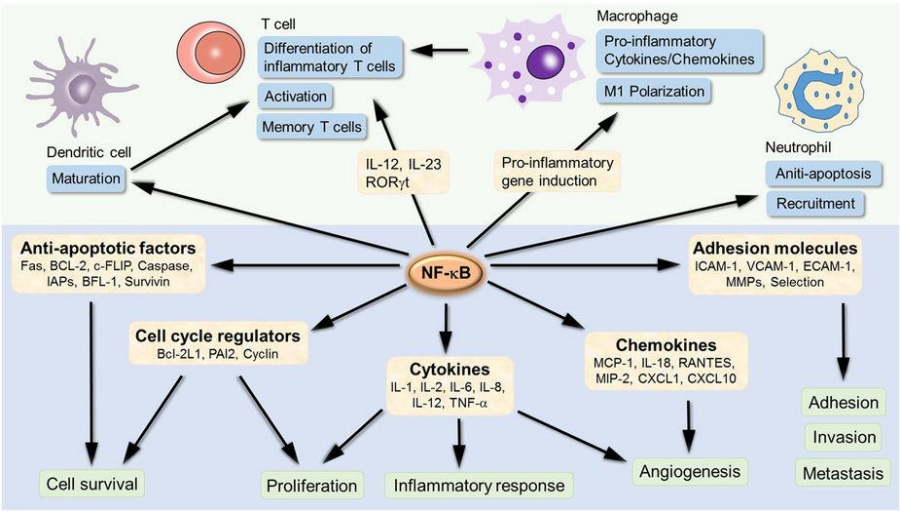


Figure 1.12: NFκB and inflammation pathways. Images taken from Liu et al. 2017.

IL – interleukin, BCL-2- B-cell lymphoma 2, IAP- inhibitor of apoptosis, c-FLIP – cellular FLICE like inhibitory protein, PAI2 – plasminogen activator inhibitor 2, TNF – tissue necrosis factor, MCP – monocyte chemoattractant protein 1, CXCL-chemokine ligand, MIP-2- macrophage inflammatory protein, ICAM-1- intracellular adhesion molecule 1, VCAM – vascular cell adhesion molecule, ECAM – epithelial cell adhesion molecule

1.4.2.2 Cytokines

Cytokines are small secreted proteins which function to regulate immune cells. Changes within the cell environment can bolster cytokine expression levels promoting both pro and anti-inflammatory responses (Dranoff, 2004). There are multiple classifications and members of the cytokine family (Table 1.2) who have varying roles within the inflammatory mediated tumour promoting environment. Prominent members of the cytokine family are interleukins (IL). ILs are small

proteins present on multiple different cell types which engage in the mediation of the innate immune response. ILs are also implicated in maintaining homeostatic equilibrium and can be classified as pro/anti-inflammatory (Boraschi et al., 2011). Various ILs have been studied for their role in cancer progression and prominent IL family member IL-6 has garnered much attention. Over-expression of IL-6 has been linked to multiple different cancer types, including prostate and lung cancer (Chang et al., 2013, Adler et al., 1999). IL-6 has demonstrated inhibitory effects on apoptosis in myeloma cancer cells (Jourdan et al., 2003) and STAT3 activation of IL-6 plays a potential role in tumourigenesis (Grivennikov et al., 2009). Another member of the cytokine family, tissue necrosis factor (TNF) is implicated in cancer progression (Balkwill, 2009). TNFα plays an essential role in immunity, and deregulation can contribute to chronic inflammation and septic shock. Modification in TNFα levels is further associated with chronic disorders such as RA and Crohn's disease (Balkwill, 2006). Elevated levels of both TNFα and IL-6 have been witnessed in patients with PrCa and cachexia (Pfitzenmaier et al., 2003). In patients with chronic lymphocytic leukaemia, TNFα serum levels were significantly higher when compared to a healthy population (Ferrajoli et al., 2002). Cytokine family growth factors like vascular endothelial growth factor (VEGF) are also implicated in cancer development. VEGF based murine models indicated a role for VEGF in organ specific tumour proliferation and the attachment of CTCs (Kaplan et al., 2005).

1.4.2.3 Chemokines

Chemokines are cytokines which respond to chemical stimulus, and encourage leukocyte migration. Production of chemokines is promoted by inflammatory cytokines and growth factors, and chemokines can coordinate targeted gene transcription and immune cell movement. Chemokines are characterised into four distinct groups, dependant on the location of the first two cysteine residues, C CC, CXC and CX3C (Balkwill, 2004). Numerous chemokines have been implicated in all stages of tumour development, and can be produced by cells during

metastatic spread (Lazennec and Richmond, 2010). The presence of CCL5 (also known as Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES)), has been detected in multiple solid tumour types including breast and prostate cancer (Aldinucci and Colombatti, 2014). CCL5 promotes growth and survival of PrCa cell lines and is present in human tissue samples (Vaday et al., 2006); in mouse models implanted with breast cancer cell lines, CCL5 was upregulated in tumour infiltrating T-cells (Owen et al., 2011).

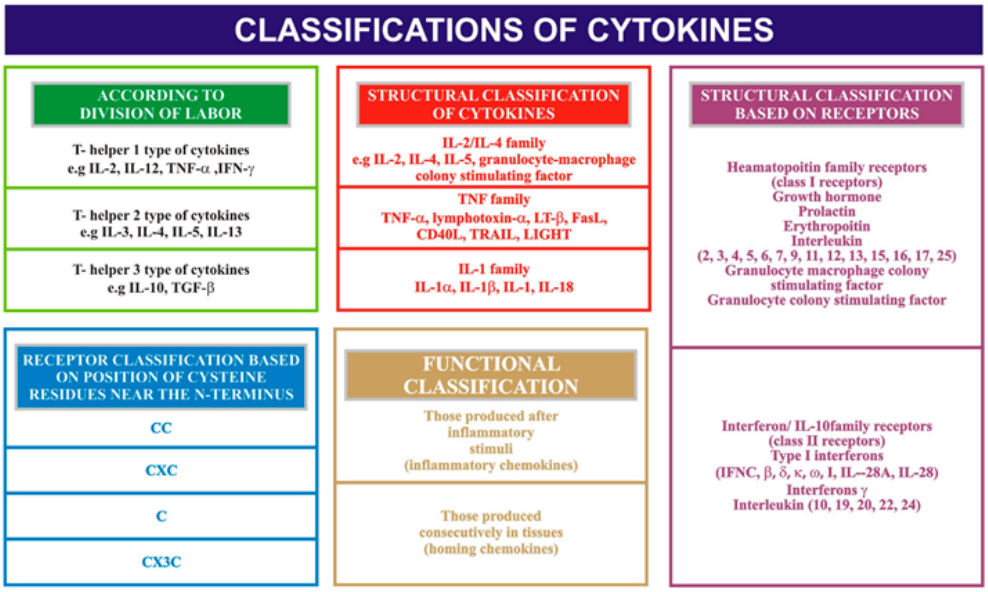


Table 1.2: Classification of the different members of the cytokine family of small proteins associated with an immune response. Adapted from Sahibzada et al. (Sahibzada et al., 2017)

TNF: tissue necrosis factor, CC, CXC: conserved cysteine residues and cytokines, IL: interleukin, TRAIL: TNF-related apoptosis inducing ligand, LIGHT: homologous to lymphotoxin, exhibits inducible expression and completes with HSV glycoprotein D for binding to Herpesvirus entry mediator, a receptor expressed on T-lymphocytes, IFN: interferon.

1.4.2.4 Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of endopeptidases with functions in wound healing, proteolytic degradation and inflammation, as

well as being implicated in vascular disorders and carcinogenesis. An extensive body of evidence outlines a role for MMPs in invasion, migration and proliferation, as well as immune surveillance (Gialeli et al., 2011). MMP2 and MMP9 are significant MMPs that exhibit proteolytic degradation by breaking down collagen and fibrinogen, key components of the extracellular matrix (Roomi et al., 2009). Furthermore, MMP9 is a poor prognostic indicator, which is strongly correlated with metastatic breast cancer (Mehner et al., 2014).

1.4.3 Inflammation and prostate cancer

Inflammation plays a role in the prostate in the non-cancerous form of prostatitis (Krieger et al., 2008). Prostatitis can be categorised into acute/chronic bacterial prostatitis, chronic pelvic pain syndrome and asymptomatic inflammation, and is thought to be prevalent in approximately 8.2% of men (Krieger et al., 2008). In addition to prostatitis, a substantial body of evidence exists documenting the relationship between inflammation and PrCa development (De Marzo et al., 2007). IL-6 expression levels can be used as a prognostic indicator of the likelihood of developing BCR in men with PrCa post radical prostatectomy (Alcover et al., 2010). Research has also shown tumorigenic and malignant provoking properties of IL-6 (Rojas et al., 2011). In addition to primary PrCa, it is widely accepted that inflammation can aid the metastatic cascade by promoting tumour cell progression (Wu and Zhou, 2009). The bone microenvironment is also thought to harbour many of the inflammatory mediators associated with cancer progression and hence may aid cancer metastasis (Roca and McCauley, 2015). Bone metastases are the most common site of metastatic spread in PrCa (88.9%) when compared to visceral metastases (16.7%) and lymph metastases (22.2%) (Roberts et al., 2013). VEGF is expressed in PrCa and is correlated with increasing grade and metastatic potential. When compared to the primary tumour site, VEGF expression levels are increased at locations of bone metastases, hypothesising that VEGF may have a leading role in the PrCa metastatic cascade (Chen et al., 2004). CXCL8 (IL-8) is a significant chemokine involved in inflammation. It has

long been understood that CXCL8 is expressed by PrCa cells and expression levels are linked with metastatic potential (Greene et al., 1997). An *in vivo* PrCa cell line study indicated a role for CXCL8 in neovascularisation, angiogenesis and resulting metastatic spread. The group also defined a relationship between CXCL8 and MMP9, with CXCL8 regulating MMP9 expression (Inoue et al., 2000). This compelling peer reviewed research furthers the understanding of the relationship between inflammation and PrCa, and paves the way for potential anti-inflammatory related treatment strategies for patients with PrCa.

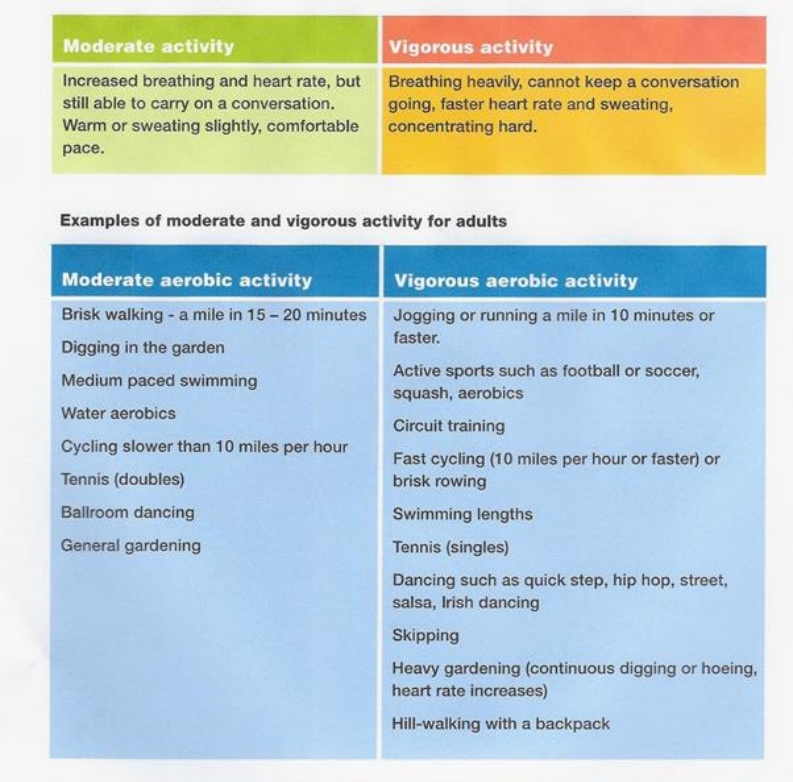
1.5 Physical activity

1.5.1 Health benefits of physical activity

The effects of physical activity (PA) have long been associated with positive health benefits, including quality of life (QoL) in cancer survivorship and lower risk of certain cancer types (NIH, 2018b). The National Guidelines on Physical Activity for Ireland state that young people (2-18 years) should engage in moderate to vigorous exercise for at least 60 min per day, while adults (>18 years) are recommended to engage in at least 30 min of moderate activity (Figure 1.12) 5 days per week (NPAGSG, 2009). The guidelines state that exercise in adults can reduce the risk of early death, high blood pressure, coronary heart disease and stroke and can improve muscular and cardio-respiratory fitness (NPAGSG, 2009). These guidelines are in accordance with the WHO's global recommendations, which also state that a further 150 min of moderate intensity exercise per week can provide additional health benefits (WHO, 2011). This infers that QoL can be greatly impacted by PA. A randomised intervention trial, comprising of aerobic and strength based exercise groups, demonstrated an improvement in health related QoL post exercise (Awick et al., 2015). Dissatisfaction in adults has also been strongly correlated with physical inactivity, suggesting a role for PA to improve general wellbeing (Strine et al., 2008). In addition to physical health, mental health has also been linked to PA. A recent meta-analysis established that exercise leads to a reduction in anxiety levels (Ensari et al., 2015), while a randomised trial indicated that multiple forms of

exercise (stretching, dance, walking) had a positive impact on reducing psychological distress (Awick et al., 2017).

The biological effects of PA have also been documented. Levels of fibrinogen post-PA are reduced, postulating that PA may reduce the levels of platelet co-factors and platelet aggregation in the bloodstream of adult subjects (Wannamethee et al., 2002). Hence PA may have a role to play in the reduction of platelet cloaking of CTCs. A study of healthy adults implicated that frequent PA may reduce levels of pro-inflammatory mediators and CRP, thus reducing the risk of systemic inflammation (Abramson and Vaccarino, 2002). Combined resistance and aerobic exercise reduced levels of TNF in obese subjects post 12 weeks of intervention, when compared to baseline levels (Ho et al., 2013). Expression of resistin and leptin are decreased post high-intensity exercise in participants with type-2 diabetes and MetS, with adiponectin levels showing an increased expression (Balducci et al., 2010). These studies, amongst others, lead to the hypothesis that PA may ameliorate underlying inflammatory symptoms in certain disease states.



Moderate activity	Vigorous activity
Increased breathing and heart rate, but still able to carry on a conversation. Warm or sweating slightly, comfortable pace.	Breathing heavily, cannot keep a conversation going, faster heart rate and sweating, concentrating hard.

Examples of moderate and vigorous activity for adults

Moderate aerobic activity	Vigorous aerobic activity
Brisk walking - a mile in 15 – 20 minutes	Jogging or running a mile in 10 minutes or faster.
Digging in the garden	Active sports such as football or soccer, squash, aerobics
Medium paced swimming	Circuit training
Water aerobics	Fast cycling (10 miles per hour or faster) or brisk rowing
Cycling slower than 10 miles per hour	Swimming lengths
Tennis (doubles)	Tennis (singles)
Ballroom dancing	Dancing such as quick step, hip hop, street, salsa, Irish dancing
General gardening	Skipping
	Heavy gardening (continuous digging or hoeing, heart rate increases)
	Hill-walking with a backpack

Figure 1.13: Definitions of moderate and vigorous activity with accompanying examples. Adapted from The National Guidelines on Physical Activity for Ireland (NPAGSG, 2009).

1.5.2. Physical activity and cancer

PA and structured exercise have long been proposed as possible interventions for patients with cancer, when preparing for, and recovering from treatment (Courneya, 2017). Aerobic exercise, resistance training and flexibility exercises, focusing on areas with limited range of movement, for patients with various cancer types, including breast, prostate and lung, may help improve physical function (Noble et al., 2012). Intense Exercise for Survival among Men with Metastatic Castrate-Resistant Prostate Cancer (INTERVAL-GAP4) is a multicentre randomised clinical trial that is incorporating aerobic and resistance exercise interventions for patients with metastatic PrCa. The primary endpoint of this study aims to improve OS in this patient population (Newton et al., 2018). Cancer related fatigue is a common side effect

associated with most aspects of a cancer diagnosis and can have a negative impact on QoL. Tailored PA programmes may present an opportunity to improve QoL (Wolvers et al., 2017). Prolonged adherence to structured aerobic exercise intervention programmes can reduce levels of cancer related fatigue and this has been demonstrated in different cancer types including breast and nasopharyngeal (Tian et al., 2016). Another debilitating side effect for patients with cancer is poor sleep quality. Results from the CARE trial, a randomised trial for women with breast cancer undergoing chemotherapy, determined that higher levels of aerobic exercise, when compared to a standard level, improved sleep quality (Courneya et al., 2014). This data establishes a possible role for PA in the management of cancer related symptoms. As previously discussed, PA can have a positive impact on mental health. Although the rates of depression vary between patients with cancer, it can greatly impact QoL (Craft et al., 2012). A meta-analysis by Craft *et al*, indicated that the side effects of depression may be reduced with exercise interventions in patients with cancer (Craft et al., 2012).

It has been extensively documented that patients with cancer, in particular those patients presenting with advanced disease, have an increased likelihood of systemic inflammation which may lead to an increased risk of mortality (Shinko et al., 2017). In patients with leukaemia, post-exercise intervention, a decrease in pro-inflammatory cytokine IL-6 and an increase in anti-inflammatory cytokine IL-10 was witnessed (Battaglini et al., 2009). Hypoxic exercise regimes have demonstrated an ability to increase immune function and NK cell number in healthy males (Wang and Weng, 2011), thus leading to the possibility of replicating this finding in a cancer population. Further research into the inflammatory status in patients with cancer post-exercise may provide valuable insight into the mechanisms and pathways by which PA impacts biological functions.

1.5.3 Physical activity and prostate cancer

The Health Professionals Follow Up Study (HPFS) began in 1986 and is comprised of 51,529 health professionals who are followed prospectively

through a series of questionnaires examining men's health (HPFS, 2016). A sub-study examining a cohort of men with PrCa from the HPFS, found that moderate PA was attributed to prolonged overall survival in men with PrCa, and vigorous activity was associated with PrCa specific survival (Kenfield et al., 2011). An 11 week walking intervention for men with PrCa resulted in an improvement in cardiovascular health, and provided a basis for the feasibility and effectiveness of this type of exercise intervention (Pernar et al., 2017). This data suggests PA to be an effective, non-invasive method for improving QoL in men with PrCa. As outlined previously in section 1.1.4, the mainstay of treatment of PrCa is ADT. The side effects of prolonged ADT treatment can include increased fat mass, decline in sexual function and psychological distress, all of which contribute to decreased QoL for patients with PrCa (Cormie et al., 2015). Improvements in the loss of lean mass, increased muscular endurance and improved balance have all been witnessed post exercise intervention in patients with PrCa (Galvao et al., 2010). Additionally, exercise intervention in the form of moderate to high intensity aerobic and resistance programmes can improve treatment toxicity from ADT (Cormie et al., 2015).

Cancer related fatigue is anticipated to affect approximately 40% of men with PrCa undergoing ADT (Storey et al., 2012). A range of different exercise methods including impact loading, resistance training and aerobic exercise, have been shown to reduce fatigue levels (Taaffe et al., 2017). A recent meta-analysis examining adverse events resulting from exercise interventions for men with PrCa, found the frequency of such events was insignificant and in line with levels in control populations (Bourke et al., 2016). This strengthens the hypothesis of PA as a safe intervention strategy for men with PrCa (Cormie et al., 2013). In patients with metastatic PrCa, 80% will experience bone metastasis (Small et al., 2003). A systematic review of PA and exercise interventions for men with advanced or metastatic PrCa, outlined the low number of studies recruiting for this population and the avoidable exclusion of patients with bone metastasis (Sheill et al., 2018 under review, Journal of Palliative and Supportive Care). Research has indicated that patients with

metastatic PrCa, and more specifically bone metastases, can participate effectively and safely in correctly monitored and prescribed exercise programmes, which also improve overall physical wellbeing (Cormie et al., 2013). An aerobic exercise intervention for this population of patients, demonstrated improved physical health post-exercise (Zopf et al., 2016). The lack of PA and exercise intervention programmes for men with metastatic PrCa highlights the need for continuing larger scale research in this area.

1.6 The ExPeCT Trial

With increasing evidence demonstrating the benefits of physical activity in patients with PrCa, the need for a randomised intervention presents itself. Smaller scale studies have shown the benefits of aerobic exercise in metastatic PrCa patients with improvements in overall physical wellbeing (Pernar et al., 2017). The ExPeCT Trial (Exercise, Circulating Tumour Cells and Prostate Cancer, CTrial-IE 15-21, ClincalTrials.gov identifier NCT02453139) was a multi-centre randomised clinical trial for men with metastatic PrCa. ExPeCT received ethical approval from five different Irish hospitals (St James's Hospital, Mater Misericordiae Hospital, Adelaide and Meath National Children's Hospital (AMNCH), Beaumont Hospital and St Luke's Hospital), and one United Kingdom site, (Guy's and St Thomas'). Accrual to ExPeCT commenced in November 2014 and closed in all sites in April 2017 (n=67). The primary endpoint of ExPeCT was to determine CTCs and platelet cloaking in ExPeCT participants, observing any impact of an exercise intervention over time. ExPeCT was comprised of multiple secondary endpoints, including quality of life assessment and subjective questionnaire, measurement of systemic and localised tumour inflammation and coagulability and expression of a lethality associated gene expression signature in participant NCBs.

ExPeCT originally aimed to recruit 200 participants over the lifetime of the study. Study power was calculated utilising data from a previous study of ovarian cancer cell lines which showed approximately 2% platelet adhesion (Egan et al., 2011). A previous study of PrCa CTCs

(Allard et al., 2004) showed a mean of 75 CTCs with a standard deviation of 333. If the SD of platelet adhesion in PrCa CTCs is proportionate to ovarian cancer cell lines (6.66%), then it was expected that a difference of 2.65% with 100 participants in both exposed and non-exposed groups would be detectable, as determined by independent t-testing. A standard deviation (SD) varying from 2% to 10% aimed to enable detection of a difference in platelet cloaking of between 0.79% and 3.9%. It should be noted that the Allard study quoted above used the CellSearch® system, which relies upon EpCAM expression and which can count only single CTCs and not microemboli or clumps. Greater numbers of CTCs were expected using the more sensitive ScreenCell® kits. Based on the assumptions outlined, it was projected that it would be possible to detect a change of 1.8% platelet cloaking between any two time points in the 100 participants in each the exercise and the control groups, determined by paired t-testing. A SD varying from 2% to 10% would enable a detectable difference of platelet cloaking of between 0.56% and 2.8%.

Inclusion criteria for ExPeCT was as follows, written informed consent obtained before any study-related procedures, age ≥ 18 years and male, histologically confirmed diagnosis of prostate adenocarcinoma, M1 metastatic disease as confirmed by CT/MRI or by bone scan, excluding patients who only have nodal metastatic disease, stable medical condition, including the absence of acute exacerbations of chronic illnesses, serious infections, or major surgery within 28 days prior to randomisation and to be capable of participating safely in the proposed exercise as assessed and signed off by a treating physician involved in ExPeCT recruitment. The exclusion criteria for ExPeCT required no history of a radical prostatectomy or no previous diagnosis of any other malignant tumour (patients with non-melanoma skin cancer or carcinoma in situ of any type are not excluded provided they have undergone complete resection).

Consented ExPeCT participants were randomised into a control arm or an exercise arm and were monitored over a six month time frame. Participants randomised to exercise attended supervised aerobic

exercise classes once weekly for the first three months, whereby overall intensity was measured using an increase in heart-rate. Exercise participants then participated in a further 3 months of home based aerobic exercise, monitored using polar heart-rate monitors. Participants randomised into the control arm, continued their standard care and were encouraged to continue their regular exercise habits. At the end of their involvement in the trial, control arm participants were offered advice on safe exercise participation. The study was run in conjunction with the Department of Physiotherapy (Trinity College Dublin) and exercise classes were supervised by a chartered physiotherapist (Grainne Sheill). At three different time points, baseline (T0), three months (T3) and six months (T6), whole blood, serum and plasma were taken from the participants and analysed for a series of systemic inflammatory markers, CTC number and platelet cloaking in accordance with the primary and secondary endpoints. Clinical history and a subjective lifestyle questionnaire were also completed at each time point.

1.7 Aims and Objectives

1.7.1 General Aims

The overall aim of this thesis is to molecularly characterise disease progression in PrCa and to determine the relationship between exercise, obesity, inflammation and metastatic PrCa.

1.7.2 Specific Objectives

The specific objectives of this thesis are:

(i) To molecularly characterise differences in patients who have reached BCR to those who have not

(ii) To compare CTCs and platelet cloaking in patients with metastatic PrCa, examining the effects of a structured exercise intervention

(iii) To compile an inflammatory and obesity related expression profile in patients with metastatic PrCa and to assess the impact of a structured exercise intervention

(iv) To assess the role of an obesity associated lethality gene signature in diagnostic biopsies and a panel of enzalutamide resistant PrCa cell lines

These objectives will broaden the understanding of the interplay between these biological states and metastatic PrCa.

Chapter 2:
Materials and Methods

2.0 Materials and Methods

2.1 Preparation and handling of materials

Reagents and chemicals used in the laboratory were of analytical grade and were handled and stored in accordance with the manufacturers' instructions. Industrial methylated spirits (IMS), ethanol (EtOH) and xylene were all purchased from the Hazardous Materials Facility, Trinity College Dublin. The Hazardous Materials Facility, Trinity College Dublin, disposed of all hazardous waste according to local protocols.

2.2 Project ethics and patient populations

FFPE tissue for the SPOP study was provided by the Irish Prostate Cancer Research Consortium (PCRC). Ethical consent was obtained from the relevant ethical committee. The PCRC is prostate cancer biobank funded by the Irish Cancer Society, consisting of FFPE material obtained from patients with localised PrCa. Additional archived FFPE tissue from localised prostate cancer cases for the SPOP study, were obtained from Orebro University Hospital. Ethical consent was obtained from the relevant ethical committee. Samples were selected based on those patients with BCR (post-prostatectomy PSA level ≥ 0.4 ng/mL, followed by a second PSA level >0.4ng/mL), patients who did not progress after surgery and patients with immediate progression after surgery (never reached an undetectable PSA). ExPeCT (Appendix I-VI) opened initially in St James's Hospital in November 2014 and ethical approval was sought from four additional Irish hospital ethics boards, including St James's Hospital and AMNCH, Beaumont Hospital, Mater Misericordiae and St Luke's Radiation Oncology network. Ethical approval was also granted in one United UK site, Guy's and St Thomas' Hospital, London. Protocol amendments, patient information leaflets and consent forms were submitted to each board and ethical approval was received for all sites. Cancer Trials Ireland provided assistance and support for ExPeCT and led initiation in AMNCH, Beaumont, St Luke's and Mater Misericordiae. All participants were assigned a unique patient identification number and de-identified patient data sheets and biological sample logs were generated for the translational aspect of the trial.

Recruitment to the ExPeCT trial ended on March 31st 2017. All samples consented at Irish sites (whole blood, serum and plasma) were processed in St James's Hospital. Filtration of CTCs, from participants consented in Guy's and St Thomas' hospital, was performed on site. Serum and plasma samples were shipped to St James's Hospital in Dublin on dry ice for analysis. All ScreenCell® filters were reviewed by an independent pathologist in Cork University Hospital.

2.3 Cell culture

Cell culture was performed aseptically in accordance with good laboratory practice in an Optimale 18 laminar flow hood (ADS Laminaire, France). The hood was exposed to UV light for approximately 30 min pre and post use. The air was allowed to circulate for 10-20 min before use. The laminar flow was sanitised with 70% (v/v) IMS in dH_2O.

2.3.1 Cell culture reagents

RPMI-1640, Minimum Essential Medium Eagle (MEM) and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Sigma Aldrich (MO, USA). F-12K Nut Mix media was obtained from Life Technologies (CA, USA). Keratinocyte serum free media (SFM) and associated supplements (2.5 µg Epidermal Growth Factor (EGF), 25 mg bovine pituitary extract) were acquired from Invitrogen (GIBCO) (BioSciences, Dublin, Ireland). All supplemental cell culture reagents, including fetal bovine serum (FBS), penicillin-streptomycin (10,000 units penicillin and 10 mg/mL streptomycin) and L-glutamine (200 mM) were attained from Sigma Aldrich. All cell culture reagents were pre-warmed in a 37°C oven for approximately 30 min prior to use.

2.3.2 Prostate cancer cell lines

All cell lines were acquired from American Type Culture Collection (ATCC) (VA, USA) (Table 2.1). 22Rv1 and LNCaP cells were maintained in RPMI-1640 media supplemented with 10% FBS and 2% penicillin-

streptomycin. DU145 cells were maintained in MEM media supplemented with 10% FBS, 2% penicillin-streptomycin and 1% L-glutamine. PC-3 cells were maintained in F-12K Nut Mix media supplemented with 10% FBS and 2% penicillin-streptomycin. VCaP cells were maintained in DMEM media with 10% FBS and 2% penicillin-streptomycin.

Table 2.1: Prostate cancer cell lines

Cell Line	Metastatic Site
PC-3	Bone
DU145	Brain
22Rv1	NA
LNCaP	Lymph node
VCaP	Vertebral metastasis

NA – not available

2.3.3 Normal cell lines

Normal cell lines were acquired from ATCC (Table 2.2). BPH-1 cells were cultured in RPMI-1640 media supplemented with 10% FBS and 2% penicillin-streptomycin. RWPE-1 and PWR-1E (immortalised with SV40 adenovirus) cells were maintained in complete keratinocyte-SFM.

Table 2.2: Normal prostate cell lines

Cell Line	Type
BPH-1	Benign prostatic hyperplasia
RWPE-1	Epithelial
PWR-1E	Epithelial

2.3.4 SGBS cell line

Simpson Golabi Behmel Syndrome (SGBS) cells were originally isolated by Professor Martin Wabitsch (Wabitsch et al., 2001). SGBS cells are a human pre-adipocyte cell line maintained in pre-adipocyte medium supplemented with 0.05 mL/mL, FBS, 0.004 mL/mL, endothelial cell growth supplement, 10 ng/mL EGF (recombinant human), 1 µg/mL hydrocortisone and 90 µg/mL heparin (PromoCell, Germany). Differentiation of the pre-adipocyte cells occurs over a 14 day period using a pre-adipocyte differentiation medium supplemented with 8 µg/mL d-Biotin, 0.5 µg/mL insulin (recombinant human), 400 ng/mL dexamethasone, 44 µg/mL 3-isobutyl-1-methylxanthine (IBMX), 9 ng/mL L-Thyroxine and 3 µg/mL ciglitazone (PromoCell).

2.3.5 Propagation of cells from storage

The appropriate media was pre-warmed at 37ºC for 30 min prior to use. A cryovial of cells was removed from -80ºC storage and thawed rapidly. The cells were added to 9 mL pre-warmed media in a 15 mL tube. Cells were centrifuged at 300 χ g for 5 min and the supernatant decanted. The resulting pellet was re-suspended in 1 mL fresh media and added to a T25 cm^3 flask containing a further 5 mL fresh media. Cells were incubated at 37ºC in a humidified atmosphere with 5% CO_2 and monitored daily. Upon reaching approximately 80% confluence, cells were transferred to a T75 cm^3 flask (Methods 2.3.6) in a total volume of 12 mL media.

2.3.6 Cell subculture

Cells were monitored daily and subcultured upon reaching 80% confluence. The media was decanted and the adhered cells washed with 3 mL 1X phosphate buffered saline (PBS) (Fisher Scientific, Ireland) to remove any residual FBS. Three mL trypsin (Sigma Aldrich) was added and the cells were incubated at 37ºC until cells became dislodged from the surface of the flask. Pre-warmed complete media was added to deactivate the trypsin and the cells were centrifuged at 300 χ g for 5 min.

The supernatant was discarded and the cell pellet was re-suspended in 12 mL fresh pre-warmed media. Cells were seeded at various densities in T75 cm^3 flasks or other appropriate tissue culture treated plastics and incubated at 37ºC in a humidified atmosphere with 5% CO_2.

2.3.7 Cell counting

Cells were prepared as per section 2.3.6 with the cell pellet re-suspended in 1 mL fresh media. Twenty µL cell suspension was added to 180 µL trypan blue (Invitrogen, CA, USA) in a microfuge tube. Trypan blue is routinely used to determine cell viability and to detect dead cells. A haemocytometer was cleaned using 70% EtOH and a glass coverslip placed over the chamber using light pressure. The chamber was allowed to fill by capillary action. Viable cells were counted within the four large corner squares and an average cell number obtained. The number of cells per mL was calculated using the following equation:

Average no. of cells counted x 10 (dilution factor) x 10^4(correction factor converts 0.1mm^3 to 1 mL) = no. of cells/mL

2.3.8 Preparation of cell stocks for storage

Cells were trypsinised as per Methods 2.3.6. Nine mL fresh media was added to deactivate the trypsin and the cells were centrifuged at 300 χ g for 5 min. Supernatant was removed and the pellet was re-suspended in 1 mL fresh media. Cells were counted according to Methods 2.2.7. Cells were re-pelleted and re-suspended at 1 x 10^6 cells per 1 mL Cell Banker (AMSBIO, UK). One mL aliquots were frozen and stored at -80ºC for short term storage and in liquid Nitrogen for long term storage.

2.3.9 Mycoplasma testing

At 70-80% cell confluence, media was removed from T75 cm^3 flasks for mycoplasma testing. Prior to use, the cell supernatant was centrifuged at 300 χ g to remove cellular debris. A polymerase chain reaction (PCR) was adapted from Young et al, 2010 using primers known to encompass

common mycoplasma species (FWD 5' GGGAGCAAACAGGATTAGATACCCT 3'; REV 5' TGCACCATCTGTCACTCTGT TAACCTC 3') (Young et al., 2010). The reaction was as follows: 12.5 µL Green 2x GoTaq (Promega, WI, USA), 0.5 µL forward primer (10 µM) (Sigma-Aldrich), 0.5 µL reverse primer (10 µM) (Sigma-Aldrich) and 10.5 µL DNase free water (Fisher Scientific). Total reaction volume was 25 µL, which included 1 µL cell supernatant. The PCR cycling conditions are outlined in Table 2.3.

Table 2.3: PCR conditions for mycoplasma testing

Temperature	Time	
95ºC	5 min	
94ºC	30 sec	40 Cycles
55ºC	30 sec	
72ºC	1 min	
72ºC	10 min	

The resulting PCR products were electrophoresed on a 2.5% agarose gel as per section 2.3.10. A known mycoplasma positive control was included and the expected band size was 270 bp. A negative control, consisting of nuclease free water replacing cell supernatant, was also included (Appendix VII).

2.3.10 Agarose gels

A 2.5% agarose gel (Sigma Aldrich) was prepared in 100 mL 1X Tris-acetate EDTA (TAE) buffer (10X TAE stock (Sigma Aldrich), 900 mL deionised water), heated in a microwave and allowed to cool briefly. Five µL SYBR Safe DNA gel stain (Invitrogen) was added and the mixture was poured onto a gel rig with combs inserted and allowed to solidify. Once set, the combs were removed and the gel was covered in 1X TAE running buffer. Six µL 25- 650bp ladder (Fisher Scientific) was added to the first

well and 15 µL sample was added to subsequent wells. If required, samples were mixed with 5 µL 6X loading dye (Fisher Scientific) before loading on to the gel. Gels were electrophoresed at 120V for 60 min. Visualisation of the gels was performed using a BIO-RAD (CA, USA) gel imaging system and analysis was carried out using the Quantity One (BIO-RAD) analysis software.

2.4 Formalin fixed paraffin embedded (FFPE) samples

2.4.1 Preparation of a FFPE cell block

Cells were trypsinised and pelleted as per Methods 2.3.6. Excess liquid was removed from the pellet using a sterile pipette. Three droplets bovine thrombin (Hemosil, NY, USA) and 6 droplets human plasma (Hemosil) were added to the pellet to allow for clot formation. The tube was mixed briefly and allowed to stand for 3 min at room temperature (RT). The cell pellet was then processed and embedded in the Histopathology Department in St James's Hospital following standard laboratory protocols. Resultant FFPE cell blocks were stored at RT.

2.4.2 FFPE sectioning

Thin sections from FFPE tissue and cell blocks were cut using a micron HM325 microtome (Medical Supply Company). Sections were cut between 3 – 5 µm in thickness, depending on the downstream application. After sectioning, tissue sections were placed in a water bath at 48^{o}C and adhered to glass slides (charged or uncharged, dependant on application). Sections were baked at 40^{o}C for 30 min followed by baking over night at 62^{o}C and stored at RT. For long term storage, sections were placed at -20^{o}C.

2.4.3 Haematoxylin and Eosin staining

For morphological analysis, FFPE sections were stained according to Haematoxylin and Eosin (H&E) staining protocols. H&E staining allows for morphological distinction of cell structures. H&E staining was carried

out in conjunction with the Department of Histopathology, St James's Hospital, Dublin, Ireland, utilizing automated machinery according to standardised protocols. Briefly, sections were deparaffinised in xylene baths and rehydrated with a series of EtOH washes. Sections were dipped in H&E stains for 30 sec – 2 min and washed with deionised H_2O. Sections were re-dehydrated in EtOH and xylene, mounted and allowed to dry. The resulting staining was viewed microscopically (Figure 2.1).

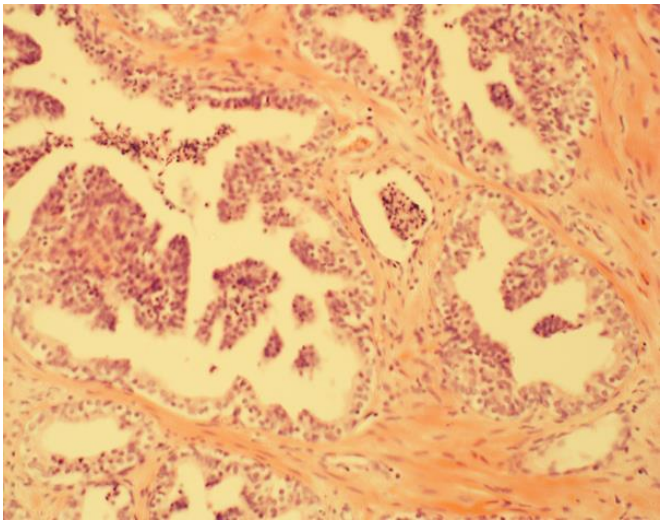


Figure 2.1: Example of H&E staining in prostate tissue.

2.4.4 Macrodissection of FFPE tissue sections

FFPE sections for macrodissection underwent H&E staining as per Method 2.4.3 Macrodissection avoids contamination of samples with cells surrounding area of interest. Sections were viewed by a pathologist and relevant tissue marked e.g. tumour area. A scalpel blade was used to scrape the marked tissue from the slide into a 1.5 mL Eppendorf (Fisher Scientific). Samples were stored at $4^{o}C$ until use.

2.4.5 DNA extraction from FFPE tissue

Tumour DNA was extracted from FFPE 5 µm tissue sections using a macrodissection scraping technique as per Method 2.4.4. DNA was

isolated using the Ambion Recoverall Total Nucleic Isolation Kit for FFPE sections (Invitrogen). To summarise, macrodissected tissue was deparaffinised in a xylene wash, rehydrated in EtOH and dried at RT. The resulting pellet underwent protease digestion using a digestion buffer and protease mix and was incubated overnight for 16 hr at 50^{o}C. Nucleic acid isolation was performed using a mix of isolation additive buffer and EtOH. Each mixture was added to a filtration column and centrifuged to bind DNA. Each column was then subjected to a series of EtOH based washes. RNase A was added to the column for nuclease digestion and incubated at RT for 30 min. The column was washed twice further to purify the DNA. Resulting DNA was eluted in elution buffer and stored -20^{o}C until use.

2.5 Nucleic acid quantification

2.5.1 Qubit

For RNA and DNA quantification using the Qubit, the RNA HS Assay and dsDNA HS Assay (BioSciences) were used. The Quant-iT working solution was prepared based on the number of samples, including two standards (Quant-iT reagent 1 x n µL, Quant-iT buffer 199 x n µL). Ten µL standard was added to 190 µL working solution and 2 µL sample to 198 µL working solution. Samples were allowed to stand at RT for 2 min and were then read on the Qubit fluorometer. Samples were logged in ng/µL.

2.5.2 Nanodrop spectrophotometer

Isolated RNA and DNA were also quantified using the nanodrop 2000 spectrophotometer (Thermo Scientific, MA, USA). The instrument was blanked using 1 µL elution buffer, cleaned and 1 µL of each sample was added in turn (cleaning between each sample). The quality of the nucleic acid was analysed based on the 260/230 and 260/280 ratios. These ratios are used as measures of nucleic acid purity. Each sample was repeated in triplicate and an average value in ng/µL calculated.

2.6 SPOP

2.6.1 Primer design for SPOP mutations

Primers encompassing eight known mutations in the SPOP gene, Y87C, Y87N, F102C, S119N, F125V, W131G, F133L and F133V, were designed (Table 2.4). Primers were purchased from Sigma Aldrich and were re-constituted as 100 µM stock solution. Working stocks were diluted 1:10 with dH_2O, aliquoted and stored at -20^oC until use.

Table 2.4: Primer pairs covering eight known SPOP mutations

Primer Name	Sequence	Mutations Covered
Forward primer 1	5'-GATCTGGTTTTTGCGTAACCTTAAA-3'	S119N, F125V, W131G, F133L, F133V
Reverse primer 1	5'- CCGTTGGCCTCATCCAAAAG-3'	S119N, F125V, W131G, F133L, F133V
Forward primer 2	5'- GTGTTTGCGAGTAAACCCCA-3'	Y87C, Y87N, F102C
Reverse primer 2	5'- CCCTTGGCATTCAGGATGGA-3'	Y87C, Y87N, F102C

2.6.2 G-Block synthesis of SPOP mutations

G-blocks (250 ng sequence verified genomic DNA) were replicated for the SPOP WT sequence and the F125V mutation (Integrated DNA Technologies, IA, USA). G-blocks were required to verify sequencing sensitivity. G-blocks were reconstituted to 10 ng/µL in Tris-EDTA (TE) buffer (Fisher Scientific) and incubated for 20 min at 50^{o}C. G-blocks were stored long term at -20^{o}C.

2.6.3 SPOP PCR

A 30 µL PCR reaction was prepared utilising one of two reaction mixes; 27 µL Platinum PCR supermix high fidelity mastermix (Invitrogen), 1 µL forward primer, 1 µL reverse primer and 1 µL sample (10 ng/µL); or 15 µL 2X GoTaq, 0.5 µL forward primer, 0.5 µL reverse primer, 13 µL nuclease free H_2O and 1 µL sample (10 ng/µL). SPOP DNA was subjected to the PCR conditions outlined in Table 2.5 on an Applied Biosystems thermocycler. The resulting PCR product was stored long term at -20^{o}C.

Table 2.5: PCR conditions for SPOP amplicons.

Temperature	Time	
94^{o}C	2 min	
94^{o}C	30 sec	} 35 Cycles
55^{o}C	30 sec	
68^{o}C	30 sec	
68^{o}C	7 min	

2.6.4 PCR-clean up

Resulting PCR products were subjected to a post-PCR clean up using the ReSource PCR Purification Kit (Source Bioscience, UK) according to manufacturers' instructions. Briefly, 5 volumes of buffer was added to the

PCR samples and the subsequent mixture added to spin columns, which bound the DNA. Flow through was discarded and EtOH based washing steps were used to purify the DNA. Purified DNA was eluted in 50 µL elution buffer and the DNA quality and size assessed on a 2.5% agarose gel as per Methods 2.3.10. DNA concentration was quantified using the nanodrop 2000 spectrophotometer (Methods 2.7.2.).

2.6.5 Sanger sequencing

Samples were prepared according to Eurofins purified PCR product specifications. Samples were prepared in 96 well plates; 150-300 bp, 1 ng/µL in a total volume of 15 µL. Primers were used at a concentration of 10 pmol/µL, with 5 µL per sample required. Base calling of chromatograms was performed manually due to the small product size.

2.6.6 TMA construction

Five µm sections, from two TMAs constructed by the Prostate Cancer Research Consortium, were donated by Prof William Watson and Dr Amanda O'Neill. The TMAs contained the Irish SPOP cohort of n=72 patient samples. TMAs containing the Swedish SPOP cohort, n=60, were constructed using an automated tissue microarrayer. Three representative tumour cores were extracted per FFPE block using 0.6 mm punches. Upon completion, each TMA was baked at 65^{o}C for 5 min and cooled on ice. Five µm sections were cut according to Methods 2.4.2 for IHC analysis. Additional 5 µm TMA sections (Dr. Maria Svensson, University Hospital Orebro) were used for cohorts of the Swedish patients. Use of all samples was in accordance with each institution's approved ethical consent.

2.7 Standard immunohistochemistry protocol for FFPE tissue sections

Tissue sections of approximately 4 µm in thickness were adhered to glass slides from FFPE blocks. The slides were then de-paraffinised and hydrated using the following series of xylene and EtOH washing steps:

Xylene bath x 3 (8 min per bath), 100% EtOH bath x 2 (1.5 min per bath), 95% EtOH bath x 2 (1 min per bath), 70% EtOH bath x 2 (1 min per bath), dH_2O bath x 3 (1 min per bath)

Subsequently, 200 µL peroxidase-blocking solution (Sigma Aldrich) was added to the sections and incubated in a humid chamber for 20 min. Excess peroxidase-blocking solution was then removed in de-ionised H_2O baths (3 X 1 min per bath). Tissue sections were transferred to antigen retrieval solution (Dako, Denmark) (pre-heated to 95-99^oC) and incubated for 25 min. Sections were then incubated at RT for a further 25 min. Excess antigen retrieval solution was removed using 1X Tris-buffered Saline (TBS) baths (3 x 1 min per bath). Two hundred µL blocking solution (250 mg bovine serum albumin (BSA) (Sigma Aldrich) and 25 mL 1 X TBS) was added to the sections and incubated for 30 min in the humid chamber. Excess blocking solution was removed using TBS baths (3 x 1 min per bath). Two hundred µL primary antibody (Table 2.6) diluted in blocking solution was added to tissue and allowed to incubate overnight at 4^oC in the humid chamber. Tissue sections were washed in TBST with 0.1% tween (TBST) (500 mL TBS, 500 µL Tween (Sigma Aldrich) baths (3 X 1 min per bath) to remove excess antibody. Two hundred µL secondary antibody (Table 2.7) was then added and allowed to incubate for 40 min in the humid chamber. Excess antibody was removed using TBST baths (3 X 1 min per bath). Two hundred µL Chromo-Dab (Dako) (20 µL DAB-Chromogen in 1 mL DAB-substrate buffer) was added to the sections. The DAB-Chromogen was allowed to incubate for 1-30 min depending on the intensity of the staining. Excess DAB-Chromogen was removed using dH_2O baths (3 x 1 min per bath). Tissue sections were counterstained with Haematoxylin for 30 sec – 2 min and washed with dH_2O to remove any excess stain. Sections were

re-dehydrated using the reverse of the deparaffinisation/hydration steps, mounted and allowed to dry.

Table 2.6: List of primary antibodies for IHC

Primary Antibody	Species	Clone
Monoclonal mouse anti-human Cytokeratin	Mouse	MNF116 (Dako)
Monoclonal mouse anti-human Epithelial Antigen	Mouse	Ber-EP4 (Dako)
Monoclonal mouse anti-human PSMA	Mouse	3E6 (Dako)
Monoclonal rabbit anti-human ERG	Rabbit	EPR3864 (abcam)
Monoclonal mouse anti-human CD42a	Mouse	ALMA.16 (BD Pharmingen)
Monoclonal mouse anti-human CD45	Mouse	PD7/26/16 (abcam)
Monoclonal Rabbit anti-human Androgen Receptor	Rabbit	SP107 (Thermo Scientific)

Table 2.7: List of secondary antibodies for IHC

Secondary Antibodies	Species
Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP	Goat
Polyclonal Goat Anti-Mouse Immunoglobulins/HRP	Goat

HRP – Horseradish Peroxidase

2.8 Blood draw

Grenier bio-one blood tubes (K2EDTA, 9NC coagulation sodium citrate 3.2% and Z serum sep clot activator) and 23G ¾" needles were obtained from Cruinn (Dublin, Ireland).

2.8.1 Blood draw from healthy donors

Healthy blood donors were classed as individuals not suffering from PrCa or any known malignant disease. Blood was drawn from these volunteers into K2EDTA tubes using 23G ¾" needles according to standard phlebotomy practices.

2.8.2 Blood draw from ExPeCT trial participants

Blood was drawn by members of the ExPeCT research team or clinical trials nurses in the respective hospital sites according to Table 2.8 K2EDTA tubes were inverted approximately 10 times to avoid coagulation prior to use on the ScreenCell® filtration device. Blood drawn for serum analysis was allowed to stand for 30-60 min at RT. Clotting

was visible after the first 30 min. The tube was centrifuged at 1000 χ g for 10 min. The supernatant was pipetted into labelled cryovials in 400 µL aliquots. For plasma analysis, blood was centrifuged at 1000 χ g for 10 min. The supernatant was pipetted into 500 µL aliquots. All cryovials were immediately placed at -80ºC. All details from the participants' blood draws were recorded in the ExPeCT trial biological sample logs (Appendix IV).

Table 2.8: Blood isolation from ExPeCT trial participants.

Type of tube	No. of Tubes	Component required
K2EDTA	4 x 3 mL	Whole blood
9NC coagulation citrate 3.2%	1 x 3.5 mL	Plasma
Z serum sep clot activator	1 x 5 mL	Serum

2.8.3 Cell line spiking into healthy whole blood

Whole blood was collected from healthy donors in K2EDTA tubes prior to cell spiking. Cell lines were subcultured according to Methods 2.3.6. Once pelleted, the cells were re-suspended in 1 mL of pre-warmed media and counted as per Method 2.3.7. The cell suspension was then diluted to obtain the required number of cells according to the downstream application. The required volume was added to 3 mL whole blood and

blood was immediately fixed and filtered according to the ScreenCell® filtration guidelines (Method 2.9).

2.8.4 Platelet cloaking of prostate cancer cell lines

Surplus human platelets were donated by the Irish Blood Transfusion Service according to an authorised clinical indemnity form. Platelets collected were viable at RT for approximately 5 hr. PrCa cell lines were subcultured (Methods 2.3.6) and prepared in the same manner as Methods 2.13.3. The cell suspension was diluted to a required volume of $1x10^6$ cells in 1 mL media. One mL platelets was added to the $1x10^6$ cells/mL suspension, immediately added to healthy donor blood and allowed to incubate at RT for various periods of time (5–45 min). Ten - 500 µL of the platelet/cell mixture was added to whole blood and the blood was immediately fixed and filtered according to the ScreenCell® filtration guidelines (Methods 2.14).

2.9 ScreenCell® CTC isolation

Three mL whole blood was drawn into K2EDTA tubes and processed according to ScreenCell® filtration guidelines (ScreenCell, Sarcelles, France). Briefly, 3 mL whole blood was added to a 15 mL conical tube. Four mL ScreenCell® fixation buffer was added and the tube was inverted approx. 10 times and allowed to stand for 8 min. The whole blood/buffer mix was added to module A (Figure 2.2) and a vacuum tube placed into module B (Figure 2.2). The blood filtered through the module into waste and 1.6 mL PBS was added once the blood passed the yellow line on module B. Once all the liquid had passed through, the ScreenCell® filter was removed, allowed to dry at RT overnight and morphologically stained using May Grunwald Giemsa staining (MGG) (Methods 2.11.1). Potential CTCs were recorded by a pathologist. ScreenCell® filters were stored long term at -20^{O}C.

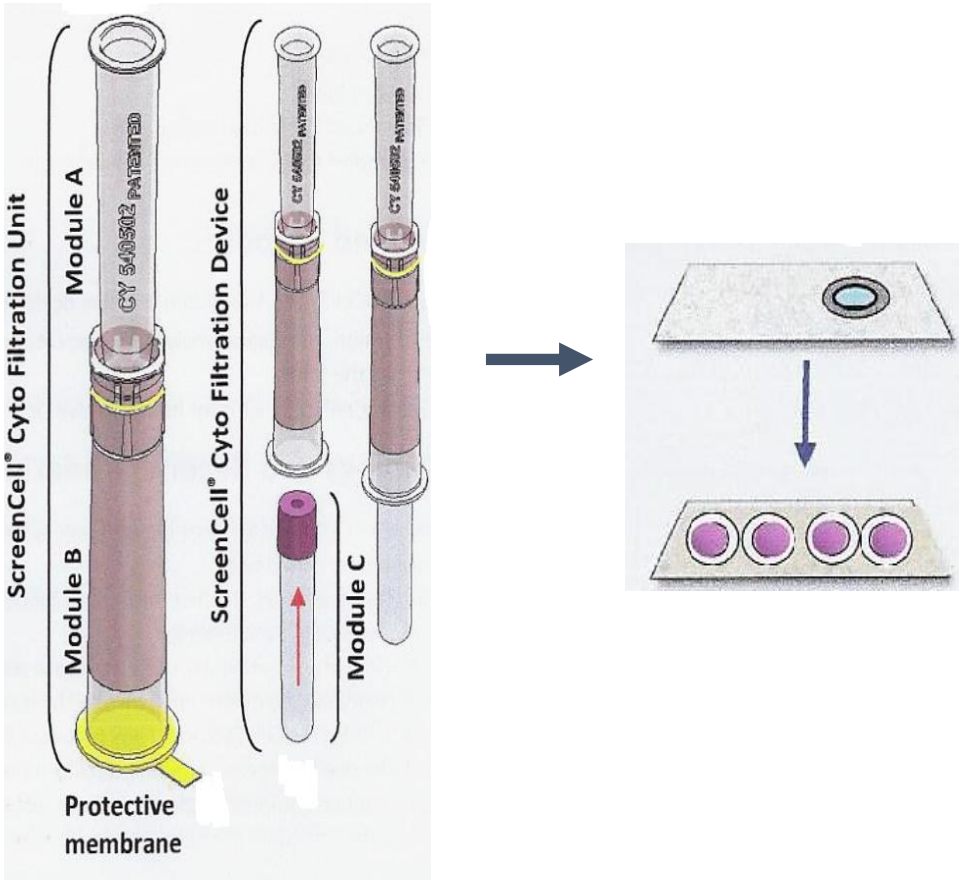


Figure 2.2: ScreenCell® Cyto filtration device, filtration unit and ScreenCell® filter (ScreenCell® insert).

2.10 Isoflux CTC isolation

PrCa cell lines were spiked into healthy donor blood according to Methods 2.8.3. CTC enrichment was carried out according to the ISOFLUX circulating tumour cell enrichment manual (Fluxion, CA, USA). In short, samples were decanted into a Leucosep tube containing Ficoll-paque. The Leucosep tubes were centrifuged at 800 χ g for 15 min. The resulting supernatant was added to a 50 mL conical tube and centrifuged at 280 χ g for 10 min. The peripheral blood mononuclear cell (PBMC) layer was kept on ice and suspended in blocking reagent. Cell suspension was transferred to a pre-prepared tube coated with binding buffer. Utilising magnets, CTC beads were added to the cell suspension and the cells were washed and supernatant removed. The cell/bead

mixture was prepared for the Iso-Flux cartridge and loaded onto the instrument to enrich for potential CTCs. The cell/bead mix was washed and Cell tracker green was added to the mixture. Using magnets, potential CTCs were viewed under FITC filter on an immuno-fluorescence microscope.

2.11 Morphological staining of ScreenCell® filters

2.11.1 May Grunwald Giemsa staining

ScreenCell® filter morphology was assessed using MGG staining. All stains were filtered prior to use. The filters were placed into a bath containing May-Grunwald (Sigma Aldrich) and incubated for 2 min 30 sec at RT with constant agitation. The filters were then placed into a second bath containing 1:1 dilution May-Grunwald: dH_2O (pH 7) for a further 2 min 30 sec. The filters were moved to a third bath containing 1:10 dilution of Giemsa (Cruinn): dH_2O (pH 7) for 10 min. Excess stain was removed by washing in dH_2O (pH 7) for 1 min and filters were allowed to air dry at RT for 20 min. For IHC protocols, MGG stain was removed by washing in a 1X TBS bath for 20 min at RT.

2.11.2 Papanicolaou staining

Papanicolaou staining was performed using an optimised protocol developed between St James's Hospital and Basel University Hospital (Table 2.9). Filters were allowed to air dry and examined using a microscope.

Table 2.9: Papanicolaou Staining Protocol

Step	Reagent	Time	Step	Reagent	Time
1	95% Alcohol	1 min	**13**	95% Alcohol	15 sec
2	70% Alcohol	1 min	**14**	95% Alcohol	15 sec
3	50% Alcohol	1 min	**15**	EA50	5 min
4	Distilled H_2O	1 min	**16**	95% Alcohol	1 min
5	Harris Haematoxylin	4 min	**17**	95% Alcohol	1 min
6	H_2O	1 min	**18**	100% Alcohol	30 sec
7	Acid H_2O	10 sec	**19**	100% Alcohol	30 sec
8	H_2O	4 min	**20**	100% Alcohol	30 sec
9	H_2O	4 min	**21**	Xylene	1 min
10	50% Alcohol	30 sec	**22**	Xylene	3 min
11	95% Alcohol	30 sec	**23**	Xylene	1 min
12	OG6	1.40 min			

2.12 Standard IHC protocol for ScreenCell® filters

All antibodies used on the ScreenCell® filters were tested on standard FFPE tissue sections prior to use. ScreenCell® filters were stained using MGG and evaluated prior to undergoing IHC. MGG stain was removed using 1 x 20 min TBST bath and the filters were rehydrated using 1 x 10 min TBS bath. IHC was performed on the filters according to Methods 2.7 with minor modifications (70 µL reagent was applied to the filters instead of 200 µL). Excess reagent was removed using 1 x 1 min baths. Primary and secondary antibodies were used in accordance with Table 2.6. Filters were washed in dH_2O and counter stained with 70 µL Haematoxylin for 30 sec. Filters were washed in dH_2O to remove excess stain and allowed to dry at RT. The resulting IHC was viewed microscopically.

2.13 Cytokine and adipokine assays using Meso-Scale® Discovery platform

Serum and plasma samples were prepared according to Methods 2.13.2. Aliquots were allowed to thaw and come to RT, and prepared according to Meso-Scale Discovery® (Meso-Scale Diagnostics LLC, USA) (Figure 2.3) assay requirements. Briefly, serum/plasma was diluted based on the recommended dilution factor for each assay. The 96 well plates were washed three times with PBS-tween (PBST). Up to 50 µL sample, calibrator and buffer was added to appropriate wells on each plate and incubated at RT with constant agitation for 2 hr. Unbound sample was removed and the plate washed 3 x PBST. Twenty five µL detection antibody was added to the plate and incubated at RT with constant agitation for 2 hr. One hundred fifty µL read buffer was added to each plate and analysed on the MSD instrument. Three individual adipokine assays (leptin, adiponectin and resistin), four single-plex assays (MCP-1, MMP9, MMP2, RANTES) and two multi-plex cytokine assays (VEGF, IL-17a, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13 and TNF-α) were carried out on all ExPeCT participant serum samples. One single-plex assay (C-peptide) was carried out on all ExPeCT participant plasma samples.

Sample concentration was estimated against a range of standards and calculated based on a standard curve (Appendix VII).

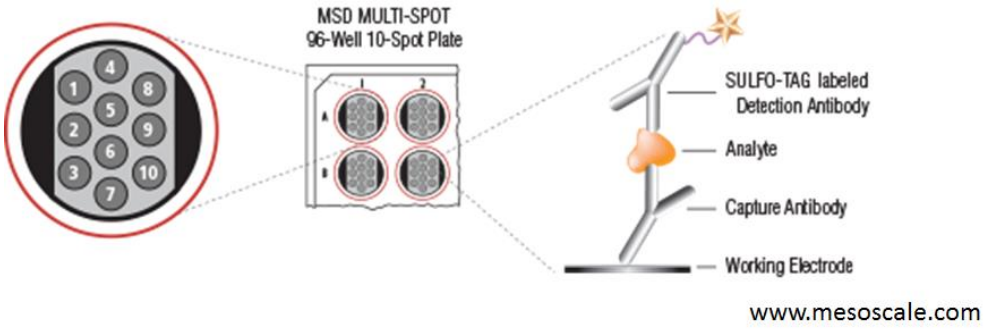


Figure 2.3: Outline of Meso-Scale® Discovery Platform Technology (Assay insert).

2.14 Laser capture microdissection

Laser capture microdissection (LCM) was performed on ExPeCT participant needle core biopsies (NCBs). NCBs were sectioned (3 µm) in conjunction with the Department of Histopathology, St James's Hospital, Dublin, Ireland and manually stained with H&E; 2 x 12 min xylene bath, 2 x 1.30 min 100% EtOH bath, 2 x 1 min 95% EtOH bath, 2 x 1 min 70% EtOH bath, 2 x 1 min dH_2O bath, 1 x 30 sec haematoxylin bath, 1 x 1 min dH_2O bath, 1 x 30 sec Fisher's tap water bath, 1 x 1 min Eosin bath, 1 x 1 min dH_2O bath and repeated alcohol baths in reverse order. Sections were not coverslipped. Tumour area on the NCBs was marked by a pathologist. Slides were loaded onto the Arcturus XT (Applied Biosystems, CA, USA) for LCM. The slide was visualised on screen and the marked area highlighted using LCM software. The infrared and ultraviolet lasers were utilised to adhere the cells of interest to the LCM caps (Figure 2.4). LCM caps were removed from the Arcturus XT and stored at RT for RNA isolation.

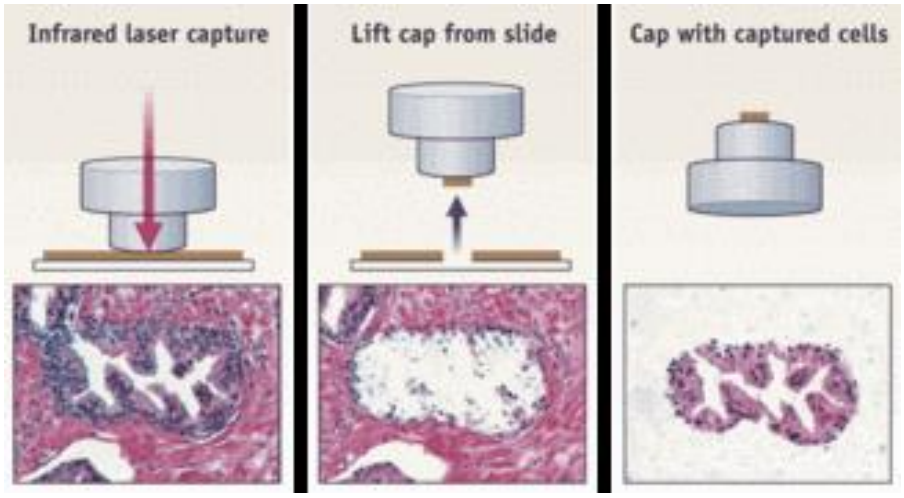


Figure 2.4: Laser Capture Microdissection. Image taken from www.bioopticsworld.com.

2.15 Gene expression analysis

2.15.1 RNA extraction from LCM caps

RNA extraction from LCM caps was completed according to the Arcturus Paradise PLUS RNA extraction and Isolation Kit protocol (Biosciences). Briefly, caps were incubated for 16 hr at 37^{o}C in proteinase K buffer mix. MiraCol purification columns were pre-coated with conditioning buffer, binding buffer and resulting cell lysis mix was added to the columns. After a series of washes, DNAse digestion was performed on the column. RNA was eluted in elution buffer into 0.5 mL tubes.

2.15.2 RNA extraction from cell line pellets

Cells were trypsinised as per Methods section 2.3.6. The cell pellet was re-suspended in 1 mL 1X PBS and centrifuged for a further 5 min at 300 χ g to wash the cells. The supernatant was removed and the resulting pellet placed on ice. RNA was extracted according to the manufacturers' instructions for the RNeasy mini kit (Qiagen, USA). Briefly, samples were added to 1 volume of EtOH and allowed to bind to a spin column. On column DNAse digestion was carried out before a series of washing and centrifuge steps. Centrifuge steps were performed at 8000 χ g for 1 min.

RNA was eluted into the RNeasy elution buffer and RNA was stored long term at -80ºC.

2.15.3 cDNA synthesis

Ten µL (10-100 ng/µL) RNA was synthesised into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A 2X RT master mix was prepared based on the number samples per run, including negative controls; 2 µL 10X RT buffer, 0.8 µL 25X dNTP mix (100 µM), MultiScribe Reverse Transcriptase 1 µL and 4.2 µL nuclease free H_2O. The tubes were centrifuged briefly to remove air bubbles and run on a thermocycler according to the conditions in Table 2.10.

Table 2.10: cDNA PCR Conditions

Temperature	Time
25ºC	10 min
37ºC	120 min
85ºC	5 min
4ºC	5 min

2.15.4 Pre-amplification

cDNA (from LCM caps only) was amplified using TaqMan® Pre-Amp Master Mix (2X) (Applied Biosystems). Equal volumes of each 20X TaqMan® gene expression assay were pooled and diluted with 1 X TE buffer to a concentration of 0.2X. Twenty five µL TaqMan® Pre-Amp Master Mix (2X), 12.5 µL pooled assay mix and 12.5 µL cDNA were combined for a final reaction volume of 50 µL. Samples were assayed according to the reaction conditions in Table 2.11 for a total of 10 cycles. Resulting products were placed on ice immediately.

Table 2.11: Pre-amplification reaction conditions

Temperature	Time	
95ºC	10 min	
95ºC	15 sec	10 Cycles
60ºC	4 min	

2.15.5 TaqMan® gene expression

Taqman® gene expression analysis was performed on the 7500 Fast Real-Time PCR machine (Applied Biosystems). The pre-amplification product (Method 2.15.4) was diluted 1:5 with 1 X TE buffer. One µL TaqMan® gene expression assay, 5 µL diluted Pre-Amp product, 10 µL TaqMan® gene expression mastermix and 4 µL nuclease free water was prepared per reaction and assayed according to the conditions outlined in Table 2.12. Negative controls and endogenous (GAPDH) positive controls were included on each run. Each assay was normalised against the endogenous control and expression quantified using the ΔΔCT method.

Table 2.12: TaqMan® gene expression PCR Conditions

Temperature	Time	
50ºC	2 min	
95ºC	10 min	
95ºC	15 sec	40 Cycles
60 ºC	1 min	

2.16 BrdU proliferation assay

Cell proliferation was measured using a Cell Proliferation ELISA BrdU (Roche Diagnostics, Basel Switzerland). This assay is based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis. Cells were seeded at 4 x 10^3 cells per well in a flat bottomed 96 well plate and incubated overnight at 37^{o}C. The media was removed and the cells treated with appropriate adipokines/drugs and media controls for a specified period of time. Following treatment, 10 µL BrdU labelling solution (1:100 dilution in sterile media, concentration 100 µM) was added to each well and incubated at 37^{o}C for 4 hr. All liquid was removed from the 96 well plate and the plate was allowed to dry at 37^{o}C for a further 10 min. Cells were fixed with 200 µL FixDenat solution for 30 min at RT. FixDenat solution was removed and 100 µL anti-BrdU-POD (1:100 anti-BrdU-POD stock with antibody dilution solution) was added to each well and incubated at RT for 90 min. The antibody conjugate was removed, wells washed 3 x 200 µL PBST and 100 µL substrate solution was added to each well for 5-10 min or until colour change was detected. Twenty five µL 1 mM H_2SO_4 was added to each well to stop the reaction and the plate was read at 450 nm with reference wavelength 690 nm. Untreated controls were set to 100% and treatments compared to the untreated control.

2.17 SGBS cells

2.17.1 SGBS co-culture

Pre-adipocyte SGBS cells were cultivated according to Methods 2.3.6. Upon 70-80% confluency, cells were trypsinised and counted as per Methods 2.3.7. Ten thousand SGBS cells were seeded into 6 well plates and allowed to attach overnight. At day 0, fresh preadipocyte growth medium was added to half of the wells, and preadipocyte differentiation media was added to the second half. Full differentiation of the cells occurred over a period of 14 days. At day 14, 10,000 cancer cells were added to the co-culture inserts and incubated at 37^{o}C for 24 hr (Figure 2.5). Media was removed from the inserts and wells, and stored at -80^{o}C

for further analysis. Cancer cells were removed from the insert for RNA extraction according to Methods 2.15.2.

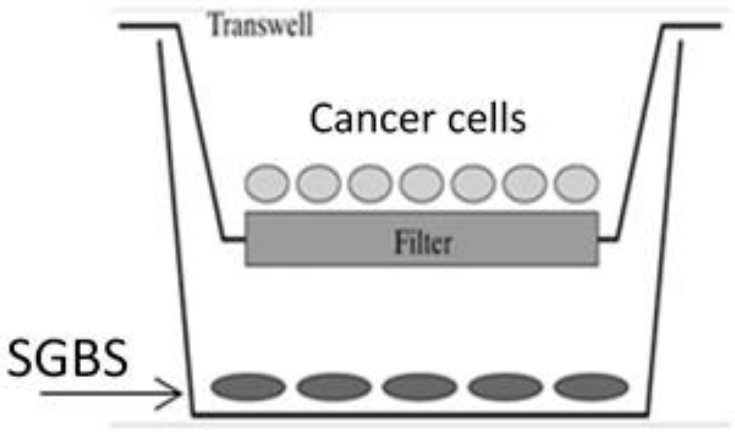


Figure 2.5: Outline of SGBS co-culture

2.17.2 Oil Red O staining

SGBS cells were assessed morphologically for differentiation at day 0, day 7 and day 14 by Oil Red O, a red lysochrome, fat soluble dye. All staining steps were carried out at RT. Cells were fixed with 10% formalin for 30 min and washed 3 times with 1 mL PBS. One mL 60% isopropanol was added to the cells and incubated for 5 min. Oil Red O was prepared as a 3 mg/mL stock in 99% isopropanol and a working solution was prepared with 3 parts Oil Red O stock and 2 parts dH_2O. The working solution was allowed to stand for 10 min and filtered prior to use. Isopropanol was removed and 1 mL Oil Red O was added to the cells and incubated for 5 min. Cells were washed 3 times with 1 mL PBS and photographed using an inverted phase contrast microscope with camera (Olympus Corporation, Tokyo, Japan).

2.17.3 RNA extraction

RNA was extracted from the SGBS cells at day 0, day 7 and day 14. Media was removed from the 6 well plates and 500 µL Tri-Reagent

added. A cell scraper was used to dislodge the cells and cells were pipetted into sterile 1.5 mL eppendorfs. Two hundred µL of chloroform was added to the sample and shaken vigorously for 15 sec. Samples were allowed to stand at RT for 15 min and centrifuged at 12,000 χ g for 15 min at 4ºC. The aqueous phase containing the RNA was removed into a fresh tube and precipitated with 500 µL isopropanol. Samples were allowed to stand for 10 min at RT and centrifuged for 8 min at 12,000 χ g. The RNA pellet was washed with 75% EtOH and centrifuged for 5 min at 7,500 χ g. The resulting pellet was resuspended in 20 µL sterile PCR grade water. Samples were then subjected to purification and DNAse digestion using the Qiagen RNeasy protocol as per Methods 2.15.2.

2.17.4 Gene expression analysis of SGBS cells

Sybr Green based gene expression analysis was performed on the 7500 Fast Real-Time PCR machine (Applied Biosystems). Ten µL Sybr Green mastermix, 1 µL forward primer, 1µL reverse primer and 8 µL nuclease free water was prepared per reaction and assayed according to the conditions outlined in Table 2.13. Negative controls and endogenous (18S) positive controls were included on each run. Each assay was normalised against the endogenous control and expression quantified using the ΔΔCT method.

Table 2.13: Sybr Green reaction conditions

Temperature	Time	
95ºC	10 min	
95ºC	15 sec	40 Cycles
60ºC	1 min	
95 ºC	15 sec	Melt Curve
60ºC	1 min	
95 ºC	30 sec	

2.18 Statistical Analysis

Statistical packages IBM SPSS Statistics 24 (IBM, Ireland) and Graphpad PRISM 7 (Graphpad, CA, USA) were used for the majority of statistical analysis in this thesis. Data is graphed as mean ± SEM. Significance was determined via one way analysis of variance (ANOVA), where the number of groups in the experiment was three or more, or a paired student t test. A probability of $p \leq 0.05$ was considered to represent a significant difference between the groups. A post hoc test was necessary after ANOVA to determine which groups were significantly different to each other. For ExPeCT patient data, excluding baseline patient characteristics, statistical analysis was completed in conjunction with Dr Bryan Stanfill, Pacific Northwest National Laboratory, Washington, USA. The specifics of each statistical test utilised, are outlined in each results section.

Chapter 3:

SPOP Mutations and Expression of ERG and PTEN in Patients with Recurrence in Prostate Cancer

3.0 Introduction

As outlined previously, the identification of molecularly distinct subtypes of PrCa may hold significant clinical relevance. The goal towards precision medicine has led to the availability of commercial gene expression biomarkers. Platforms such as Decipher™, a microarray for RNA marker profiling in FFPE tissue, and Prolaris ®, a test for expression of 46 genes, have demonstrated potential for use in a clinical setting (Clinton et al., 2017). In PrCa SPOP mutations have been identified as occurring in approximately 6-13% of PrCa cases (Barbieri et al., 2012), with comparable levels present across different demographics and ethnicities (Blattner et al., 2014). The structure of the SPOP gene, consists of a N-terminal MATH domain, a bric-a-brac, tramtrack and broad complex (BTB)/POZ domain, a three-box domain and a C-terminal localisation sequence (Figure 3.1) (Mani, 2014). The N-terminal MATH domain is responsible for recruiting proteins for substrate binding (Geng et al., 2013). Interestingly, all of the currently known somatic missense SPOP mutations have been identified as being present within the N-terminal MATH domain (Barbieri et al., 2012).

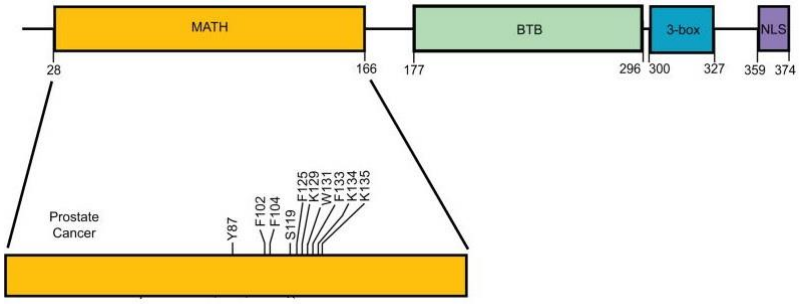


Figure 3.1: SPOP domains. Image taken from Mani et al. 2014, depicts SPOP domains and the position of common mutations found in PrCa.

BTB: bric-a-brac, tramtrack and broad complex, NLS: nuclear localisation sequence.

SPOP mutants may impede ubiquitination and degradation in a dominant-negative manner (Theurillat et al., 2014). Steroid receptor

coactivator 3 (SRC-3), a member of the p160 receptor coactivator family, is known as a master regulator of transcription factors, aiding cancer cell proliferation and survival in a number of cancers, including PrCa (Ma et al., 2011). SPOP WT substrate binding of SRC-3 encourages protein degradation and suppression of the SRC-3 protein, thus promoting tumour suppressor capabilities (Geng et al., 2013). However, SPOP mutants do not possess the ability to promote ubiquitination or the degradation of SRC-3 (Geng et al., 2013). Another transcriptional coactivator from the bromodomain and extraterminal (BET) family, BRD4, stimulates oncogenic drivers and functions, and has been linked with SPOP substrate binding (Dai et al., 2017). SPOP WT pinpoints BRD4 for destruction, suggesting that it inhibits cancer progression through this pathway. SPOP mutants behave in an opposing manner to the WT by impairing the binding ability of BRD4, leading to increased levels of BRD4 in circulation (Zhang et al., 2017). Furthermore, the levels of endogenous BRD4 expression were significantly correlated with advanced PrCa and a shorter time to BCR post radical prostatectomy (Dai et al., 2017), highlighting the role of SPOP mutants in disease progression.

The clinical significance of SPOP mutations is conflicting in the literature. In a study by Blattner et al. it was reported that there were no significant correlations between the presence of SPOP mutations and BCR or Gleason grade (Blattner et al., 2014). Conversely, Garcia-Flores et al. demonstrated a significant relationship between the presence of SPOP mutations and a worse prognosis (Garcia-Flores et al., 2014). Recent evidence has established that mutant SPOP behaves as a regulator of prostate tumorigenesis in mouse models (Blattner et al., 2017). Analysis of the genomic landscape of PrCa, highlighted the presence of clinically relevant molecular alterations, including SPOP, ETS genes and PTEN, in 90% of metastatic PrCa cases (Robinson et al., 2015), highlighting potential for therapeutic targets. One such genomic alteration, PTEN loss, is present in approximately 50% of metastatic PrCa (Murphy et al., 2016). PTEN deletions are reported as being present more frequently in advanced PrCa when compared to levels

present in SPOP mutant early stage PrCa (Blattner et al., 2017). A combination of SPOP mutants and PTEN loss *in vivo* were recently described as capable of stimulating prostate neoplasia, thus aiding the growth of invasive PrCa (Blattner et al., 2017). However, mutual exclusivity of SPOP mutants and ERG have been well annotated (Barbieri et al., 2012). Expression of ERG is present in approximately 50% of all PrCa cases (Ayala et al., 2015), with ERG fusions attributed with metastasis and disease progression (Adamo and Ladomery, 2016). A study by An et al. determined that SPOP mutations and ERG fusions can increase ERG protein levels by diminishing the interaction between ERG and SPOP WT (An et al., 2015). This finding was hypothesised as a potential explanation of the mutually exclusive relationship, as the presence of both concurrently would be redundant (An et al., 2015).

While further research is required to examine these findings, the role of SPOP mutations, ERG rearrangements and PTEN loss in PrCa may hold insight into disease progression and provide new targets for therapy. The aims of this chapter were to (a) examine the presence of eight annotated SPOP mutations in a cohort of Swedish and Irish patients with PrCa who met the criteria for BCR, (b) assess the mutual exclusivity of ERG and SPOP mutants and (c) determine PTEN expression in the same cohort of patients. The overall objective was to identify a distinct molecular sub-type of PrCa and to assess the clinical relevance of this sub-type.

3.1 Results

3.1.1 SPOP Patient Cohort

Patients were included in this study based on clinical criteria for BCR; post-prostatectomy PSA level ≥0.4 ng/mL followed by a second PSA level >0.4 ng/mL (Stephenson et al., 2006). A number of patients who never met the criteria for BCR (progression free) and patients who progressed immediately after surgery (never reached an undetectable PSA) were also included as a comparison. The study was comprised of two groups, an Irish cohort provided by the PCRC (n=72) and a Swedish cohort provided by Orebro University Hospital (n=60). Ethical consent was obtained from the relevant ethics committees at each site for this study. Independent t-tests, Fisher's tests, Mann-Whitney U tests and Chi-Squared analysis were performed, as appropriate, to assess differences in baseline characteristics between groups across all samples. No significant differences were noted in patient characteristics between groups in either the Swedish (Table 3.1) or Irish cohort (Table 3.2).

Table 3.1: Patient Characteristics of Swedish Cohort.

		All Cases (n=60)	BCR (n=35)	No BCR (n=10)	p-value
Age at Diagnosis	Mean Median	63.5 (51.6 - 73.5) 64.3 (51.6 - 73.5)	63.5 (51.6 - 73.5) 63.9 (51.6 - 73.5)	63.8 (56.3 - 68.9) 64.1 (56.3 - 68.9)	0.861 0.883
PSA at Diagnosis (ng/mL)	Mean Median Missing	13.4 (3.2 - 45) 9.85 (3.2 - 45) 1	12.7 (3.2 - 34) 9.2 (3.2 - 34) 1	10.6 (7.1 -20) 9.9 (7.1 - 20) 0	0.27 0.71

Table 3.1 Continued: Swedish Cohort, Patient Characteristics.

		All Cases (n=60)	BCR (n=35)	No BCR (n=10)	p-value
T- Stage	T1b T1c T2 T3	3 (5%) 23 (38.3%) 30 (50%) 4 (6.7%)	2 (5.7%) 9 (25.7%) 21 (60%) 3 (8.6%)	0 7 (70%) 3 (30%) 0	0.098
Gleason Score	6 7 8 9 Missing	7 (11.7%) 34 (56.7%) 15 (25%) 3 (5%) 1	4 (11.4%) 21 (60%) 8 (22.9%) 2 (5.7%)	2 (20%) 6 (60%) 2 (20%) 0	0.858
Gleason Score Group	6 3+4 4+3 8-10 Missing	7 (11.7%) 14 (23.3%) 20 (33.3%) 18 (30%) 1	4 (11.4%) 10 (28.6%) 10 (31.4%) 10 (28.6%)	2 (20%) 2 (20%) 4 (40%) 2 (20%)	0.822
Cancer Specific Death	Yes No	11 (18.3%) 49 (81.7%)	8 (22.9%) 27 (77.1%)	0 10 (100%)	0.168
Biochemical Relapse	Yes No Progression	35 (58.3%) 10 (16.7%) 15	na na	na na	
Follow up BCR	Months (median)	36.1 (4.3-159.1)	na	na	
Follow-Up	Months (median) Years (median)	124.7 (28.6 - 260.8) 10.4 (2.4 - 21.7)	147.3 (28.6-260.1) 11.98 (2.38 - 21.74)	135 (98-208) 11.26 (8.13 - 17.33)	0.581 0.581

T- stage – tumour stage, no baseline data available for n=15 immediate progression

Table 3.2: Patient Characteristics of Irish Cohort.

		All Cases (n=72)	BCR (n=40)	No BCR (n=32)	p-value
Age at Surgery	Mean Median	61.75 (42 - 71) 63 (42 -71)	62.18 (42 - 71) 63 (42 -71)	61.23 (43 - 69) 64 (43 -69)	0.253
PSA at Surgery (ng/mL)	Mean Median Missing	8.64 (1-18.8) 7.98 (1-18.8) 2	8.52 (1 - 18.5) 7.9 (1 -18.5) 2	8.86 (1.9 - 18.8) 7.98 (1.9 - 18.8) 0	0.228
T- Stage	T2a/b T2c T3a SMI	5 (6.9%) 22 (30.6%) 35 (48.6%) 10 (13.85)	2 (5%) 8 (20%) 23 (57.5%) 7 (17.5%)	3 (9%) 14 (44%) 12 (38%) 3 (9%)	0.108
Gleason Score	6 7 8 9	16 (22.2%) 36 (50%) 11 (15.3%) 9 (12.5%)	9 (22.5%) 19 (47.5%) 7 (17.5%) 5 (12.5%)	7 (22%) 17 (53%) 4 (12.5%) 4 (12.5%)	0.682
Gleason Score Group	6 3+4 4+3 8-10	16 (22.2%) 19 (26.4%) 17 (23.6%) 20 (27.8%)	9 (22.5%) 9 (22.5%) 10 (25%) 12 (30%)	7 (22%) 10 (31%) 7 (22%) 8 (25%)	0.740
Time to BCR (months)	Mean Median	na na	18.1 (2 -96) 11.5 (2 -96)	na na	
Progression Free (months)	Mean Median	na na	na na	83.13 (44 - 116) 86 (44 - 116)	

na – not applicable, SMI – seminal vesicle invasion, T-stage – tumour stage

3.1.2 SPOP Mutant Amplification using PCR

Two sets of primer pairs (Table 2.4) were designed to encompass the eight known SPOP mutations: Y87N, Y87C, F102C, S199N, F125V, W131G, F133L and F133V using PCR as per Methods 2.6.3. FFPE tissue samples (5 μM sections, adhered to glass slides) were provided for each patient by the PCRC and Orebro University Hospital. Tissue sections were stained with H & E and the relevant tumour area marked by a pathologist. Marked area was removed using macrodissection and DNA processed according to Methods 2.4.5. DNA was quantified using the nanodrop, and PCR was performed using 1 μL (10 ng/μL) of each sample per primer pair. The resulting PCR products underwent post-PCR clean-up and all samples were examined on a 2.5% agarose gel to ensure bands were present at the correct size. Estimated sample size for primer sets 1 and 2 was 153 bp and 132 bp, respectively (Figure 3.2). All samples were visible on the agarose gel, despite a low concentration of DNA in a subset of tumours.

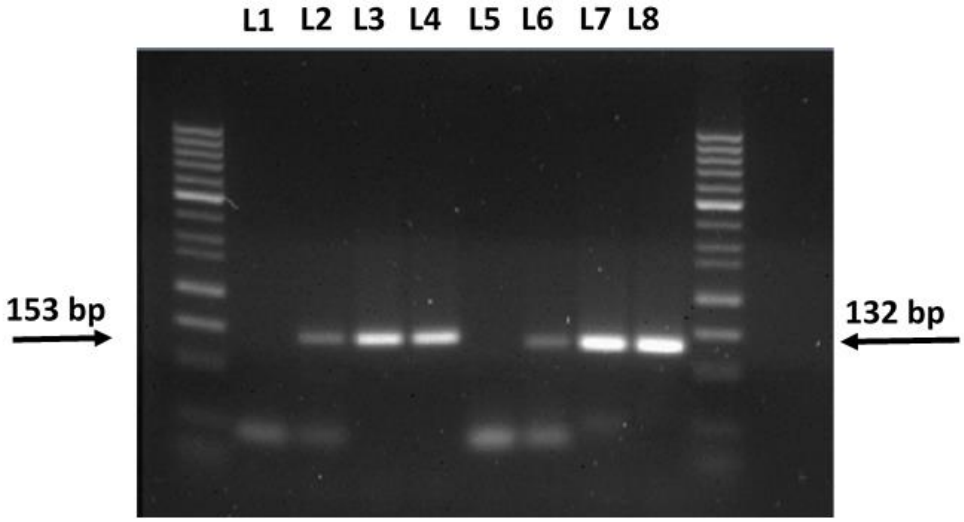
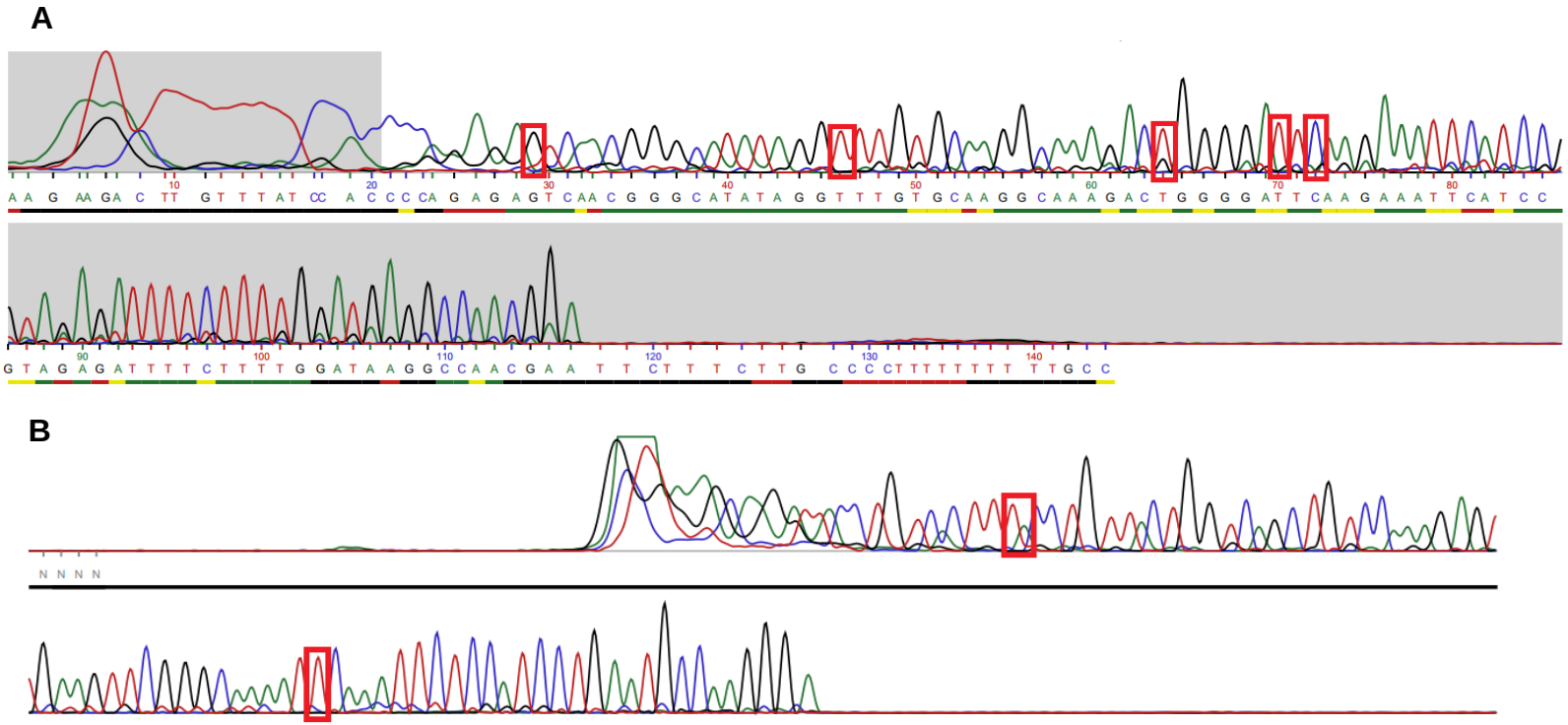


Figure 3.2: Representative image of agarose gel of both primer pairs post PCR clean-up. Arrows depict band size of primer pair set 1, 153 bp (L2-L4), and primer pair set 2, 132 bp (L6-L8). No bands were present in L1 and L5, which were negative PCR controls. A molecular weight size ladder is also included.
L- Lane

3.1.3 Sanger Sequencing of SPOP PCR products

Sequencing sensitivity was assessed using G-blocks, with 50% mutant/WT mix deemed as the sensitivity threshold. Samples were prepared and shipped for Sanger Sequencing according to the Eurofins protocol for purified DNA samples (1 ng/µL in a total volume of 15 µL) in 96 well plates (Methods 2.6.5). Two chromatograms (Figure 3.3) per sample were received, utilising each primer set to span all mutations of interest. Due to the small size of the PCR product, manual base calling was required. Each sequence was compared to the WT SPOP sequence and assessed for the presence of any of the eight SPOP mutations (Figure 3.3). No mutations were noted in either cohort.


A
B
N N N N

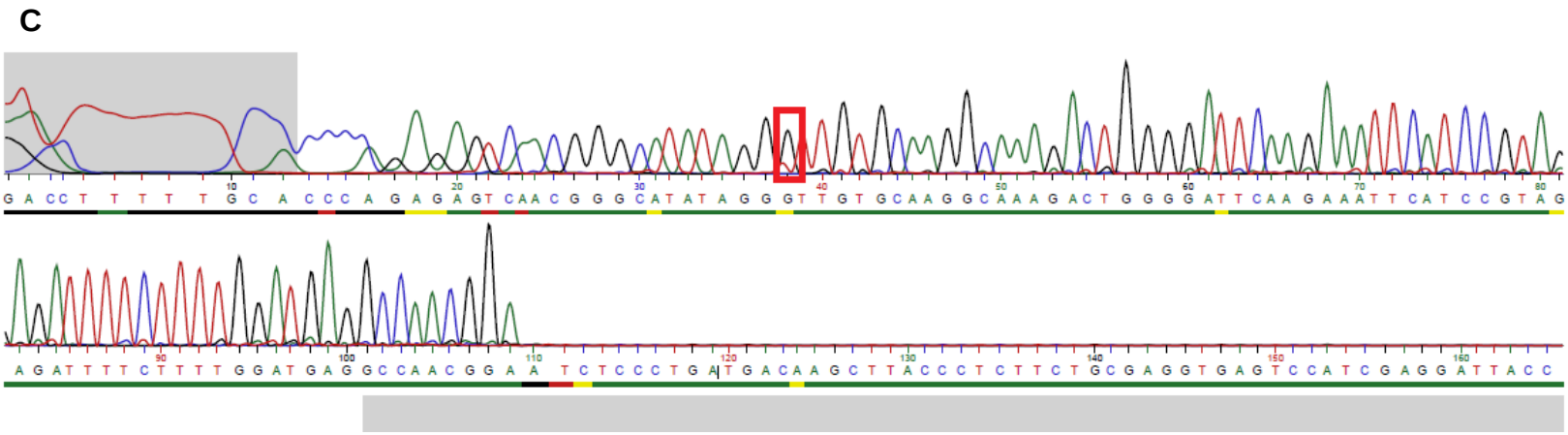


Figure 3.3: Representative image of Sanger sequencing chromatograms.

A) Red boxes highlight position of WT sequence of SPOP mutations of interest (S119N – c.356G>A, F125V – c.373T>G, W131G - c391T>G, F133V – c.397T>G and F133L – c.399C>G).

B) Example of sequence for manual calling due to short sequence length. Red boxes highlight position on WT sequence of SPOP mutations of interest (Y87N – c.259T>A, Y87C – c.260A>G and F102C – c.305T>G).

C) Red box highlights the position of mutation F125V – c.373T>G. Synthetic G-blocks were constructed with 80% mutant, WT mix to demonstrate sequencing sensitivity.

Red peaks associate with T, black with G, green with A and blue with C.

3.1.4 Tissue Microarrays

Sections of previously constructed TMAs from the Irish cohort were provided by the PCRC. TMAs containing the Swedish cohort were constructed using an automated TMA machine. IHC staining was completed using standard protocols as per Methods 2.7 (ERG clone EPR3864, PTEN clone D4.3X). ERG IHC of the Swedish cohort was completed in conjunction with University Hospital Basel. TMA scoring was completed in conjunction with pathologists Prof Stephen Finn (ERG), Dr Danielle Costigan (ERG) and Dr David Cormican (PTEN).

3.1.4.1 ERG

ERG scoring was based on a three tier system; a negative score of 0 (no presence of positive ERG nuclear staining), heterogeneous score of 1, (presence of both negative and positive ERG nuclear staining) and a positive score of 2 (positive ERG nuclear staining) (Figure 3.4). Data was graphed to show the range of expression within the cohorts (Figure 3.5).

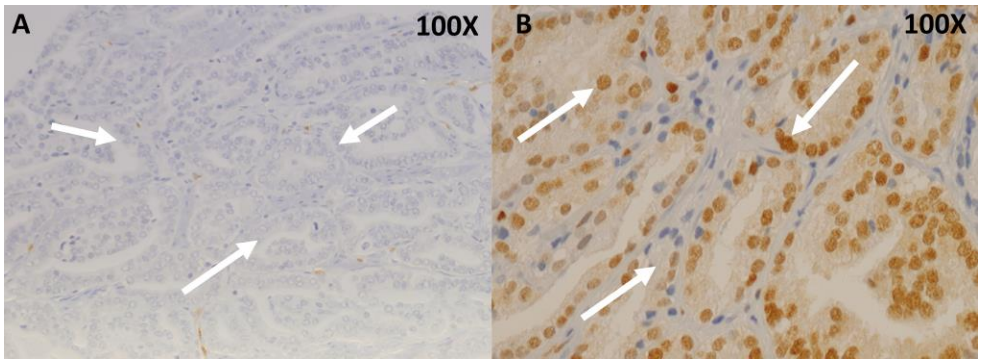


Figure 3.4: Representative ERG TMA core images.

A) Negative ERG nuclear staining.

B) Positive ERG nuclear staining.

Arrows illustrate positive and negative ERG staining. Images are at 100X.

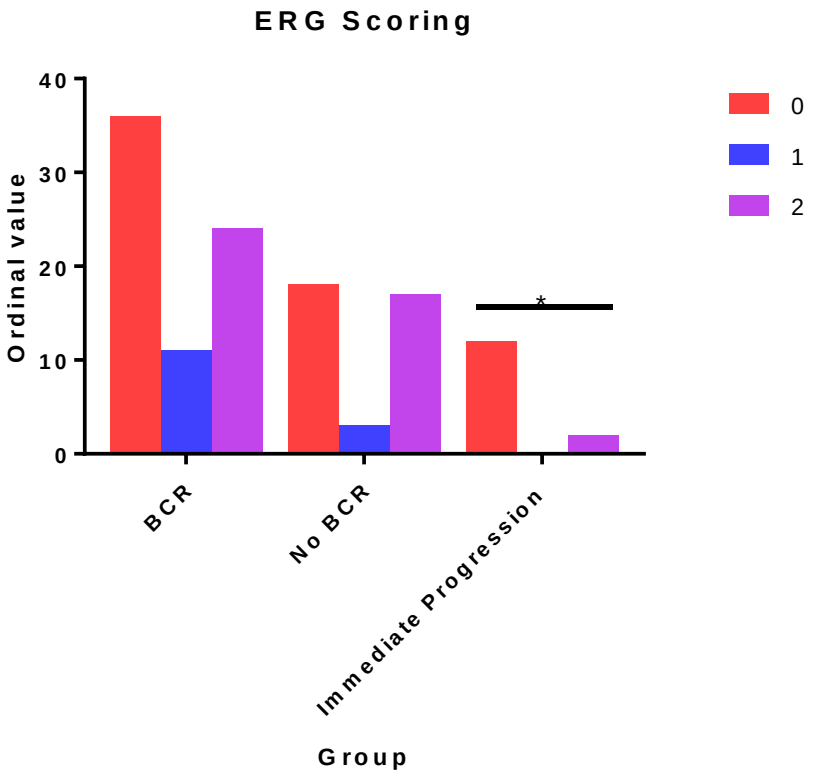


Figure 3.5: ERG expression in all patient groups.

* denotes significant correlation between ERG expression and the immediate progression group

Data grouped as ordinal variables, 0 – no ERG expression, 1 – heterogeneous ERG expression, 2- strongly positive ERG expression.

BCR n=75, *no BCR* n=42 and *immediate progression* (never reached an undetectable PSA post-surgery) n=15.

3.1.4.2 PTEN

PTEN imaging was based on graded intensity of staining, 0-3 (Figure 3.6). PTEN scoring was completed using the H-score system. The H-Score is calculated from the percentages of nuclei classified as 3+, 2+, 1+ (the three positive categories, where 3+ has the highest staining intensity) multiplying them with their grade: H-Score = (Percentage of 3+) x 3 + (Percentage of 2+) x 2 + (Percentage of 1+).Thus the H-Score is a value between 0 and 300 (0 if there are only negative cells, and 300 if all cells are positive with an intense stain), giving an indication of the ratio of positive cells while factoring in staining intensity (Detre et al., 1995) (Figure 3.7).

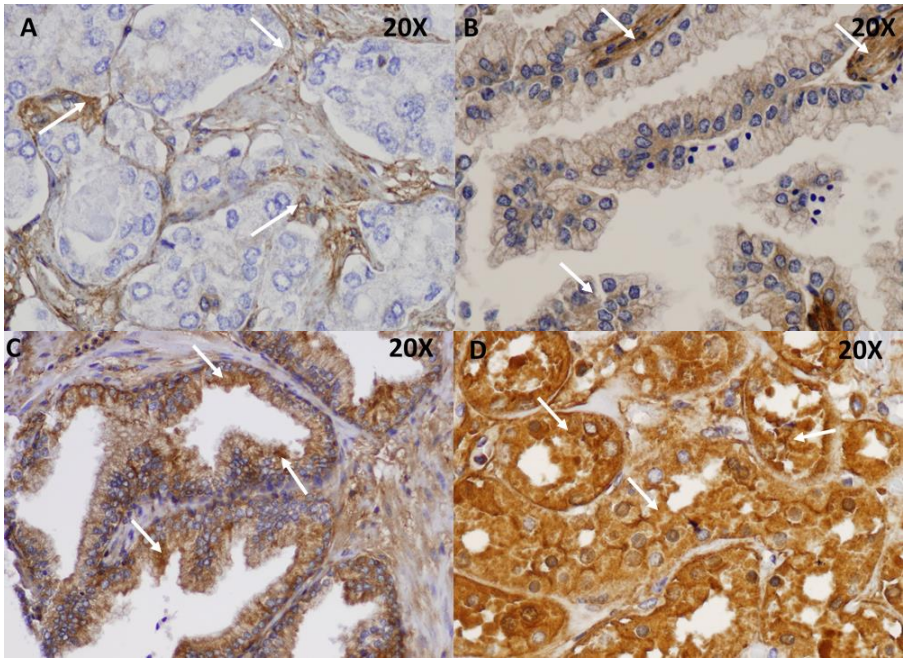


Figure 3.6: Representative TMA core images of cytoplasmic PTEN staining.

Staining intensity was graded 0-3.

A) PTEN grade 0 staining.

B) PTEN grade 1 staining.

C) PTEN grade 2 staining.

D) PTEN grade 3 staining.

Arrows depict grade of staining and all images are at 20X as indicated.

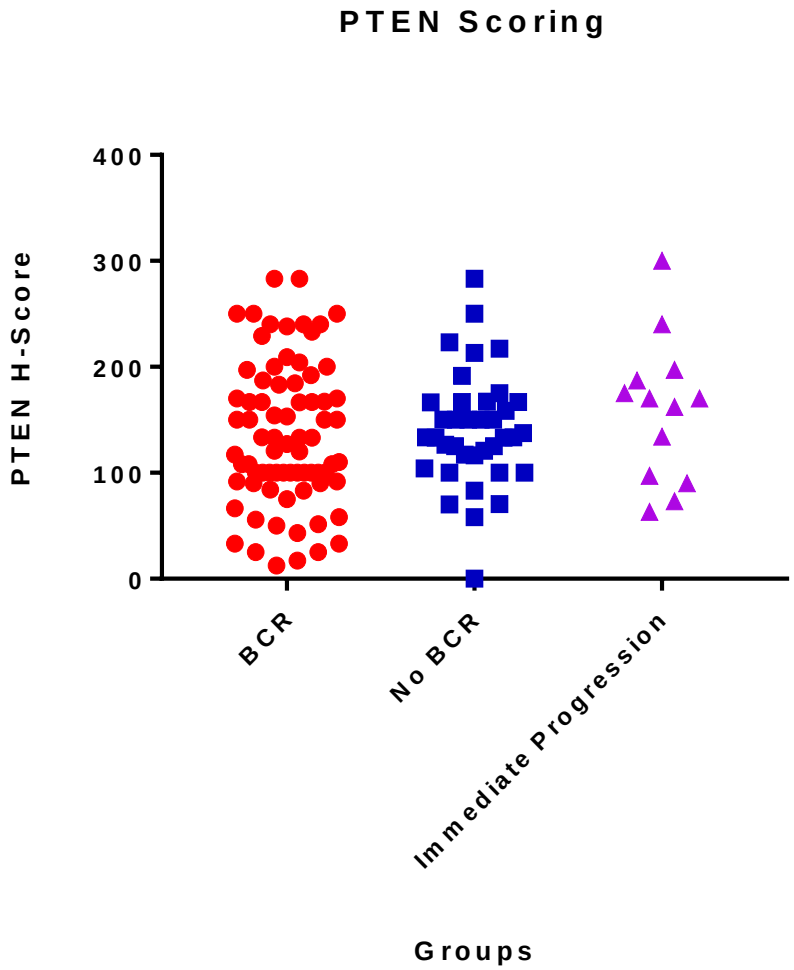


Figure 3.7: PTEN scoring across patient groups.

Data graphed as continuous variables. *BCR* n= 75, *no BCR* n= 42 and *immediate progression* (never reached an undetectable PSA post-surgery) n=15.

3.1.5 Statistical Analysis of ERG and PTEN Expression

Statistical analysis of ERG and PTEN data between groups was performed in conjunction with Dr Jessica Carlsson (Department of Urology, Orebro University, Sweden).

3.1.5.1 Statistical analysis of ERG expression

The difference in ERG expression was compared between groups using a Fisher's exact test as ERG expression was defined as a categorical variable. There was no significant association between ERG score and BCR status (p=0.059). Nominal regression was performed, using *no BCR* as the reference category and a significant correlation between ERG expression and *immediate progression* was detected (p=0.029). The nominal regression analysis was adjusted for age and Gleason grade, and the significant correlation between ERG and *immediate progression* remained true (p=0.033) (Table 3.4). No significant correlation between *no BCR* and *BCR* was detected in either model. PTEN was added to the model, and ERG expression between *no BCR* and *immediate progression* remained significant (p=0.028) (Table 3.5). Kaplan Meier survival analysis was performed to test for any correlation between time to recurrence and ERG expression (Figure 3.8). No significant effect was observed (p=0.405).

Table 3.3: Comparison of ERG expression between patient groups.

	OR	95% CI	p-value
BCR	1.417	0.611-3.283	0.417
Progression	6.137	1.203-31.320	**0.029**

OR- odds ratio, CI- confidence interval, BCR - post-prostatectomy PSA level ≥0.4 ng/mL followed by a second PSA level >0.4 ng/mL, progression – immediate progression after surgery (never reached an undetectable PSA post-surgery)

Table 3.4: Comparison of ERG expression, age and Gleason grade between groups.

	OR	95% CI	p-value
BCR	1.382	0.582-3.277	0.463
Progression	6.064	1.154-31.865	**0.033**

OR- odds ratio, CI- confidence interval, BCR - post-prostatectomy PSA level ≥0.4 ng/mL followed by a second PSA level >0.4 ng/mL, progression – immediate progression after surgery (never reached an undetectable PSA)

Table 3.5: Correlation between ERG expression, PTEN, age and Gleason grade between groups.

	OR	95% CI	p-value
BCR	1.414	0.605-3.303	0.424
Progression	6.278	1.221-32.294	**0.028**

OR- odds ratio, CI- confidence interval, BCR - post-prostatectomy PSA level ≥0.4 ng/mL followed by a second PSA level >0.4 ng/mL, progression – immediate progression after surgery (never reached an undetectable PSA)

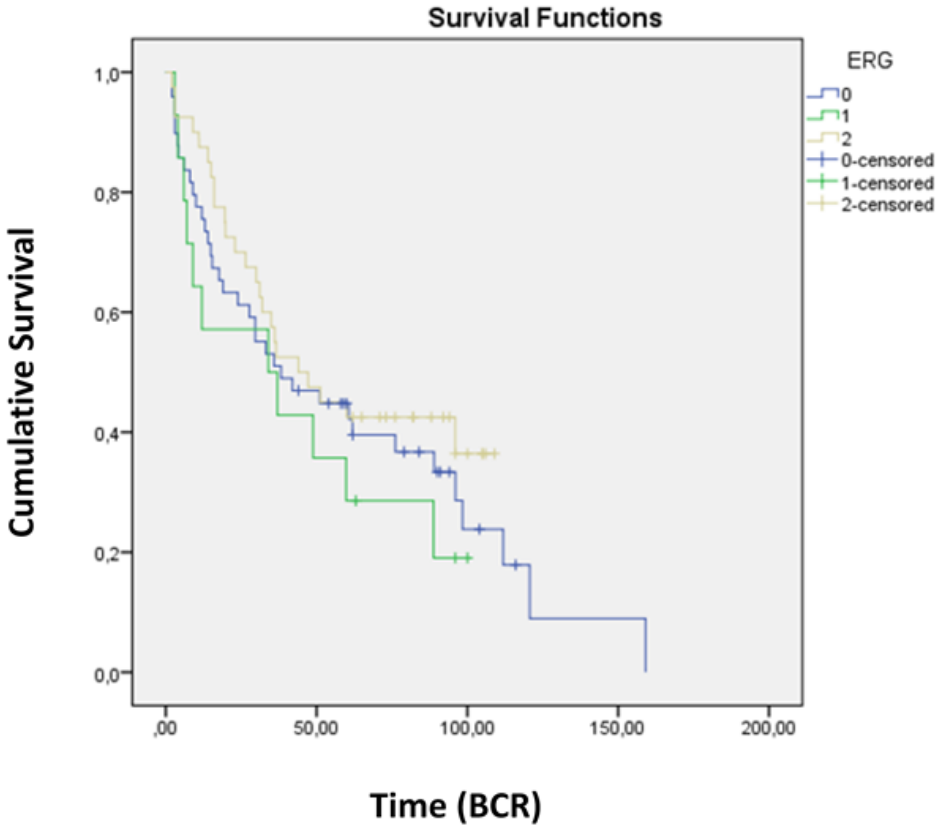


Figure 3.8: Kaplan Meier survival analysis of ERG expression over time between groups.

There was no significant association between time to recurrence and ERG expression observed (p=0.405).

Data grouped as ordinal variables, 0 – no ERG expression, 1 – heterogeneous ERG expression, 2- strongly positive ERG expression

BCR n=75, *no BCR*=42 and *immediate progression* (never reached an undetectable PSA post-surgery) n=15. Censored refers to cases lost during follow up.

3.1.5.2 Statistical analysis of PTEN expression

PTEN scoring was analysed as a continuous variable and was tested for normality. PTEN score was normally distributed (p=0.223) (Figure 3.9) and an ANOVA was performed to assess for an association between groups and PTEN expression. Results from the ANOVA demonstrated no statistical significance in PTEN expression between groups (p=0.997).

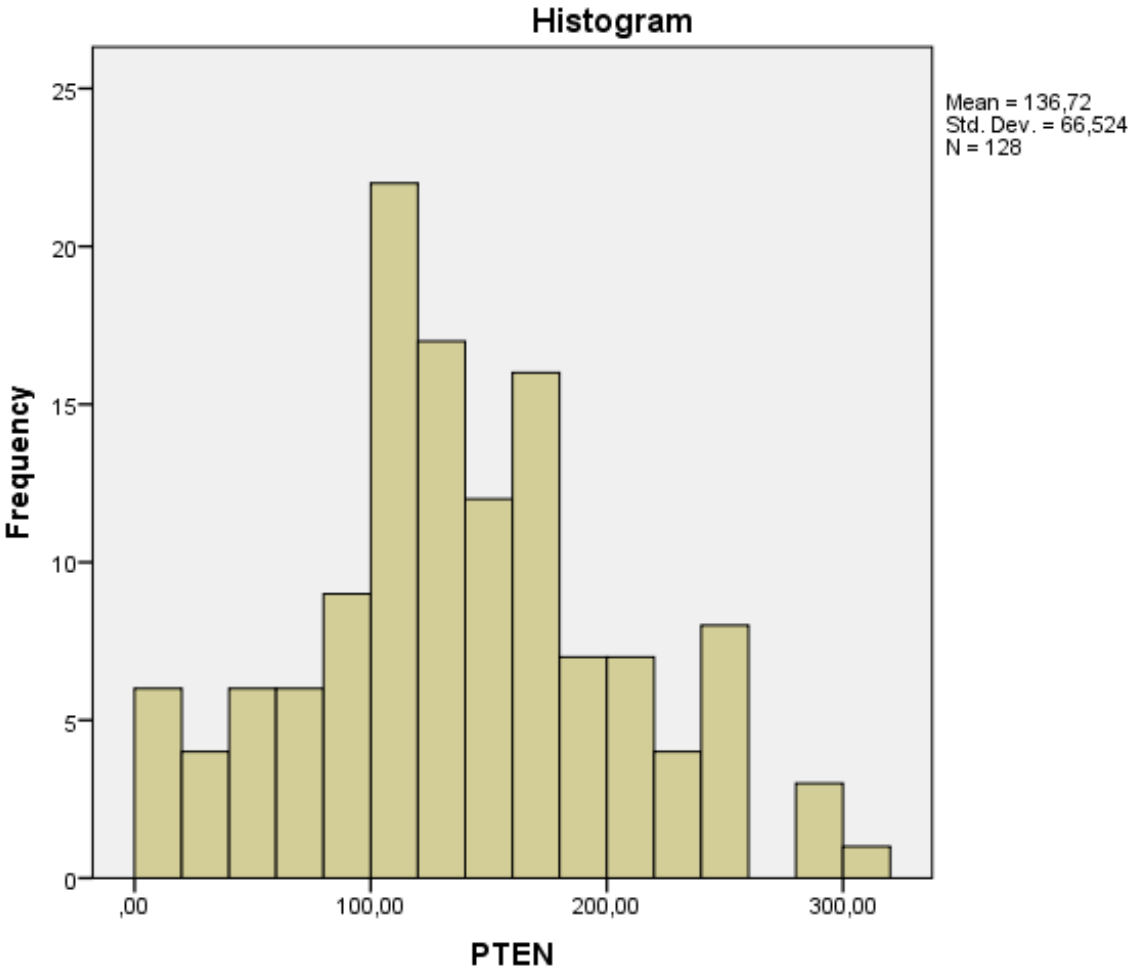


Figure 3.9: Histogram normality plot of PTEN expression. PTEN expression was normally distributed (p=0.223).

Nominal regression was performed using *no BCR* as the reference. No significant difference in PTEN expression between *no BCR* and *BCR,* or *no BCR* and *immediate progression* was established (Table 3.6). Age and Gleason grade were added to the model, however no significance was ascertained after this adjustment (Table 3.7). Kaplan Meier survival analysis was performed to test for any correlation between time to recurrence and PTEN expression (Figure 3.10). No significant effect was observed (p=0.162).

Table 3.6: Association between PTEN expression and patient groups.

	OR	95% CI	p-value
BCR	1.145	0.523-2.507	0.734
Progression	0.787	0.238-2.609	0.696

OR- odds ratio, CI- confidence interval, BCR - post-prostatectomy PSA level ≥0.4 ng/mL followed by a second PSA level >0.4 ng/mL, progression – immediate progression after surgery (never reached an undetectable PSA)

Table 3.7: Association between PTEN, age and Gleason grade between groups

	OR	95% CI	p-value
BCR	1.082	0.489-2.392	0.846
Progression	0.690	0.203-2.346	0.552

OR- odds ratio, CI- confidence interval, BCR - post-prostatectomy PSA level ≥0.4 ng/mL followed by a second PSA level >0.4 ng/mL, progression – immediate progression after surgery (never reached an undetectable PSA)

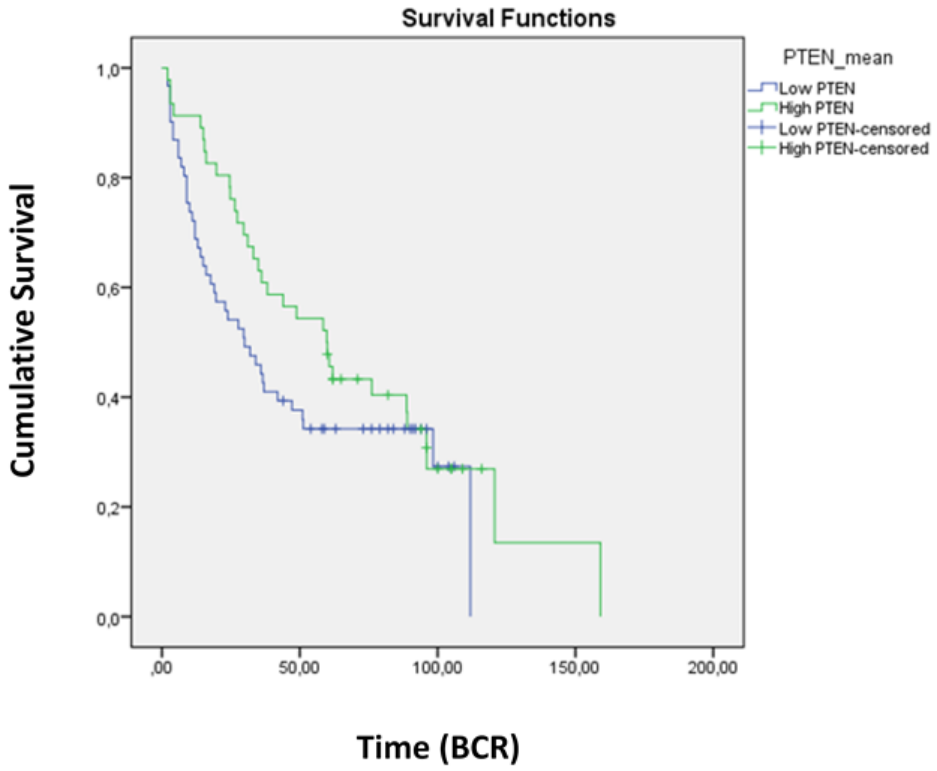


Figure 3.10: Kaplan Meier survival analysis of PTEN expression over time between groups.

There was no significant association between time to recurrence and PTEN expression observed (p=0.162).

BCR n=75, *no BCR*=42 and *immediate progression* (never reached an undetectable PSA post-surgery) n=15.

Censored refers to cases lost during follow up. Low and high PTEN refer to samples above or below the mean PTEN value of all samples.

3.2 Discussion

Molecularly distinct subtypes of PrCa may provide an insight into disease progression and hold the key to improving patient outcomes. The overall aim of this chapter was to determine the presence of a panel of common SPOP mutations in a cohort of patients with and without disease progression (as defined by BCR). In addition to SPOP mutations, common genomic alterations (ERG and PTEN) were also assessed to provide a more advanced molecular profile. The patient cohort for this study included patients from Sweden and Ireland, who were progression free (no BCR), met chosen criteria for BCR (BCR) and never reached an undetectable PSA, i.e. immediately progressed after surgery (immediate progression). In the Swedish cohort of patients, no statistically significant differences were noted between groups in terms of general characteristics; however, a trend towards differences in tumour stage between *BCR* and *no BCR* was apparent. In the Irish cohort of patients, no patients met the criteria for *immediate progression* and were divided based on no BCR and BCR. There was no statistically significant difference between groups. The trend towards a difference in tumour stage, that a more advanced tumour stage may be associated with BCR in this cohort of patients. A meta-analysis examining tumour volume and percent tumour volume in BCR after prostatectomy, indicated significant correlations with BCR (Meng et al., 2015). Further in-depth retrospective analysis into tumour characteristics in this cohort, may provide insight into BCR.

The presence of eight annotated SPOP mutations was assessed in each participant in this cohort. No mutations were detected across both the Irish and Swedish patient populations. SPOP mutations are present in a modest proportion of PrCa cases, approximately 6-13% (Barbieri et al., 2012). The detection of SPOP mutations has generally been determined in patients who are treatment naive prior to radical prostatectomy, inferring a role of SPOP as an early driver in PrCa (Barbieri et al., 2012, Garcia-Flores et al., 2014). Additionally, the prevalence of SPOP mutations in a diverse population of samples, which included patients from America and Europe, determined no significant

differences based on demographic or ethnicity (Blattner et al., 2014). Therefore, the lack of SPOP mutations may infer the presence of other common genomic alterations which can act as a driver for progression. One such example of this is the documented mutual exclusivity between SPOP and ERG (Barbieri et al., 2012). The approximation of SPOP mutations in the general PrCa population is low; therefore increased numbers within future cohorts may increase the probability of detecting SPOP mutations. A limitation of this analysis may be the sensitivity of the sequencing approach. The positive controls outline a sensitivity threshold of 50%, which may infer the lack of detection of heterogeneous mutations present at lower levels. The use of more highly sensitised assays such as competitive allele-specific TaqMan PCR (Cast-PCR), next generation sequencing or exome sequencing, which have demonstrated the ability to detect rare mutations, may increase the sensitivity threshold and improve the detection rate of SPOP mutations.

ERG expression levels were examined across groups in this cohort of patients. ERG expression was assayed by IHC and categorised into expression levels as per standard accepted pathology protocols. In the *no BCR* patient group, the most frequent category of expression was heterogeneous, with similar expression frequency reported in the *BCR* group. Contrastingly, the patient group with *immediate progression* reported no expression of ERG as the most frequent occurrence. Although it did not reach statistical significance, a trend towards an association between ERG expression and BCR status was observed, suggesting a potential relationship between the two variables. This correlation has previously been reported in the literature, with the presence of ERG expression significantly associated with an increased risk of BCR (Huang et al., 2014). A statistically significant correlation between ERG expression and *immediate progression* after radical prostatectomy, when compared to *no BCR* was reported. This finding remained consistent with the addition of co-variates such as age and Gleason grade. There was no significant association between ERG expression and *BCR*, when compared with *no BCR*. Kaplan Meier survival analysis was in agreement with this outcome, as no significant

relationship between ERG expression and overall time to progression was established. This analysis suggests a potential role for ERG expression as a predictor of disease progression in this profile of patients with PrCa. The presence of ERG fusions, have previously been significantly associated with disease recurrence in patients with PrCa (Nam et al., 2007). The data witnessed in this study is in contrast to the literature, with lack of ERG expression the most common category in the cohort of patients with *immediate progression*. A limitation of this analysis may be the small number of patients within this cohort (n=15), with larger validation cohorts required to confirm these findings and to further elucidate the potential of ERG expression in disease monitoring.

PTEN expression was scored using the recognised H-score system and thus was assessed as a continuous variable. The range of expression of H-score is from 0-300 and expression levels appeared similar across all three comparative groups. No statistical significance was observed between PTEN expression and BCR status. When examined as a potential predictor of recurrence, no significant association was reported between PTEN status and *BCR*, or PTEN status and *immediate progression*. This observation remained constant, with Kaplan Meier survival analysis suggesting no significant relationship between PTEN status and overall time to recurrence. A recent systematic review of PTEN expression and BCR, reported a significant association between positive expression of PTEN and lower risk of BCR, with PTEN loss associated with increased likelihood of BCR (Xie et al., 2017). The majority of patients in this study reported some level of PTEN expression, which suggests compliance with the literature. PTEN and ERG expression levels were combined in the same statistical model, with the significant relationship between ERG expression and *immediate progression* remaining constant. This finding may suggest a role between ERG negative cancers and a shorter time to *immediate progression* after radical prostatectomy. The elucidation of the molecular profiles associated with disease progression in PrCa may aid in the development of improved therapeutic targets and improve the overall survival of

patients at risk of developing metastatic cancer and for those living with advanced disease.

Chapter 4:

Circulating Tumour Cells and Platelet Cloaking

4.0 Introduction

CTCs represent a morphologically distinct subset of cancer cells, which aid in metastatic spread. A study by Park et al. identified recognisable differences in characteristics between CTCs isolated from patients with metastatic CRPC and prostate cancer cells lines (Park et al., 2014). CRPC CTCs demonstrated a significant size difference in comparison to the cultured cells, in addition to an irregular, heterogeneous shape and a greater nuclear to cytoplasmic ratio (Park et al., 2014). As outlined previously in section 1.2, due to the heterogeneous nature of CTCs, there is no standard detection method. CellSearch® is the only FDA approved (enumeration only) isolation system, based on EpCAM coated immunomagnetic beads, and morphological analysis is generally combined with additional staining, resulting in an EpCAM and cytokeratin positive, and CD45 negative phenotype (Ligthart et al., 2013). A study by Adams et al. replicated CellSearch® output, by utilising a size based filtration method coupled with further staining criteria (Adams et al., 2015). Multiple studies using the size based filtration system, ScreenCell®, have demonstrated efficient capture and isolation of CTCs in ovarian, colorectal, lung and prostate cancers, with some cases identifying increased CTC yields when compared to CellSearch® (Kruspe et al., 2017, Nicolazzo et al., 2017, Coco et al., 2017, Wark et al., 2017). Therefore size based filtration, may allow for the capture of a heterogeneous population of CTCs.

The clinical significance of CTCs in advanced PrCa lies in their potential for use as both prognostic and diagnostic liquid based markers. Recent studies have examined CTC number in terms of overall survival (OS) in patients with PrCa. SWOG 0421 was a phase III trial for patients with metastatic CRPC randomly assigned to docetaxel with or without atrasentan (Goldkorn et al., 2014). In both study arms, high levels of CTCs at baseline were associated with an elevated PSA and increased bone pain, with an increase in CTC number over time highly correlated with worse OS (Goldkorn et al., 2014). Data from two prospective clinical trials for men with metastatic CRPC undergoing chemotherapy also determined that a 30% decrease in CTC number was associated with improved OS following treatment with abiraterone and chemotherapy,

suggesting CTCs as a surrogate marker to monitor treatment response (Lorente et al., 2016). The detection of TMPPRSS2-ERG fusions and AR gene status have been positively identified in CTCs in the blood of men with metastatic CRPC (Danila et al., 2011, Podolak et al., 2017). Furthermore, synaptophysin expression, a recognised marker of neuroendocrine differentiation in PrCa, was identified in CTCs isolated from patients undergoing treatment with abiraterone acetate or enzalutamide, with increased expression associated with resistance to these therapies (Pal et al., 2017). Additionally, a landmark paper by Antonarakis et al. detected the presence of AR-V7 on CTCs isolated from patients who demonstrated resistance to enzalutamide and abiraterone (Antonarakis et al., 2014). These data suggests the possibility of using CTC number to predict OS and treatment response for patients with metastatic PrCa.

The mechanisms, by which CTCs extravasate undetected by the immune system, is one that is poorly understood. One proposed mechanism relates to the 'platelet cloaking' of cancer cells. Platelets are understood to play a role in increasing the invasive potential of cancer cells (Bambace and Holmes, 2011). This was determined using ovarian cancer cell line, SK-OV-3 (Cooke et al., 2015). This study demonstrated significant platelet adhesion and activation when combined with cancer cells, suggesting a reliance of SK-OV-3 on platelets to aid extravasation (Cooke et al., 2015). This study also assessed the link between aspirin use and platelet inhibition. The introduction of SK-OV-3 cells to platelets treated with aspirin, caused a significant decrease in invasive activity of the cancer cells (Cooke et al., 2015). In CVD, aspirin is utilised as an anti-platelet agent, whereby it inhibits COX-1 function, essential for platelet aggregation (Gurbel et al., 2007). This leads to the hypothesis that aspirin use may play a role in inhibiting platelet cloaking of cancer cells, which may in turn aid the detection of CTCs by the immune system and increase CTC destruction in circulation. This increasing body of evidence on the relationship between platelets and cancer cells, highlights the need for further investigation into the possible role platelets have in the development of metastatic PrCa.

A substantial body of evidence exists highlighting the benefits of physical activity in metastatic PrCa, however little is known about the impact of physical activity on CTCs. Cardiovascular/aerobic exercise can increase endothelial shear stress, which can improve vascular function (Niebauer and Cooke, 1996). A microfluidic device using cells derived from breast, lung and ovarian cancers, replicated physical activity using relevant high shear stress conditions that induced the destruction of CTCs in circulation by necrosis and apoptosis (Regmi et al., 2017). It was hypothesised that cancer patients could mimic high shear stress conditions with exercise to target CTCs in this manner (Regmi et al., 2017). Based on the current literature, the ExPeCT trial was developed to improve our understanding of CTCs, platelet cloaking and the effect of exercise in a population of men with metastatic PrCa. The primary endpoint for ExPeCT was the isolation and enumeration of CTCs and platelet cloaking in both study arms (Figure 4.1). The aims for this chapter were a) to assess size based *vs*. EpCAM based isolation methodology for effective CTC isolation, b) to enumerate CTCs and platelet cloaking present in all participants, c) to correlate baseline CTC and platelet cloaking numbers with baseline BMI, d) to determine the impact of exercise on CTC number and platelet cloaking and e) to investigate any association between clinical data and CTCs/platelet cloaking.

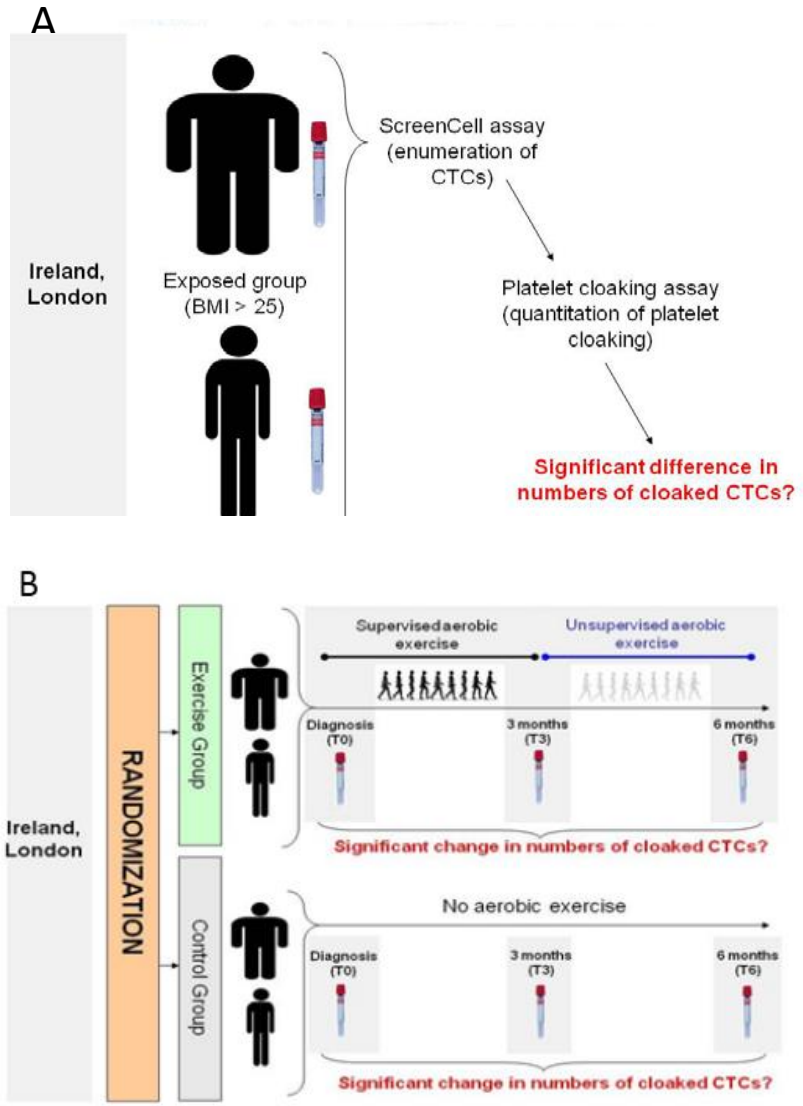


Figure 4.1: Overview of CTCs and platelet cloaking as the primary endpoint in the ExPeCT trial.A) Outlines the enumeration of CTCs and platelet cloaking in all participants and the correlation to baseline BMI and B) summarises the comparison of CTCs and platelet cloaking between ExPeCT exercise and control groups.

CTCs from all sites were isolated using the same protocol and enumeration was performed by the same pathologist in Cork University Hospital.

4.1 Results

4.1.1 CTC platform comparison

A pilot CTC platform comparison study was carried out using two contrasting techniques; Isoflux which utilises EpCAM coated immunomagnetic beads and ScreenCell®, a size based filtration method. Approximately 10, 50 and 100 PC-3 cells were added to healthy donor blood. The blood and tumour cell mix was then prepared according to the specified protocol instructions for both ScreenCell® and Isoflux (Methods 2.9-2.10). Standard MGG staining was performed on ScreenCell® filters. CellTracker Green was added to cells isolated from the Isoflux platform. Cells were visualised microscopically and enumerated (Figure 4.2). PC-3 cells spiked onto the Isoflux platform yielded capture efficiency rates ranging from 66%-80% with an overall capture efficiency rate of 74% (Table 4.1). PC-3 cells spiked onto ScreenCell® filters generated capture efficiency rates ranging from 70%-93%, with an overall capture efficiency rate of 80% (Table 4.1).

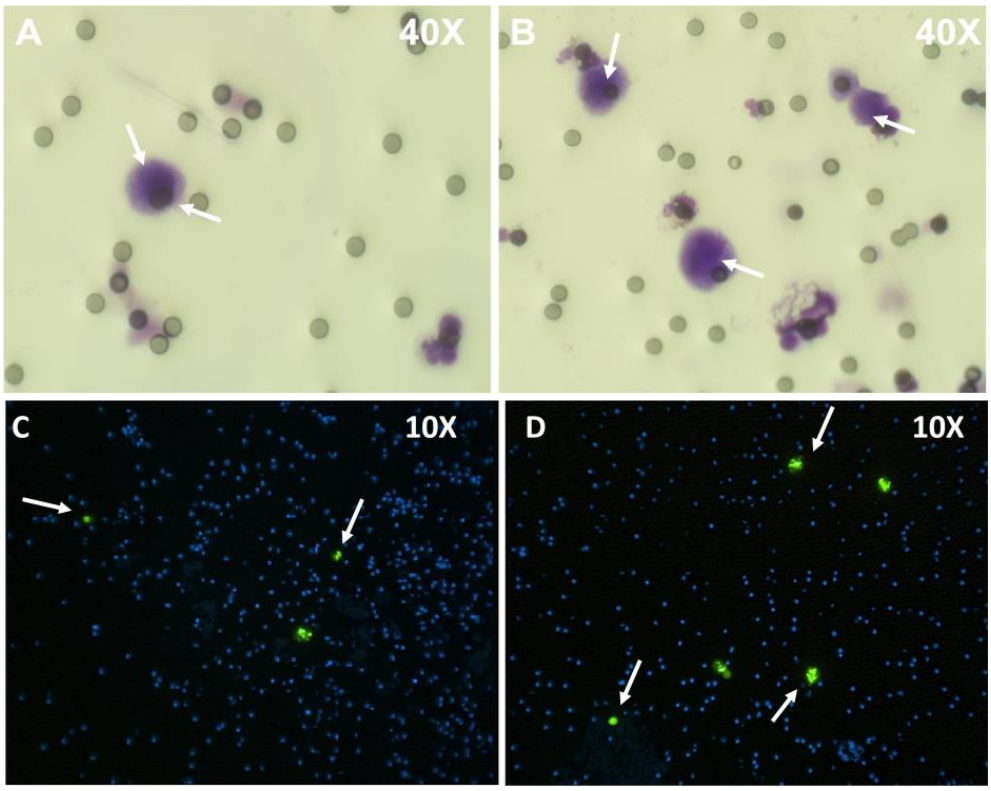


Figure 4.2: Representative images of PC-3 cells captured using ScreenCell® filters and Isoflux EpCAM immunomagnetic bead technology.

A) ScreenCell® filter with 50 PC-3 cells spiked into blood.

B) ScreenCell® filter with 100 PC-3 cells spiked into blood.

Arrows depict PC-3 cells stained with MGG in both images.

C) Isoflux, glass slide with 10 PC-3 cells spiked into blood.

D) Isoflux, glass slide with 50 PC-3 cells spiked into blood.

Arrows depict PC-3 cells visualised with CellTracker Green under a 10X FITC filter. Auto fluorescence of pores is evident on both Isoflux images (blue) under merged FITC and DAPI filters.

Table 4.1: Capture efficiency rates of CTC detection platforms using whole blood spiked with PrCa cells. PC-3 cells spiked onto the Isoflux platform and the resulting capture efficiency rates and PC-3 cells spiked on the ScreenCell® filters and the resulting capture efficiency rates.

Platform	Amount of Cells Spiked (approx.)	Number of Cells Recovered (approx.)	Capture Efficiency Rate (%)
Isoflux	10	8	80
	50	33	66
	100	75	75
ScreenCell®	10	7	70
	50	38	76
	100	93	93

4.1.2 Optimisation of morphological staining and cover slipping on ScreenCell® filters

ScreenCell® protocols recommend the use of MGG staining for morphological identification of CTCs over H & E staining. MGG staining was performed on PC-3 cells spiked into healthy donor blood and filtered according to the ScreenCell® protocol. Multiple fixative solutions were utilised to adhere coverslips to the ScreenCell® filters. However, each fixative damaged the integrity of the stain and as a result, filters were assessed without coverslips. Secondary morphological staining was attempted using papanicolaou morphological staining on PC-3 cells. The resulting stains were compared and MGG chosen for effective reproducibility and powerful morphological identification of PrCa cells.

4.1.3 Immunohistochemistry optimisation using PrCa Cell lines

A range of cytoplasmic and nuclear markers were chosen for the positive identification of CTCs, post morphological analysis, based on their expression in PrCa. These markers included cytokeratin, EpCAM, PSMA, ERG, CD45 and AR. IHC optimisation was performed, using prostate tissue for EpCAM, PSMA, ERG and AR, and tonsil for CD45 (Figure 4.3). Upon successful optimisation, cell blocks were made from PrCa cell lines: PC-3, DU145, LNCaP, VCAP and 22Rv1, and stained for the various markers (Figure 4.4). PrCa cells lines were chosen based on their expression of the antigen of interest and spiked into healthy donor blood and filtered onto ScreenCell® membranes. The filters were stained with MGG to identify their morphology and to ensure capture of the PrCa cells. MGG staining was removed during washing steps as per Methods 2.16.1 and IHC performed as per Methods 2.7 (Figure 4.5). Positive antigen identification was performed in conjunction with pathologist Dr Tatjana Vlajnic. IHC staining was difficult to reproduce for all markers, however cytokeratin and EpCAM were somewhat more successful

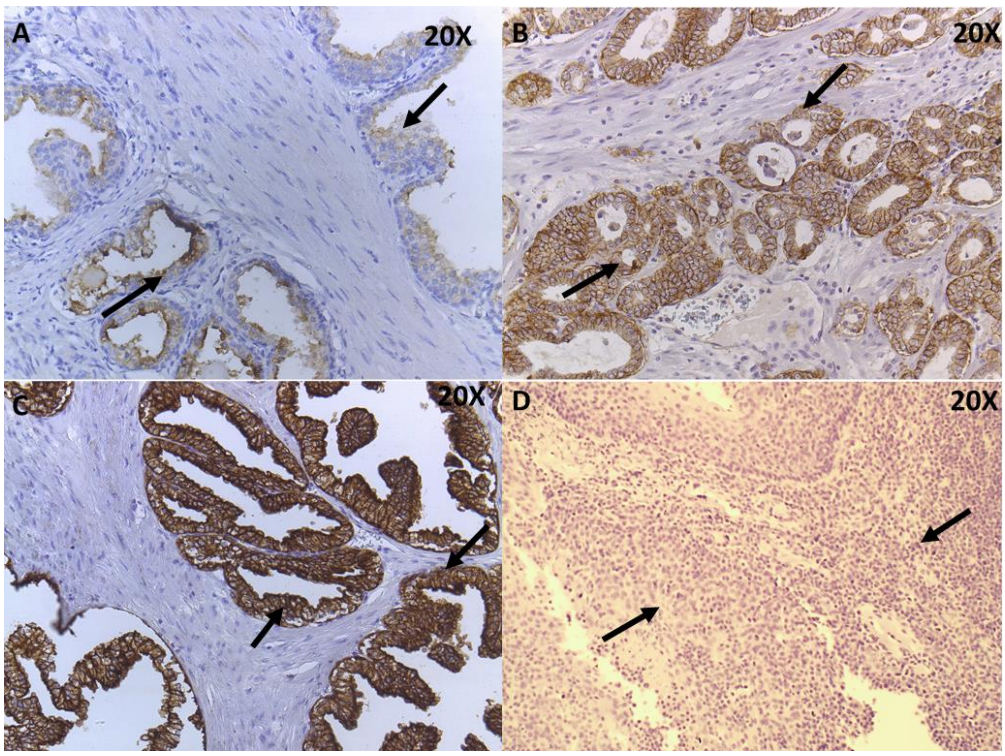


Figure 4.3: IHC staining using prostate specific markers on human tissue.

A) Arrows demonstrate positive cytoplasmic PSMA staining (20X).

B) Arrows portray positive cytoplasmic EpCAM staining (20X).

C) Arrows depict positive cytoplasmic cytokeratin staining (20X).

D) Representative negative image (Tonsil). Arrows illustrate negative CD45 staining.

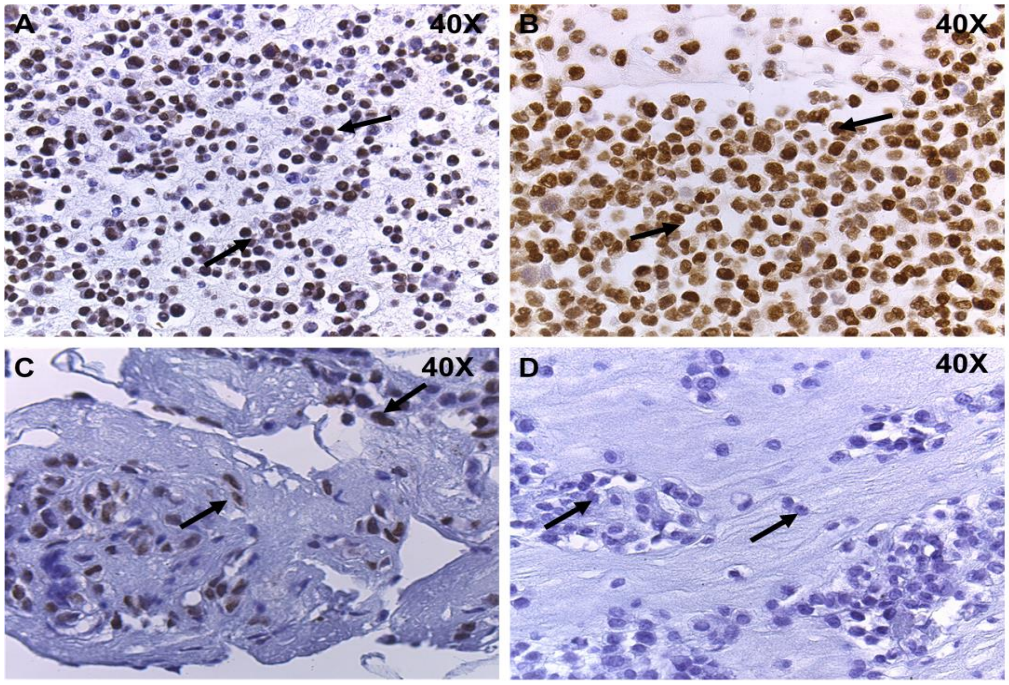


Figure 4.4: Representative IHC staining of prostate cancer cell blocks.

A) Arrows depict AR positive staining in 22RV1 cells.

B) Arrows portray positive ERG staining in VCaP cells.

C) Arrows illustrate positive AR staining in LNCaP cells.

D) Representative negative image. Arrows demonstrate negative AR staining in DU145 cells.

Negative controls were included for every IHC experiment.

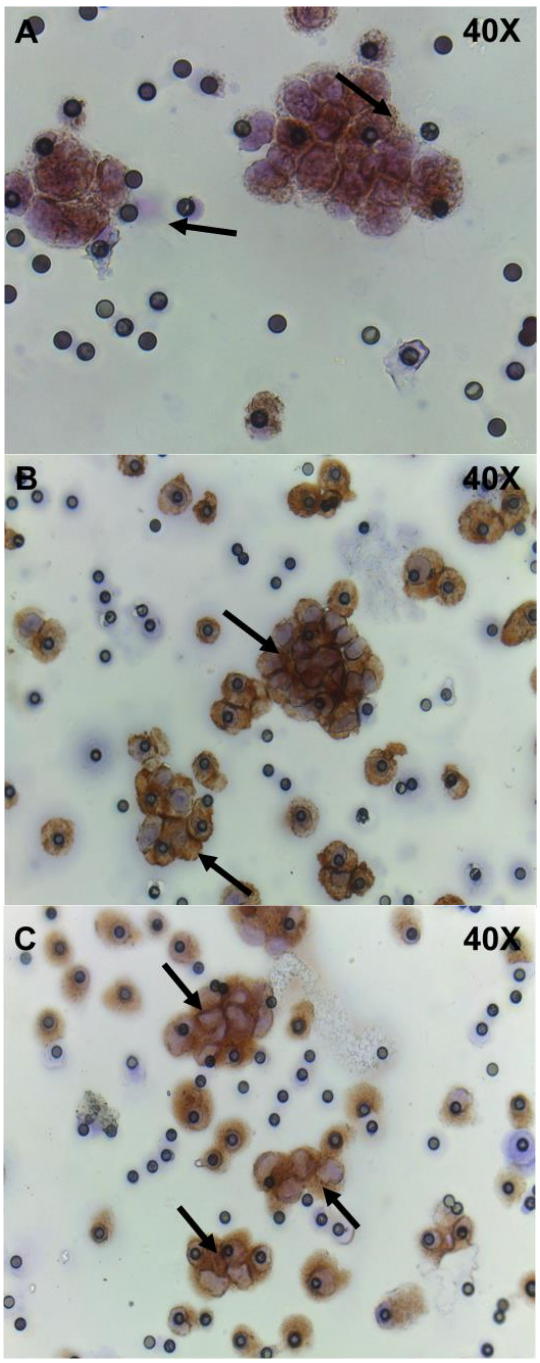


Figure 4.5: Representative IHC staining of prostate cancer cell line LNCaP on ScreenCell® filters.

A) Positive EpCAM staining.

B) Positive cytokeratin staining.

C) Positive PSMA staining.

All images were at 40X, with positive staining depicted by arrows.

4.1.4 Platelet cloaking of PrCa lines

Surplus human platelets were acquired from the Irish Blood Transfusion Service according to an authorised clinical indemnity form. One mL platelets were mixed with 1 x 10^6 cells/mL DU145 to create a tumour/platelet suspension and allowed to incubate at RT for 10, 15, 30, 45 and 60 min. Ten µL, 50 µL, 100 µL, 250 µL and 500 µL of this mix was added into 3 mL healthy donor blood and filtrated according to the ScreenCell® protocol. Filters were stained with MGG and analysed by a pathologist using clinically accepted cytology-based morphological criteria for the presence of platelet cloaking. A 50 µL cell/platelet suspension incubated at RT for 45 min produced optimum results. Platelets were present throughout the filters in aggregates on the membrane as well as in aggregates surrounding tumour cells (Figure 4.6).

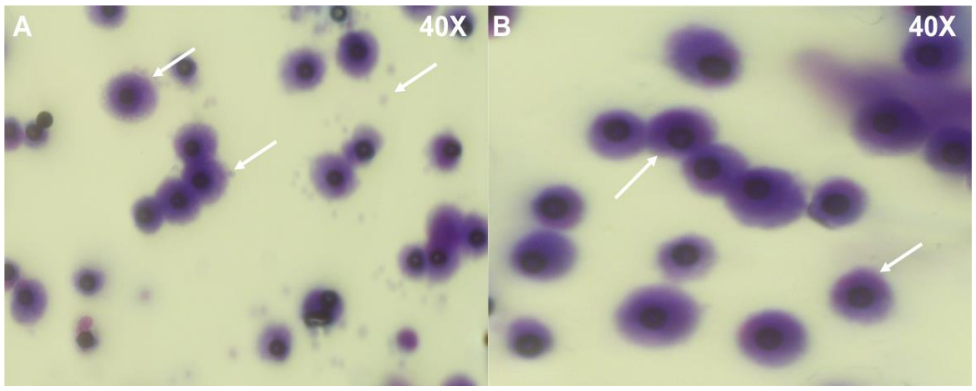


Figure 4.6: Platelet cloaking in normal and tumour prostate cells.
A) arrows demonstrate platelets cloaking DU145 tumour cells and platelets adhered to the ScreenCell® filter and B) arrows depict normal prostate cell line, RPWEI.

Cloaking of DU145 cells was compared with a normal cell line, RPWEI. The total number of cells in four fields of view was counted by two independent observers, along with the number of cells displaying platelet cloaking, and the results averaged. A percentage of platelet cloaking was calculated based on the number of cloaked cells per total number of cells. Normal prostate cells displayed reduced cloaking compared to tumour cells (p=0.0571) (Figure 4.7). IHC was performed on the platelet cloaked filters for positive identification using CD42a. Results were sub-optimal with non-specific staining present, regardless of known platelets being present.

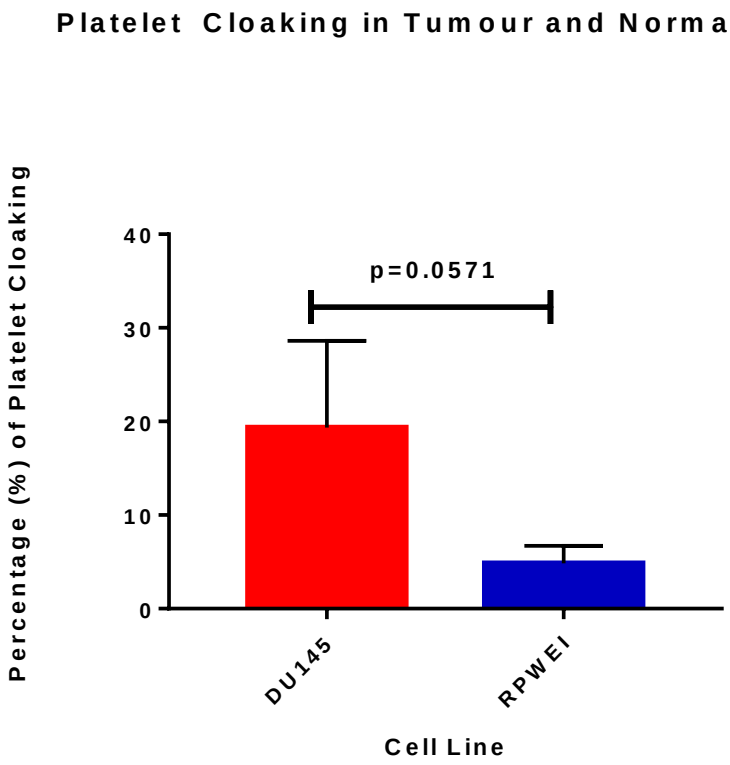


Figure 4.7: Percentage of platelet cloaking in tumour and normal prostate cells.

Data is graphed as mean ± SEM (n=3). Analysis was performed using a student's two tailed *t*-test.

4.1.5 ExPeCT trial participants

Patients with metastatic PrCa who met the inclusion criteria for ExPeCT were consented in five Irish sites and one UK site. All consented participants were randomised into either the exercise or control arms (and those participants randomised to the exercise arm underwent six months of aerobic exercise (Figure 4.8)). Whole blood (16 mL) was collected into k2EDTA tubes at three time points, T0, T3 and T6, for all participants, with a maximum of four ScreenCell® filters processed per patient. The differences between exercise and control groups for participant characteristics were evaluated using independent t-tests and Wilcoxon Signed Rank tests. Table 4.2 outlines age, Gleason grade and PSA over time of ExPeCT participants. No significant differences were observed between groups. Table 4.3 outlines clinical variables, BMI and waist circumference, in all participants at each time point. No significant differences were witnessed between groups. Table 4.4 demonstrates routine clinical blood test results, haemoglobin, platelet count and white cell count, for all participants. No significant difference was observed between groups at each time point.

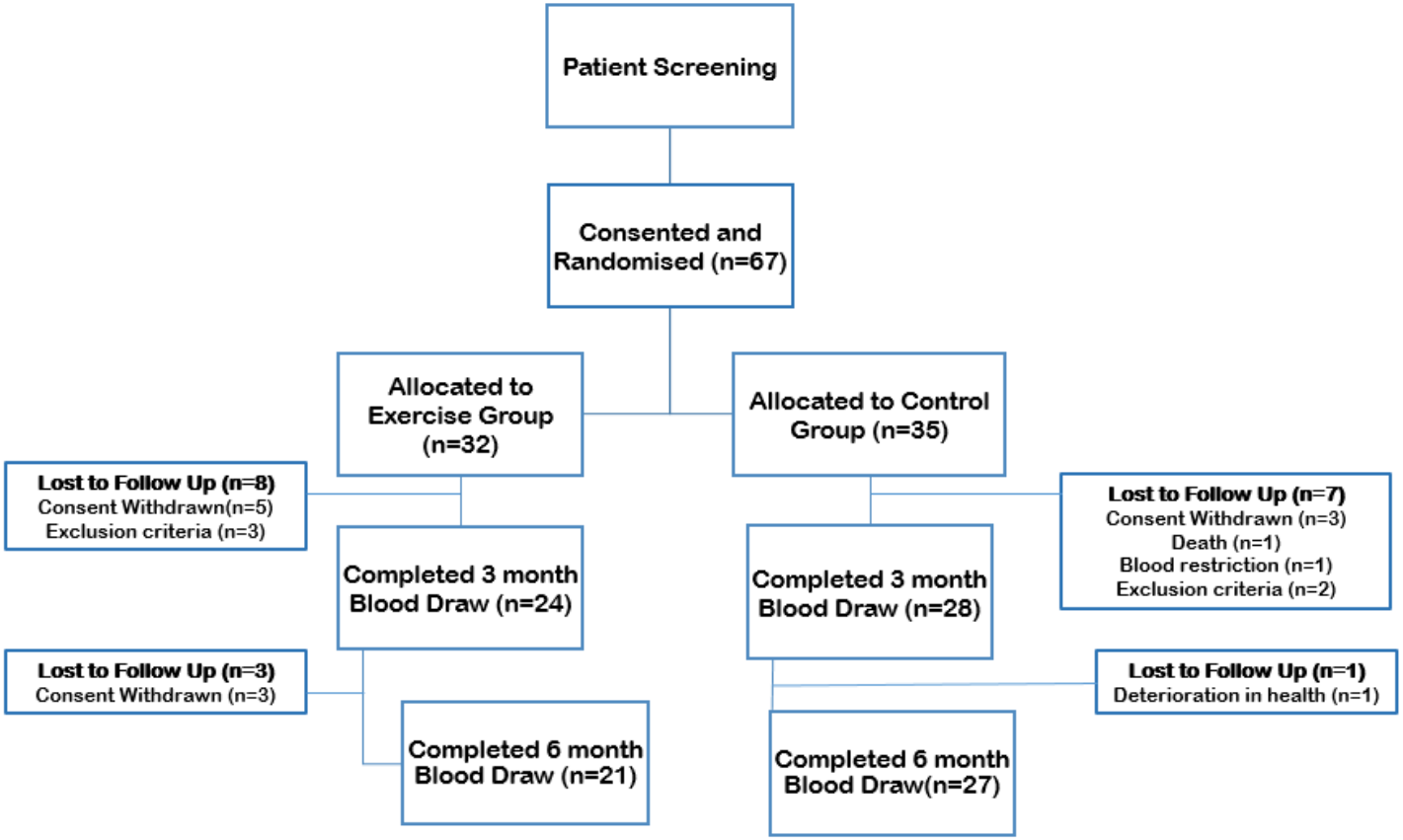


Figure 4.8: Flowchart of ExPeCT participant involvement.

Table 4.2: ExPeCT Participant General Characteristics.

		All cases (n=61)	Exercise (n=29)	Control (N=32)	p-value
Age at Baseline (T0)	Mean Median Missing	69.8 69 (51-86) 0	70.1 69 (56-86) 0	69.6 69 (51-84) 0	0.734
Time Since Diagnosis (months)	Mean Median Missing	34 25 (4-128) 3	36 25 (4-120) 2	33 12 (4-128) 1	0.976
Gleason Score	6 7 8 9 Missing	0 7 (13.2%) 20 (37.7%) 26 (49.1%) 8	0 3 (12.5%) 11 (45.8%) 10 (41.7%) 5	0 4 (13.8%) 9 (31%) 16 (55.2%) 3	0.535
PSA (ng/mL) Baseline T0	Mean Median Missing	42.6 1.45 (0.01-116.3) 2	20.2 2.32 (0.03-272) 0	64.2 0.63 (0.01-1438) 2	0.737
PSA (ng/mL) Three Months (T3)	Mean Median Missing	10.4 0.97 (0.01-116.3) 11	10.8 3.5 (0.03-82.6) 7	10 0.55 (0.01-116.3) 4	0.709
PSA (ng/mL) Six Months (T6)	Mean Median Missing	13.1 1.91 (0.01-178) 15	17.8 5.1 (0.03-178) 10	9.8 1 (0.01-97.2) 5	0.492

n=61, as 6 consented participants did not meet inclusion criteria

Table 4.3: ExPeCT Participant Waist Circumference and BMI.

		All cases (n=61)	Exercise (n=29)	Control (n=32)	p-value
BMI at Baseline (T0)	Mean Median Missing	29.2 28.4 (21-38.9) 0	28.5 28.1 (21-38.9) 0	29.9 29 (22.7-37.3) 0	0.165
BMI at Three Months (T3)	Mean Median Missing	29.1 28.5 (21.7-39.1) 11	28.5 28.3 (21.7-39.1) 6	29.6 28.6 (21.7-39.1) 5	0.271
BMI at Six Months (T6)	Mean Median Missing	29.3 28.9 (21.7-39.1) 14	28.6 28.8 (21.7-39.1) 7	29.94 28.9 (22.1 - 39.1) 7	0.199
Waist Circumference (cm) Baseline (T0)	Mean Median Missing	102.3 101 (72-126) 0	100.5 100.3 (72-124) 0	104 101 (80-126) 0	0.387
Waist Circumference (cm) Three Months (T3)	Mean Median Missing	101.3 99 (76-128) 11	100.5 97.25 (76-120) 5	102 99 (79-128) 6	0.681
Waist Circumference (cm) Six Months (T6)	Mean Median Missing	101.8 99.8 (76-128) 13	101.3 95 (76-119) 7	102.2 100 (80-128) 6	0.586

Table 4.4 ExPeCT Participant Clinical Blood Measurements.

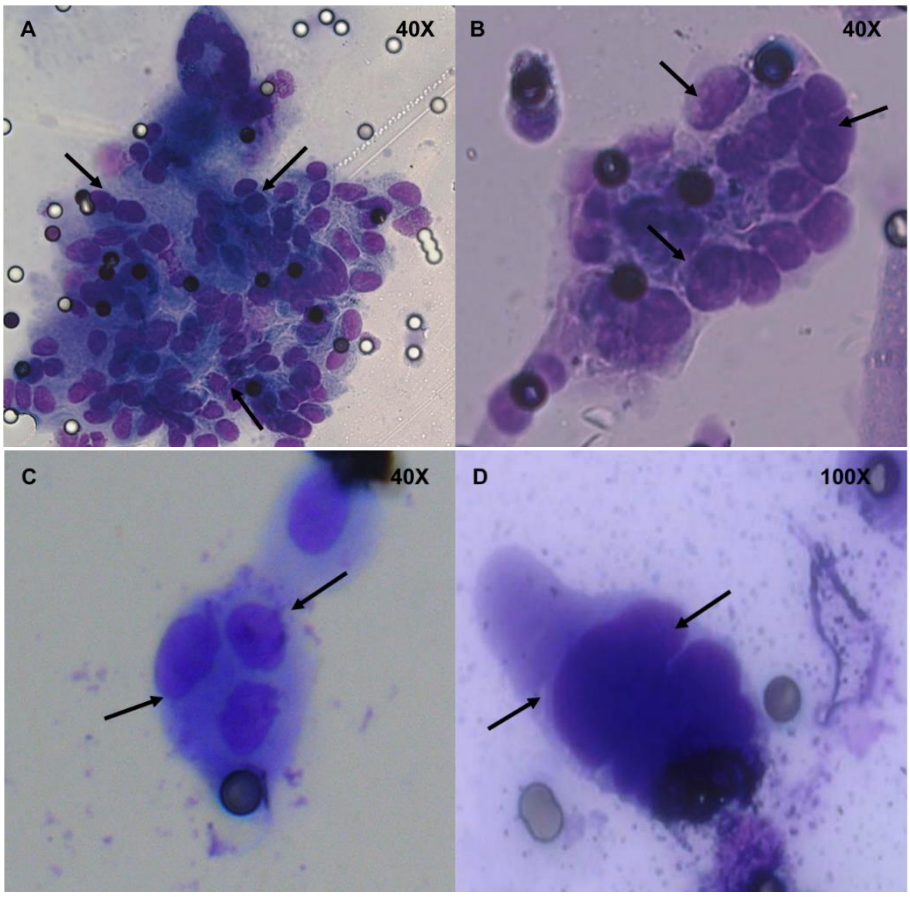
		All cases (n=61)	Exercise (n=29)	Control (n=32)	p-value
Haemoglobin (g/L) Baseline (T0)	Mean Median Missing	82 112 (7.1-112) 5	83.3 118 (10.7-159) 2	80.8 107 (7.1-150) 3	0.419
Haemoglobin (g/L) Three Months (T3)	Mean Median Missing	73.2 107 (10.1-150) 11	69 15.3 (13.5-149) 7	76.4 108.5 (10.4-150) 4	1.000
Haemoglobin (g/L) Six Months (T6)	Mean Median Missing	62.8 1.91 (10.1-148) 15	58.4 14.5 (10.1-148) 10	65.7 13.8 (10.4-148) 5	0.979
White Cell Count Baseline (cells/cmm^3) (T0)	Mean Median Missing	7.7 6.6 (2.1-37.5) 5	7.2 6.7 (4.3-15) 2	8.1 6.1 (2.1-37.5) 3	0.932
White Cell Count Three Months (cells/cmm^3) (T3)	Mean Median Missing	6.6 5.9 (3-17) 11	6.5 6 (3.6-13.7) 7	6.6 5.8 (3-17) 4	0.823
White Cell Count Six Months (cells/cmm^3) (T6)	Mean Median Missing	6.7 6.3 (3-13.1) 15	6.7 6 (3.6-10.4) 10	6.7 6.4 (3-13.1) 5	0.794

Table 4.4 Continued: ExPeCT Participant Clinical Blood Measurements.

		All cases (n=61)	Exercise (n=29)	Control (n=32)	p-value
Platelet Count Baseline (plat/cmm^3) (T0)	Mean Median Missing	218.5 204.5 (67-356) 5	226.6 219 (156-331) 2	210.9 198 (67-356) 3	0.527
Platelet Count Three Months) (plat/cmm^3) (T3)	Mean Median Missing	218.4 212.5 (27-374) 11	217.9 213.5 (27-309) 7	218.9 212.5 (136-374) 4	0.681
Platelet Count Six Months (plat/cmm^3) (T6)	Mean Median Missing	221.7 211.5 (136-385) 15	225.6 213 (164-385) 10	218.9 211 (136-374) 5	0.942

4.1.6 Morphological analysis of ExPeCT participant ScreenCell® filters

After ScreenCell filtration, each ExPeCT filter was allowed to dry overnight at RT before storage at 4ºC until use. All filters were stained with MGG for morphological identification and subsequently stored long term at -20ºC. Imaging and analysis of putative CTCs, platelet cloaking and CTC clusters adhered to filters (Figure 4.9) was performed in conjunction with pathologists Dr Brian Hayes and Dr Tatjana Vlajnic.



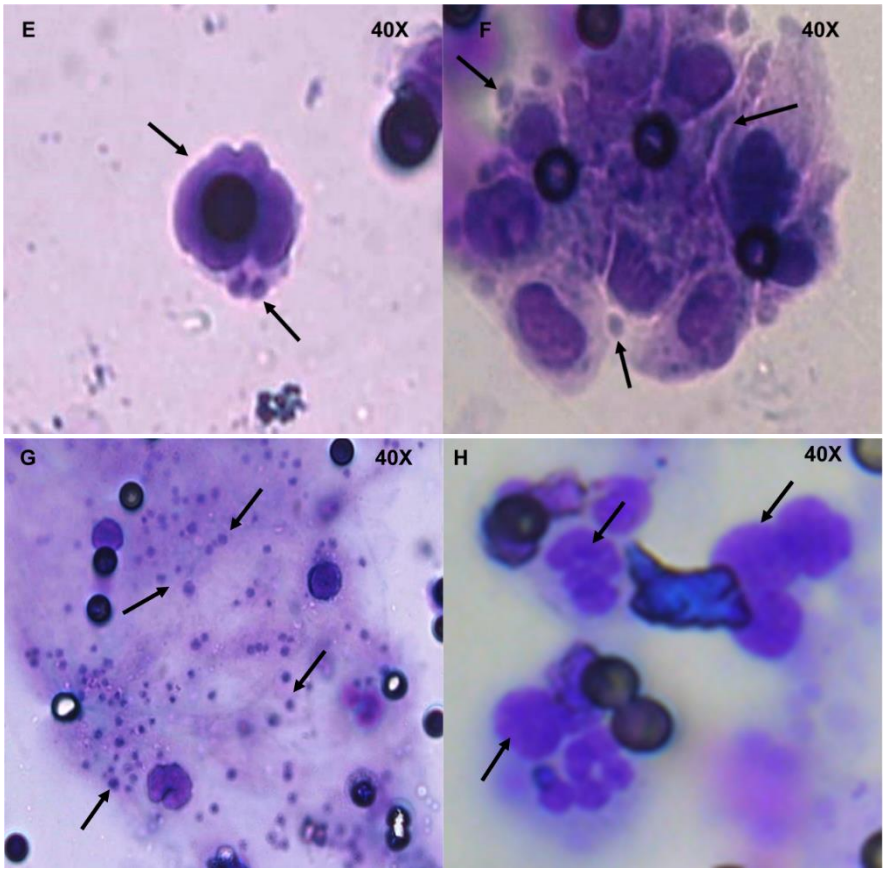


Figure 4.9: MGG morphological staining of representative ExPeCT filters.

A) and B) arrows depict large groups of putative CTCs (CTC clusters), C) arrows indicate a group of four putative CTCs, D) arrows illustrate a large putative CTC with cytoplasm attached, E) and F) arrows depict suggested platelet cloaking of CTCs, G) arrows represent platelets present as a large group on the filter and H) arrows detail inflammatory cells present in a separate plane of focus. All images are at 40X or 100X as indicated.

4.1.8 Immunohistochemistry on ExPeCT participant filters

IHC optimised on filters with PrCa cell lines (section 4.1.3), was examined on a number of ExPeCT participants' filters that had confirmed presence of CTCs, as examined morphologically by a pathologist. A number of markers including EpCAM, cytokeratin and CD45 were tested (Figure 4.10). The staining was sub-optimal despite numerous optimisation attempts. Internal positive controls present on the filters, e.g. CD45+ inflammatory cells, also appeared negative suggesting the filters were not conducive to IHC protocols. Multiple different protocols were attempted using different techniques, e.g. antigen retrieval in pressure cooker *vs.* microwave, overnight primary incubation *vs.* 1 hr primary incubation, blocking stages over a range of time, however none produced reproducible staining. As morphological staining was evident microscopically using MGG, CTCs, platelet cloaking and CTC clusters were all enumerated in this manner based on classical cytology techniques. Enumeration of CTCs from all sites was carried out in association with Dr Brian Hayes, who was blinded to the randomised nature of each filter.

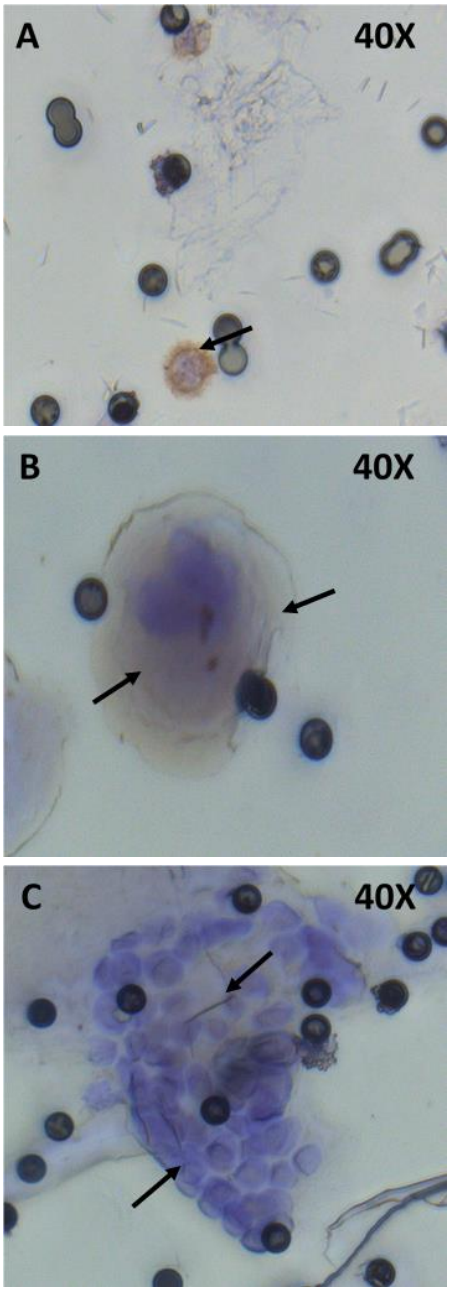
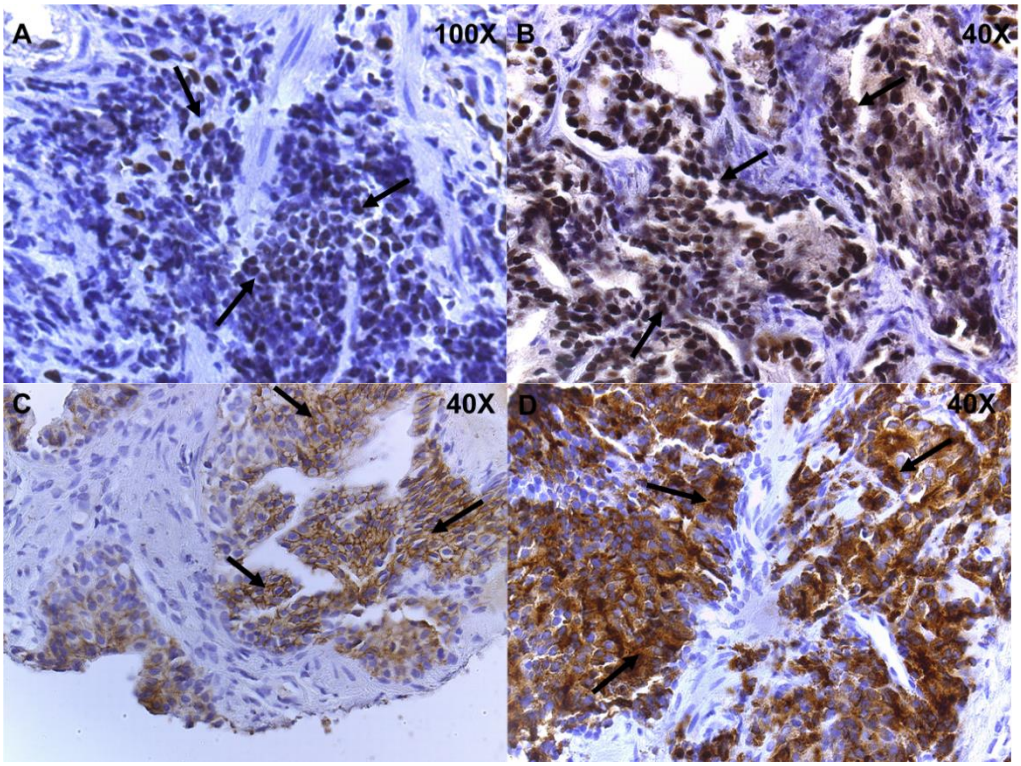


Figure 4.10: IHC optimisation on ExPeCT ScreenCell® filters.
A) Weakly positive CD45 staining on inflammatory cells.
B) Weak EPCAM staining on macrophage.
C) Negative EPCAM IHC staining on ExPeCT participant CTC cluster.
Arrows depict negative and weakly positive staining.

4.1.7 ExPeCT biopsy immunohistochemistry

A pilot study, using randomly selected ExPeCT participant NCBs, investigating the expression of cytokeratin, EpCAM, PSMA, ERG and AR, was performed. The aim of the study was to examine expression levels of these common PrCa related markers in ExPeCT NCBs and compare it to subsequent CTC analysis. Biopsies with sufficient tumour tissue were chosen from 8 participants and IHC was carried out (Figure 4.11). The biopsies were scored in conjunction with Dr Tatjana Vlajnic and the resulting scores are outlined in Table. 4.3. Cytokeratin, EpCAM and PSMA scoring was marked on levels of positive (pos) staining (levels 1-3) and ERG and AR staining was marked as positive or negative (neg) with heterogeneous (het) staining noted.



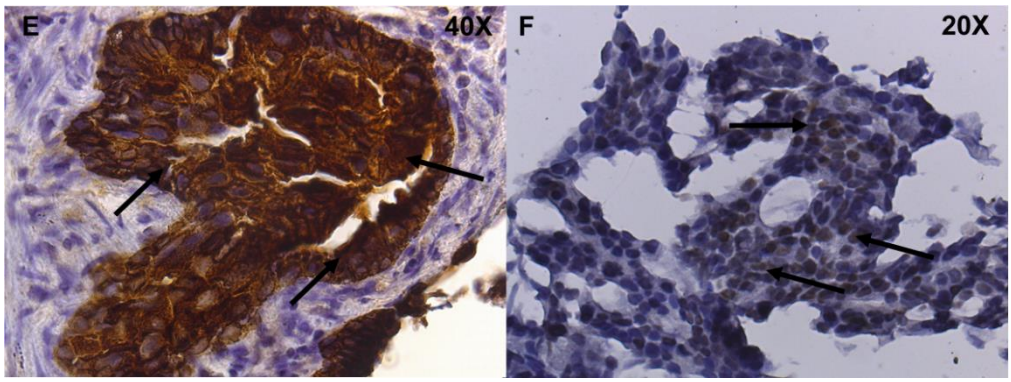


Figure 4.11: IHC staining of ExPeCT NCBs.

A) Positive ERG staining.

B) Positive AR staining.

C) Positive EpCAM staining.

D) Positive PSMA staining.

E) Positive cytokeratin staining.

F) Positive ERG staining.

Images are at 20X, 40X and 100X as indicated and arrows depict positive staining.

Table 4.4: IHC scoring of ExPeCT participant NCBs.

ExPeCT ID	Cytokeratin	EpCAM	PSMA	ERG	AR
EXP001	Pos (3)	Pos (1-2)	Pos (1-2)	Pos (het)	Pos (het)
EXP003	Pos (3)	Pos (2)	Pos (1-3)	Neg	Pos (het)
EXP004	Pos (3)	Pos (2)	Pos (2-3)	Neg	Pos
EXP005	Pos (3)	Pos (2-3)	Pos (1-3)	Neg	Pos
EXP006	Pos (3)	Pos (1-2)	Pos (1-3)	NA	NA
EXP007	Pos (2)	Pos (2-3)	Pos (3)	NA	NA
EXP008	Pos (3)	Pos (3)	Pos (2-3)	NA	NA
EXP011	Pos (3)	Pos (3)	Pos (2-3)	Pos (het)	Pos (het)

Pos – positive, Neg – negative, Het – heterogeneous, NA –insufficient tissue

4.1.9 CTC Enumeration in ExPeCT participant filters

4.1.9.1 Estimated CTC counts

Statistical analysis for ExPeCT participant CTC and platelet cloaking data was completed in conjunction with Dr Bryan Stanfill (Study statistician). A generalised mixed linear model with a negative binomial response was fit to the individual CTC counts with additive effects for time, location and a time-by-location interaction. Person random effects were included to account for the repeated measures structure of the experiment. Based on large differences in CTC number between sites (London n=34, Ireland n=27), the two locations were added to the same model, but an interaction term between time and location was included so that different trends could be captured. Missing data was removed from the datasets before analysis meaning it reduced the degrees of freedom in the analysis but did not affect the parameter estimates. Estimated individual CTC count from a maximum of four ScreenCell® filters per participant at each time point was included (Figure 4.12, Tables 4.5a and Table 4.5b). There was no significant change in CTC number over time between the exercise and control groups (p=0.2360), however decreases and increases in CTC number over time were noted within each group, in both the Irish and London sites (Table 4.6). Clinical variables, BMI, waist circumference, weight, height, PSA, haemoglobin and white cell count were added to the model individually to assess any correlation with CTC number. Statistical analysis of multiple comparison may have associated limitations, including the presence of false positives, which was taken into consideration throughout this study. There was no significant change between clinical variables and CTC number between groups over time. A positive correlation between white cell count and CTC number was observed over time within both the exercise and control groups (p=0.0002) (Table 4.7).

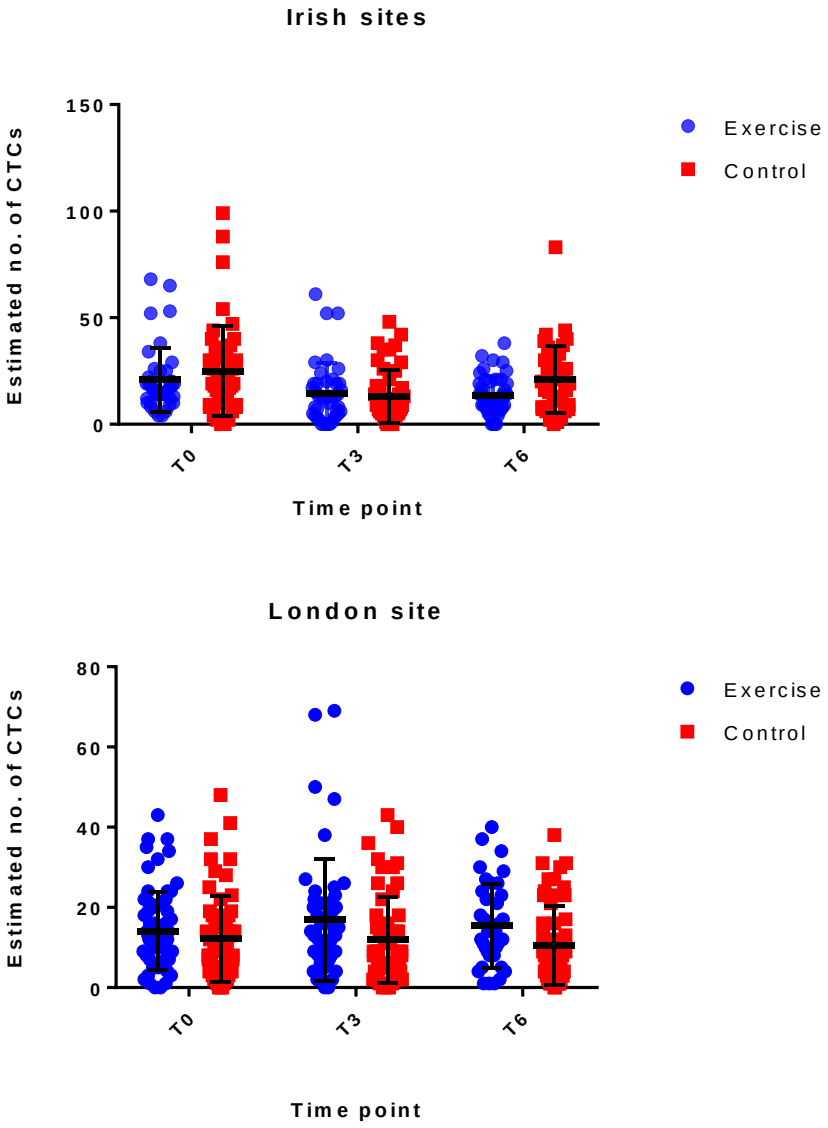


Figure 4.12: Estimated CTC count over time control *vs.* exercise, in Ireland and London.

Data graphed as mean ± SD

Exercise – Ireland n=13, London n=17

Control – Ireland n=14, London n=17

Table 4.5a: Averaged CTC counts for all patients, control and exercise, in the Irish sites. A maximum of four filters was averaged per patient (3 mL whole blood).

Site: Ireland	Group	Avg CTC Count T0	Avg CTC Count T3	Avg CTC Count T6
EXP001	Exercise	3	7.3	15
EXP002	Control	11	6.67	8.25
EXP003	Control	4	5.67	No Sample
EXP004	Exercise	25.5	10.75	1.75
EXP005	Exercise	7	4	4.75
EXP006	Control	20.3	1.25	18.3
EXP007	Control	28.75	14.75	22.5
EXP008	Control	21	9	6.75
EXP009	Exercise	13.75	4.75	9
EXP010	Control	3	4	No sample
EXP011	Exercise	15.25	4	12.75
EXP012	Withdrew	Withdrew	Withdrew	Withdrew
EXP013	Exercise	18.25	17.25	10
EXP014	Exercise	16	12	16.25
EXP015	Control	41.75	31.75	50
EXP016	Exercise	13.5	13.3	16
EXP017	Control	8	6.3	7.3
EXP018	Control	13	14.25	6
EXP019	Control	29.5	40.5	30.75
EXP020	Control	87.67	No sample	33
EXP021	Excluded	Excluded	Excluded	Excluded
EXP022	Exercise	53	24	15.3
Exp023	Excluded	Excluded	Excluded	Excluded
EXP024	Control	38.3	20	22
EXP025	Exercise	16.3	12.67	12.3
EXP026	Exercise	50.3	55	33.3
EXP027	Control	14.3	4	14.67
EXP028	Control	23.3	4.3	27.3
EXP029	Exercise	14.3	21.3	11.3
EXP030	Exercise	16	8.7	25

Table 4.5b: Averaged CTC counts for all patients, control and exercise, in the London site. A maximum of four filters was averaged per patient (3 mL whole blood).

Site: London	Group	Avg CTC Count T0	Avg CTC Count T3	Avg CTC Count T6
G001	Control	1	RIP	RIP
G002	Control	0 (1 filter)	8.75	0.5
G003	Exercise	8.75	18.75	11.5
G004	Control	15.25	33.75	30
G005	Exercise	8	4.25	13.5
G006	Control	3.25	4.5	5.5
G007	Exercise	10.25	19.25	15
G008	Exercise	13.25	15.25	27.25
G009	Control	8	3.75	1.5
G010	Control	0.67	0.75	2
G011	Excluded	Excluded	Excluded	Excluded
G012	Exercise	16	19.5	Withdrew
G013	Exercise	13.25	6.5	Withdrew
G014	Exercise	22.3	9.25	5.5
G015	Control	10.25	7.75	3.75
G016	Control	33.75	26	17.75
G017	Control	18	5.75	19.5
G018	Excluded	Excluded	Excluded	Excluded
G019	Exercise	31.25	1.5	20.75
G020	Control	10.67	Withdrew	Withdrew
G021	Exercise	5.5	Withdrew	Withdrew
G022	Control	7.5	11.6	12
G023	Control	4.25	10.5	4
G024	Exercise	8.25	Withdrew	Withdrew
G025	Control	14.75	8.5	23
G026	Exercise	27.25	27	33.25
G027	Exercise	2.25	Withdrew	Withdrew
G028	Control	9.5	Withdrew	Withdrew
G029	Exercise	19	Withdrew	Withdrew
G030	Exercise	20	58.5	11.67
G031	Control	9	8.5	7.25
G032	Excluded	Excluded	Excluded	Excluded
G033	Exercise	10.25	9.25	3.3
G034	Control	10.75	26.5	13.5
G035	Control	29.25	9.25	7.75
G036	Exercise	11	13.75	7.75
G037	Exercise	15.5	Withdrew	Withdrew

Table 4.6: Difference in CTC count over time control *vs.* exercise, in Ireland and London. An interaction term between time and location was included so that different trends between the Irish and London sites could be captured. Change in CTC number over time was compared between group, control and exercise, and within group. CTC number was compared at each time point.

	Estimate	SD	z_value	p_value
Exercise-Control	0.1935	0.1729	1.1193	0.2630
Time 3-Time 0, Dublin	-0.4938	0.0949	-5.2053	**0.0000**
Time 6-Time 0, Dublin	-0.3097	0.0950	-3.2592	**0.0011**
Time 6-Time 3, Dublin	0.1841	0.0981	1.8773	0.0605
Time 3-Time 0, London	-0.5722	0.1902	-3.0092	**0.0026**
Time 6-Time 0, London	-0.6897	0.1919	-3.5932	**0.0003**
Time 6-Time 3, London	-0.6837	0.2091	-3.2692	**0.0011**

Table 4.7: Correlation between estimated CTC number and clinical measurements. CTC number was compared to clinical variables of interest, and correlations assessed both between group, control and exercise, and within group.

	Estimate	SD	z-value	p-value
BMI	-0.0190	0.0847	-0.2241	0.8227
Weight	0.0778	0.0840	0.9263	0.3543
Height	-0.0055	0.0638	-0.0870	0.9307
Hb	0.2348	0.1836	1.2791	0.2009
WCC	0.1613	0.0428	3.7721	**0.0002**
PSA	-0.0760	0.0491	-1.5468	0.1219
WCir	0.0167	0.0674	0.2470	0.8049

4.1.9.2 Average CTC number per 3 mL blood

An average number of CTCs per 3 mL blood was estimated for each ExPeCT participant in addition to the estimated CTC count. All variables were measured in the same manner as per section 4.1.9.1. The same trend in CTC number over time between groups, in the Irish sites and London site, was evident as per the estimated CTC count (Figure 4.13). There was no significant change in CTC number over time between groups, in either the Irish or London sites (p=0.8010), however significant decreases and increases were noted within group in both sites (Table 4.8). As with the estimated CTC count, when clinical variables were added to the model there was no significant correlation between groups. However, within both groups, a positive correlation was identified between CTCs and white cell count (p<0.0000) (Table 4. 9).

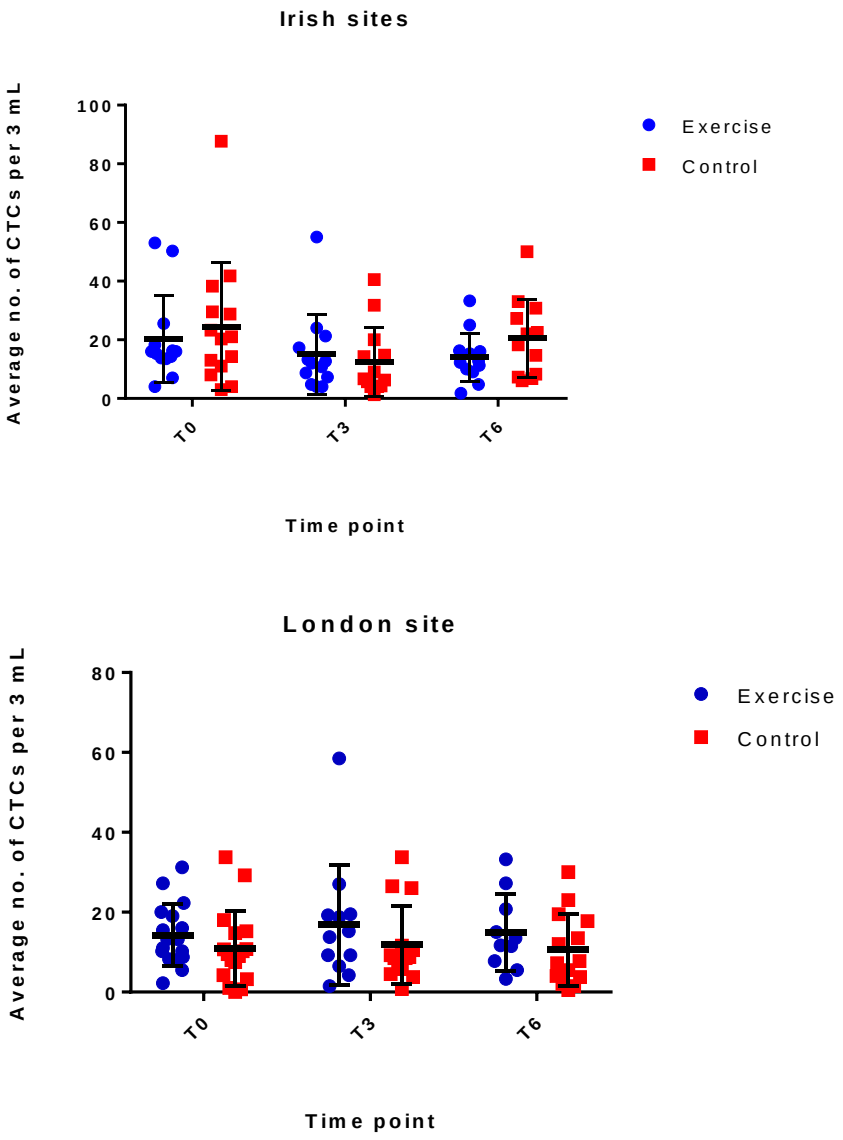


Figure 4.13: Average CTC count in 3 mL blood over time, exercise *vs.* control, at Irish and London sites.

Data graphed as mean ± SD

Exercise – Ireland n=13, London n=17

Control – Ireland n=14, London n=17

Table 4.8: Changes in CTC number over time, control and exercise, Ireland and London. Change in CTC number over time was compared between group, control and exercise, and within. CTC number was compared at each time point.

	Estimate	SD	z_value	p_value
Exercise-Control	0.6975	2.7671	0.2521	0.8010
Time 3-Time 0, Dublin	-7.5993	2.3815	-3.1909	**0.0014**
Time 6-Time 0, Dublin	-6.0952	2.4140	-2.5249	**0.0116**
Time 6-Time 3, Dublin	1.5041	2.4140	0.6231	0.5332
Time 3-Time 0, London	-8.8821	3.4260	-2.5926	**0.0095**
Time 6-Time 0, London	-10.6626	3.4720	-3.0710	**0.0021**
Time 6-Time 3, London	-11.6606	4.0781	-2.8593	**0.0042**

Table 4.9: Correlation between average CTC number in 3 mL blood and clinical variables. CTC number was compared to clinical variables of interest, and correlation assessed both between group, control and exercise, and within group.

	Estimate	SD	z_value	p_value
BMI	0.0066	1.5772	0.0042	0.9967
Weight	0.9746	1.5192	0.6415	0.5212
Height	-1.1135	1.2275	-0.9071	0.3643
Hb	-1.0644	4.0998	-0.2596	0.7951
WCC	6.2496	1.0595	5.8985	**0.0000**
PSA	-1.0302	1.1805	-0.8727	0.3828
WCir	1.1037	1.4496	0.7614	0.4464

4.1.10 Platelet cloaking in ExPeCT trial participants

Platelet cloaking was enumerated in all sites by pathologist Dr Brian Hayes using recognised clinical cytology criteria. This was based on the presence/absence of platelets adhered to identified CTCs. Platelet cloaking was modelled using logistic regression, as a binary response (presence/absence), which estimated the probability that an individual in each group (exercise/control) at each time point will give a blood sample with platelet cloaking. The presence of platelet cloaking, over time, was assessed in both groups (Figure 4.14). A trend towards an increase in platelet cloaking in the control group over time (p=0.1005) was observed (Table 4.10). Clinical variables were added to the model one at a time, to test for any association with platelet cloaking. There was no significant correlation between clinical variables and platelet cloaking noted (Table 4.11).

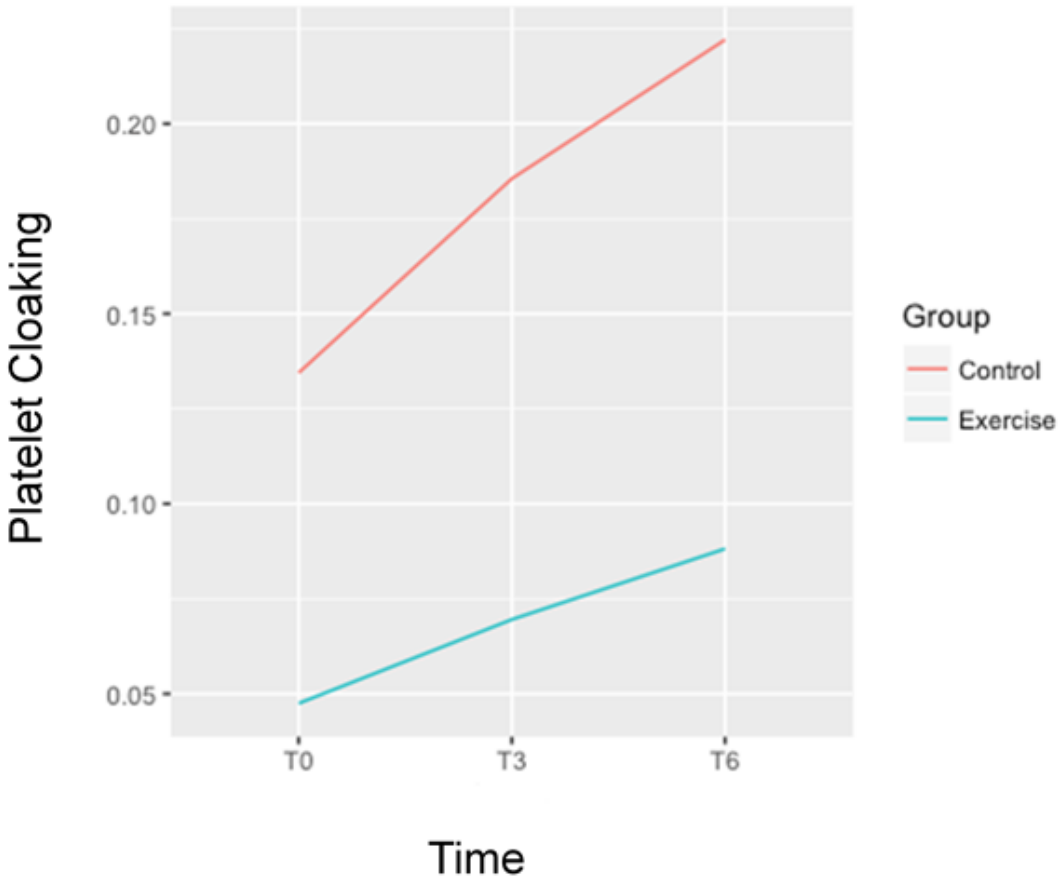


Figure 4.14: Platelet cloaking over time, exercise *vs.* control in all ExPeCT participants.

Data graphed as presence/absence using binary variables (maximum of 4 filters per patient).

Table 4.10: Changes in platelet cloaking over time in ExPeCT exercise and control groups.

	Estimates	SD	z_value	p_value
Exercise-Control	-1.0986	0.6689	-1.6424	0.1005
Time 3-Time 0	0.3605	0.6197	0.5817	0.5607
Time 6-Time 0	0.5976	0.6134	0.9743	0.3299
Time 6-Time 3	0.2370	0.6031	0.3931	0.6943

Table 4.11: Correlation between platelet cloaking and clinical variables.

	Estimates	SD	z_value	p_value
BMI	0.3362	0.2897	1.1606	0.2458
Weight	-0.0828	0.3150	-0.2629	0.7926
Height	-0.2373	0.2320	-1.0227	0.3064
Hb	-0.1859	0.3579	-0.5194	0.6035
WCC	-0.5642	0.5557	-1.0153	0.3100
PSA	0.0729	0.2400	0.3036	0.7615
WCir	0.1760	0.3003	0.5862	0.5577

4.1.11 CTC clusters in ExPeCT trial participants

CTC clusters were assessed in the same manner as platelet cloaking, in that the data was modelled using logistic regression, as a binary response (presence/absence), which estimated the probability that an individual in each group (exercise/control) at each time point will give a blood sample with the presence of a CTC cluster. The difference in the presence of CTC clusters over time was analysed in each group over time (Figure 4.15). There was no significant difference in the presence of CTC clusters over time between groups, however a decrease in the number of CTC clusters within group was observed (p=0.1663) (Table 4.12). As with the platelet cloaking model, clinical variables were added one at a time to test for any correlation with CTC clusters. A positive correlation between CTC cluster and PSA was noted within groups (p=0.0393) (Table 4.13).

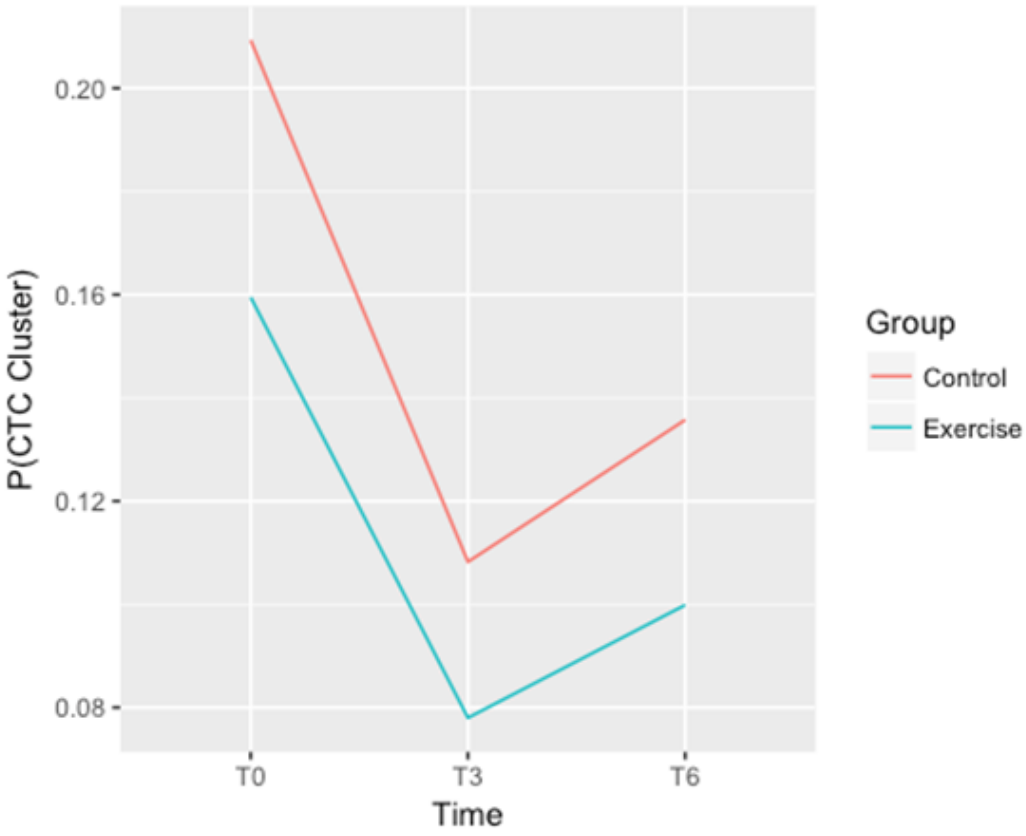


Figure 4.15: CTC cluster presence over time, exercise and control.

Data graphed as presence/absence using binary variables (maximum of 4 filters per patient).

Table 4.12: Presence of CTC cluster over time, control and exercise.

	Estimates	SD	z_value	p_value
Exercise-Control	-0.3477	0.5914	-0.5879	0.5566
Time 3-Time 0	-0.8125	0.5870	-1.3842	0.1663
Time 6-Time 0	-0.5043	0.5678	-0.8881	0.3745
Time 6-Time 3	0.3083	0.6451	0.4779	0.6327

Table 4.13: Correlation between CTC cluster and clinical variables.

	Estimates	SD	z_value	p_value
BMI	-0.3565	0.2907	-1.2261	0.2202
Weight	-0.3410	0.2847	-1.1975	0.2311
Height	-0.4179	0.2993	-1.3960	0.1627
Hb	0.4398	0.3351	1.3123	0.1894
WCC	-0.2158	0.3594	-0.6004	0.5483
PSA	1.5848	0.7690	2.0607	**0.0393**
WCir	-0.4442	0.2863	-1.5514	0.1208

4.1.12 BMI, CTCs, platelet cloaking and clinical variables in ExPeCT trial participants

At baseline (T0), all ExPeCT participants were divided into two groups based on their BMI, irrespective of their randomised trial arm. The two groups were based on normal weight (BMI < 25) and overweight or obese (BMI ≥ 25). Fifty participants were part of the BMI ≥ 25 group, while 11 participants were part of the BMI < 25 group. The mean number of CTCs at baseline was compared between groups and no significant difference was noted (p=0.88) (Table 4.14). In addition to CTCs, the presence of platelet cloaking was also compared between BMI groups, with no significant differences noted (p=0.785) (Table 4.15). A linear regression model was fitted to correlated baseline clinical variables within BMI group. A significant correlation was found between haemoglobin and BMI ≥ 25, with high BMI being associated with a decrease in haemoglobin levels (p=0.022) (Table 4.16). Additionally, a significant relationship between WCC and BMI was noted, with high BMI associated with an increase in WCC (p<0.000).

Table 4.14: Correlation between CTCs and BMI.

	Mean	Std Dev	p-value
High BMI (≥ 25)	16.347	15.693	0.88
Low BMI (< 25)	15.455	10.912	

Table 4.15: Correlation bewteen presence of platelet cloaking and BMI.

	Probability of Platelet Cloaking	p-value
High BMI (≥25)	0.120	0.785
Low BMI (<25)	0.091	

Table 4.16: Correlation between BMI < 25, BMI ≥ 25 and clinical variables.

BMI Group	Variable	Mean	Std. Dev.	Reg. Parameter	p-value
Low BMI	BMI	22.818	1.181	-2.939	0.423
High BMI	BMI	30.476	3.722	1.093	0.634
Low BMI	Hb	74.278	58.192	0.896	0.835
High BMI	Hb	85.061	59.980	-5.325	**0.022**
Low BMI	Height	175.609	8.551	4.127	0.251
High BMI	Height	167.805	25.550	-2.437	0.287
Low BMI	PSA	18.492	32.129	-3.463	0.352
High BMI	PSA	48.416	210.781	-2.118	0.357
Low BMI	Waist.Cir.	86.636	8.358	4.162	0.247
High BMI	Waist.Cir.	105.431	11.259	2.619	0.261
Low BMI	WCC	7.644	3.069	0.557	0.897
High BMI	WCC	7.757	5.325	10.629	**0.000**
Low BMI	Weight	69.630	6.642	2.751	0.506
High BMI	Weight	89.389	12.007	0.828	0.719

4.2 Discussion

The aim of this chapter was to assess the role of CTCs and platelet cloaking in men with metastatic PrCa, focussing on the implications posed in terms of clinical and prognostic significance and examining the effect of a structured exercise intervention. To investigate this, a series of *in vitro* experiments were conducted, alongside a clinical trial, ExPeCT, which aimed to assess CTCs in this population as a primary endpoint.

The first step was to determine the best method for isolation and enumeration of CTCs. An EpCAM based isolation technique, which employs immunomagnetic beads coated in an EpCAM antibody, Isoflux, was compared to a size based filtration method, ScreenCell®. Size based filtration methods work on the basis that CTCs are large enough not to pass through filter pores, while other blood cells will flow through into waste collection. ScreenCell® demonstrated a better capture efficiency rate (80%) after cell spiking experiments, in comparison to Isoflux (74%). To eradicate bias, an EpCAM positive cell line was chosen for these experiments, however, the size based platform performed at a more efficient rate. As outlined previously, the 'gold standard' or only FDA approved method for CTC isolation, CellSearch® an EpCAM based detection method, does not capture CTCs which may have undergone EMT, leading to inaccurate CTC isolation counts Comparison of platforms in renal cell carcinoma determined inferior recovery of CTCs in EpCAM dependant platforms than non-EpCAM specific platforms, highlighting the heterogeneous expression of CTCs (Maertens et al., 2017). Furthermore, comparisons between CellSearch® (EpCAM based) and ISET (size based) technologies in metastatic lung, breast and prostate cancers, highlighted limitations associated with utilising EpCAM as a sole marker for CTC isolation (Farace et al., 2011). CTCs may hold relevant biological information into the metastatic spread of prostate cancer, thus it is imperative that the most efficient method is utilised. In spite of multiple challenges associated with the isolation and positive identification of CTCs, based on these results and the current literature, size based detection may be preferable in metastatic PrCa.

Once captured, identification of potential CTCs was performed with MGG, which effectively demonstrated the distinct morphology of CTCs. Additional analysis, to identify markers expressed by CTCs, is commonly accepted as the next stage in positive CTC identification (Lu et al., 2016). A number of markers, known to be associated with PrCa (ERG, PSMA and AR) were optimised in addition to widely used CTCs markers (CD45, cytokeratin and EpCAM). Staining was effective when optimised on FFPE sections and PrCa cell lines, however difficulties arose when IHC was performed on ScreenCell® filters. In spite of optimisation and different methods, not all markers produced reproducible staining on PrCa cell lines, with positive internal controls also demonstrating negative staining. This effect was also witnessed in a panel of ExPeCT participant filters, with poor quality IHC staining observed. The poor reproducibility of the staining may be due to the filters not being conducive to the chemistry involved in IHC. Alternative methods for additional marker staining, including fluorescent in situ hybridisation and immunofluorescence, have been described as effective when utilised on ScreenCell® filters (Yanagita et al., 2018, Nicolazzo et al., 2017). These protocols may provide secondary options for further positive identification of CTCs in metastatic PrCa in future studies, however they were beyond the scope of this project. Therefore, morphological identification, based on clinical cytology protocols, was identified as acceptable by two independent pathologists.

Platelet cloaking of prostate cells was analysed using platelets applicable for research purposes. A range of times were assessed for the optimum time to cloaking, with platelets adhering to cells after an incubation period in whole blood of 45 min. This suggests an interaction between cells and the platelets, allowing time for platelets to locate the cells and adhere to them. The level of platelet cloaking was ascertained between normal and tumour prostate cells, with platelets found to be more likely to adhere to tumour cells. Some platelet cloaking was apparent in normal cells, which may be due to the presence of general cell surface markers that may attract platelets. When CTCs are released into the bloodstream, they secrete factors, such as tumour associated

proteinases or thrombin, which can promote the onset of tumour cell induced platelet aggregation (Lou et al., 2015), and may provide an explanation for the trend in platelet cloaking observed between normal and tumour cells. These data are promising as it is the first time platelet cloaking has been determined in prostate cell line studies. Future experiments, investigating the effect of aspirin on platelet cloaking in these cells may provide further insight into the tumour cell/platelet relationship.

The primary endpoint of the ExPeCT trial was to determine the impact of a structured exercise intervention on CTCs and platelet cloaking in a population of men with metastatic PrCa. A total of 67 participants were consented for the trial, with 61 participants completing baseline analysis. Participants were randomised into either the control or exercise group and assessed over a period of six months. General baseline characteristics were compared between groups. There was no significant difference between exercise and control groups in terms of age, time since diagnosis and Gleason score, suggesting a reflective profile of the general population in terms of metastatic PrCa. Routine clinical blood data was also captured over time and no significant difference was observed between exercise and control groups at each time point. This outcome was mirrored in the clinical variables, BMI and waist circumference, no significant differences between groups at each time point. The lack of difference observed over time in the exercise group in terms of BMI and waist circumference may suggest the need for a prolonged period of exercise, with an increased rate of intensity. Additionally, although BMI is an accepted clinical marker, it may be a flawed approach for measuring weight post exercise intervention. Future studies including technology, such as CT or DEXA scans, measuring total body fat composition, may provide more accurate information

Morphological analysis of ExPeCT participant filters demonstrated evidence of CTCs present on ScreenCell® filters. The presence of large, irregular shaped nuclei without any attached cytoplasm was present on multiple filters, indicating that the cytoplasm may have been sheared or lost, during the filtration process. This result highlights the need for further

identification protocols such as nuclear markers, to positively identify putative CTCs without cytoplasm. In addition to single CTCs (with and without cytoplasm), CTC clusters and platelet cloaking were also identified on participant filters. The levels of platelet cloaking were low, which may not be a true reflection of the metastatic profile. The aforementioned processing related shearing of cytoplasm, may have contributed to low numbers of platelet cloaking, as the platelet cloaking was exclusively observed adhered to CTC cytoplasm. A pilot study, utilising 8 randomly chosen participant NCBs, was undertaken with the aim of assessing any biological changes in disease progression, from diagnostic biopsy to CTC. All 8 participants were cytokeratin, EpCAM and PSMA positive, with heterogeneous AR and ERG expression noted. However, as discussed previously, reproducible IHC staining was not possible with the ScreenCell® filters, hence, the expression profile generated from the diagnostic NCBs could not be identified on CTCs. Future studies which incorporate the optimisation of additional protocols for CTC marker expression, such as ERG and AR, may allow for a NCB and CTC comparison study which may provide further insights into metastatic tumour biology.

To determine statistically significant differences between the exercise and control groups, CTC enumeration was compared between both groups over time. CTC numbers were compared as estimated CTC counts and as the average number of CTCs per 3 mL blood, with similar results observed. The location effect, produced large differences and subsequently the model was divided based on site specific data. Differences between baseline characteristics, BMI, waist circumference and age, were assessed between sites based on the location effect, however no statistically significant differences were determined. This suggests that the two cohorts were of similar profile upon recruitment. All filters were assessed utilising the same isolation protocol and were analysed under strict criteria by the same independent pathologist. Systemic treatment was not an inclusion criteria for the trial and may provide insight into the large differences in CT number observed between

locations. It may also be possible that site specific factors, such as lifestyle and socio-economic status, may have had a role to play.

In both analyses, no significant difference in CTC number was noted between exercise and control groups over time. Interestingly, significant changes were detected within both groups. In the Irish sites (n=27), there was a significant decrease in the number of CTCs in both the control and exercise arms between T0 and T3, with a significant increase in CTC number within group observed for T3 to T6. The London site (n=34), experienced different effects based on the method of analysis, with CTC number decreasing and increasing significantly within group over time. The significant alterations in CTC number may be due in part to the systemic therapy of each participant. Specific therapeutic use was not an exclusion criteria for ExPeCT, with participants undergoing a wide range of treatment, including ADT, chemotherapy and second generation anti-androgens. Thus the changes in CTC numbers may be due in part to the evolution of therapy or the eventual onset of treatment resistance that occurs over time. CTC number has previously been correlated with overall survival in patients with metastatic PrCa (De Laere et al., 2018), hence investigation into treatment outcome, is an approach that warrants further consideration. Additionally, although the impact of exercise was not significant in this instance, larger scale studies which include the use of resistance training and the possibility of longer interventions, may allow for further understanding of the impact of exercise.

CTC number was correlated with multiple clinical parameters to determine any significance with disease outcomes. A significant positive correlation between white cell count and CTC number was identified, regardless of location effect. This relationship has not been previously documented in the literature and may signify crosstalk between CTCs and immune cells. CTC counts have previously been compared to clinical variables to assess the impact on overall survival for patients with metastatic PrCa, with significant correlations established between CTC numbers, PSA levels and haemoglobin status (Olmos et al., 2009). These findings were not observed in the current study and larger scale

studies, designed to assess CTC number and white cell count, are required to gain further insight into this proposed relationship.

In addition to CTC number, the presence or absence of platelet cloaking and CTC clusters was determined across all ExPeCT participants. A decrease in CTC cluster number from T0-T3, followed by an increase from T3-T6, was in accordance with trends witnessed in single CTC number estimates. However, these values did not reach statistical significance. CTC cluster number was significantly correlated with PSA, suggesting a link with a more aggressive disease state. CTC number and high PSA levels have previously been associated with worse outcomes in patients with metastatic PrCa (Goldkorn et al., 2014). A trend associated with platelet cloaking was discernible. The exercise intervention did not have a significant impact on the presence of platelet cloaking over time, however the likelihood of platelet cloaking being present was greater within the control group. Platelet cloaking and clinical data were correlated; however, no significant relationships were observed. The hypothesis of platelet cloaking is a novel area of research that is emerging as a significant factor in aiding the extravasation of CTCs to distal organs. The analysis of platelet cloaking of CTCs in metastatic PrCa has been assessed in this study for the first time and may contribute to the understanding of platelet tumour cell interactions.

Obesity in advanced PrCa is correlated with worse outcomes (Efstathiou et al., 2007). Based on this, ExPeCT participants were divided depending on their BMI and their BMI status compared to clinical variables and CTC counts. No significant differences were observed between the low and high BMI groups, in terms of platelet cloaking and CTC number. CTC number and BMI has previously been investigated in breast cancer, with BMI not independently associated with CTC number (Fayanju, 2017). Clinical variables were also compared with BMI group. A significant association was noted between high BMI and haemoglobin levels, as well as a significant relationship between high BMI and white cell count. These findings may signify a role between obesity and blood cells, which when combined with the relationship between CTCs and white cell count, may suggest a synergistic role in advanced disease.

Notably, the number of participants who were classed as low BMI (normal weight BMI < 25) was small, with only 11 participants eligible for this classification. The low number of patients present within the normal weight group, may be a factor impacting the study. These numbers are likely reflective of this population, as 3 in 4 adults over 50 in Ireland are classed as obese (DOH, 2016), in addition to the prevalence of central adiposity as a common side effect of ADT (Crawford and Moul, 2015).

A limitation of this research is the number of patients accrued. ExPeCT was initially powered based on the primary endpoint of CTCs and platelet cloaking for a total of 200 participants. To address this, the ExPeCT research team completed ethical applications to open the trial in four additional Irish sites. Reasons for the low number of patients recruited to ExPeCT included the advancing age of patients with metastatic PrCa, co-morbidities that rule out the ability to partake safely in an exercise intervention, living far away from the hospital site and the possibility of additional hospital appointments. These are similar reasons to those reported in previous clinical trials. However, ExPeCT was the first trial of its kind in Ireland for patients with metastatic PrCa. Adherence rates were high, especially within the Irish cohort, and the structured intervention was tailored to each individual's abilities. The CTC and platelet cloaking data from this endpoint of the trial is promising, and may provide an insight into the metastatic cascade and the nature of aggressive disease in this population of men.

Chapter 5:

Insulin Secretion and Adipokine Status in ExPeCT Trial Participants

5.1: Introduction

As outlined previously, obesity and MetS may have a role to play in aggressive PrCa (Grotta et al., 2015). This is strengthened by results from the REDUCE study, a clinical trial for men with PrCa (n=6427), which noted an association between obesity and greater risk of high-grade PrCa (Vidal et al., 2014). In other studies, obese patients displayed significantly lower serum adiponectin levels and higher serum leptin levels, with leptin further correlated to the likelihood of disease progression (Kang et al., 2018). A recent meta-analysis, determined polymorphisms and genetic variants within adiponectin and leptin and their related receptors, were linked with PrCa progression (Hu et al., 2016). Thus, these adipokines may hold particular relevance in patients with metastatic PrCa (Table 5.2).

Resistin is a novel adipokine, with *in vitro* studies demonstrating a link between high resistin and cell proliferation (Table 5.2) (Kim et al., 2011). Links between resistin and inflammation have also been established. Treatment with resistin in isolated human mononuclear cells can stimulate production of TNFα and lL-6, suggesting a role for resistin in the modulation of chronic systemic inflammation (Tsiotra et al., 2013). In addition, resistin levels are markedly increased in patients with MetS when compared to those without (Malo et al., 2011), postulating a role between resistin and the development of MetS. Hyperinsulinemia, elevated insulin levels associated with insulin resistance, is strongly linked with MetS and cancer (Orgel and Mittelman, 2013). Circulating C-peptide levels are considered an appropriate marker for hyperinsulinemia (Jones and Hattersley, 2013). While investigation into the relationship between C-peptide and cancer has been ongoing for several years, conflicting reports exist on the association between C-peptide and PrCa. Di Sebastiano et al. identified a significant link between high Gleason score and C-peptide level (Di Sebastiano et al., 2017), however, analysis of data obtained from the HPFS, determined no correlation between C-peptide levels and risk of PrCa (Lai et al., 2014). This highlights the need for further investigation into C-peptide in patients with PrCa (Table 5.2).

Table 5.1: Analytes for the assessment of insulin secretion and adipokine status in ExPeCT trial participants.

	Analyte of Interest	**Link to PrCa**
Insulin Secretion	C-peptide	Conflicting evidence of role in PrCa (Di Sebastiano et al., 2017, Lai et al., 2014)
Adipokine	Adiponectin	Decreased proliferation *in vitro* in PrCa cell lines (Gao and Zheng, 2014)
	Leptin	Increased PrCa aggressiveness (Lopez Fontana et al., 2011)
	Resistin	Elevated proliferation *in vitro* in PrCa cell lines (Kim et al., 2011)

All analytes were assessed in serum, with the exception of C-peptide, which was assessed in plasma.

Multiple studies have investigated the relationship between physical activity and cancer (Shephard, 2017). The impact of physical activity on obesity and C-peptide in cancer survivors has been investigated, with physical activity impacting circulating levels (Irwin et al., 2005). Based on the evidence in the literature, ExPeCT aimed to assess the impact of an exercise intervention on adipokine status and insulin secretion in patients with metastatic PrCa (Figure 5.1). The specific aims for this chapter were to assess changes between exercise and control groups over time in terms of a) adipokine expression profiles, b) C-peptide levels as a surrogate marker of insulin secretion levels and c) to correlate clinical data, CTCs and platelet cloaking with insulin secretion and adipokine status.

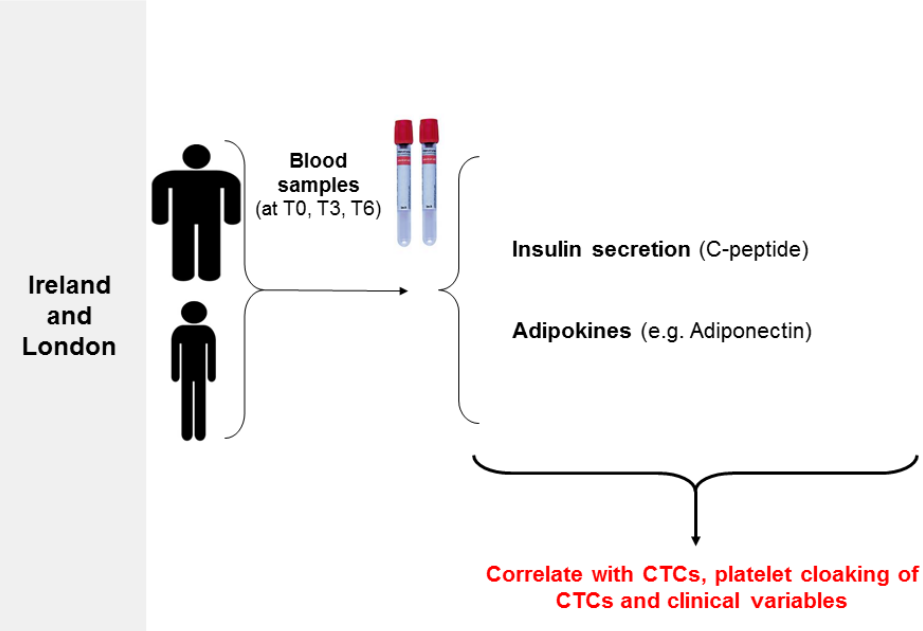


Figure 5.1: Image outlines adipokine and insulin secretion assessment in ExPeCT trial participants.

5.1 Results

5.1.1 ExPeCT trial participants

The number of participant serum/plasma samples included in this analysis is outlined in Figure 5.2. Reasons for the exclusion of samples included an insufficient volume of sample, consent withdrawal and death.

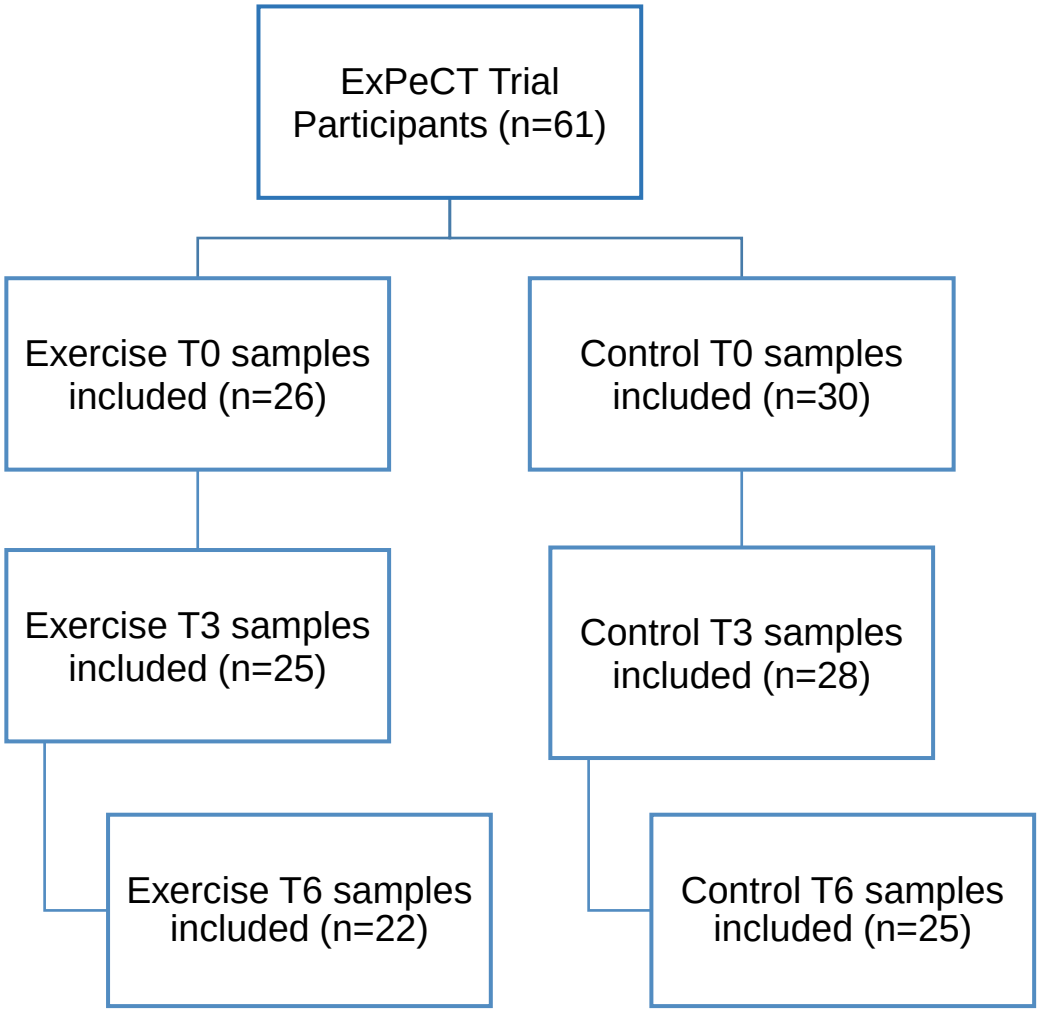


Figure 5.2: Flowchart outlining ExPeCT participant serum and plasma samples for analysis.

5.1.2 Adipokine expression and insulin secretion in ExPeCT participants

Statistical analysis was performed in conjunction with Dr Bryan Stanfill (Study statistician). Data was transformed on the log base 2 scale and analysed using a linear mixed model with Gaussian response and graphed on the normal scale. Clinical variables were added to the model one at a time to test for any correlation with the analyte of interest. A summary of significant correlations is outlined in Table 5.2.

Table 5.2: Summary of significant findings between adipokines, C-peptide and clinical variables

Analyte	Clinical variable	p-value
Adiponectin	CTCs	0.0400
	BMI	0.0109
	Weight	0.0049
	Waist Circ	0.0079
Leptin	BMI	0.0001
	Weight	0.0000
	WCC	0.0491
	Exercise-Hb	0.0002
Resistin	CTCs	0.0000
	Platelet Cloaking	0.0004
	Height	0.0139
	WCC	0.0000
C-peptide	CTCs	0.0384
	Platelet cloaking	0.0229
	Weight	0.0336

5.1.3 Adipokine status in ExPeCT trial participants

5.1.3.1 Adiponectin

Changes in adiponectin concentration over time is outlined in Figure 5.3. There was no significant change in adiponectin concentration (p=0.3449) over time (T0-T3, T3-T6, T0-T6), between the exercise and control groups. However, a significant increase in concentration (p=0.0234) was noted within both groups over time (T0-T6) (Table 5.3).

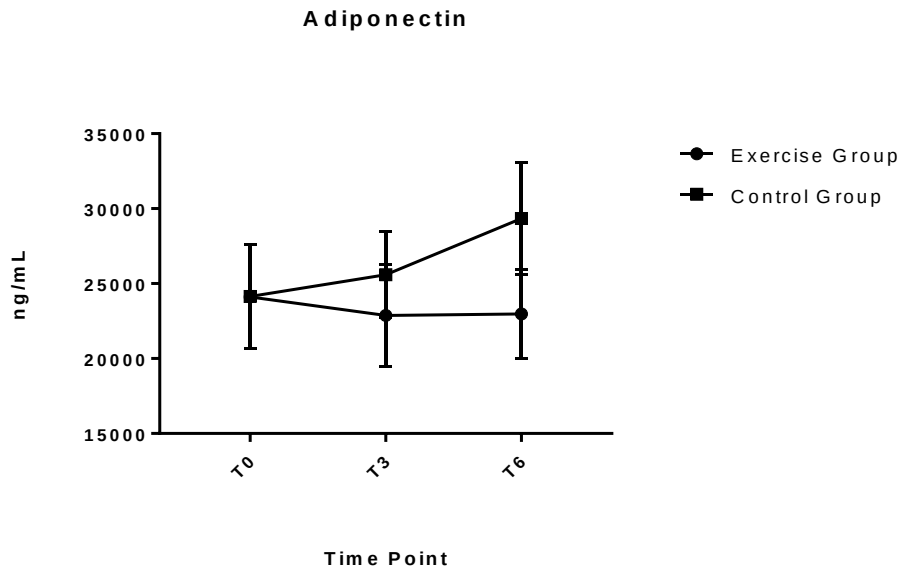


Figure 5.3: Adiponectin concentration over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=25.

(Data range; exercise T0 3694.2-57081.8 ng/mL, control T0 2406.3–85285.7 ng/mL, exercise T3 2736.1-56412.4 ng/mL, control T3 4986.4-55640.8 ng/mL, exercise T6 3974.8-55104 ng/mL and control T6 4325.7-68478.5 ng/mL).

Table 5.3: Changes in adiponectin expression over time, within and between, ExPeCT control and exercise groups.

	Estimate	SD	z Value	p Value
Exercise-Control	-0.2978	0.3153	-0.9446	0.3449
T3-T0	0.1187	0.0942	1.2605	0.2075
T6-T0	0.2205	0.0973	2.2667	**0.0234**
T6-T3	0.1018	0.0982	1.0369	0.2998

An inverse association between adiponectin and CTC count (p=0.0400) was present in both groups (Table 5.4).

Table 5.4: Association between CTC number, platelet cloaking presence and adiponectin expression.

	Estimate	SD	z Value	p Value
Average CTCs	-0.0105	0.0051	-2.0534	**0.0400**
Control - Cloaking	-0.1817	0.1805	-1.0067	0.3141
Exercise - Cloaking	0.3952	0.2287	1.7280	0.0840

In addition to CTCs and platelet cloaking, BMI, weight, height, haemoglobin (hb), white cell count (WCC), PSA and waist circumference (Waist Circ) were added to the model. No significant changes were noted between groups over time. An inverse association between adiponectin

and BMI (p=0.0109), weight (p=0.0049) and waist circumference (p=0.0079) was identified within each group (Table 5.5).

Table 5.5: Correlation between adiponectin expression within groups over time and clinical variables.

	Estimate	SD	z value	p value
BMI	-0.3557	0.1398	-2.5448	**0.0109**
Weight	-0.3952	0.1404	-2.8139	**0.0049**
Height	-0.0355	0.1529	-0.2318	0.8167
Hb	0.2038	0.1319	1.5448	0.1224
WCC	-0.1243	0.0681	-1.8239	0.0682
PSA	0.0528	0.0987	0.5344	0.5931
Waist Circ	-0.2745	0.1033	-2.6574	**0.0079**

5.3.2 Leptin

Changes in leptin concentration over time is outlined in Figure 5.4. There was no significant change in leptin expression between groups (p=0.9285) over time. Additionally, no significant change in leptin expression was noted within groups over time (Table 5.6).

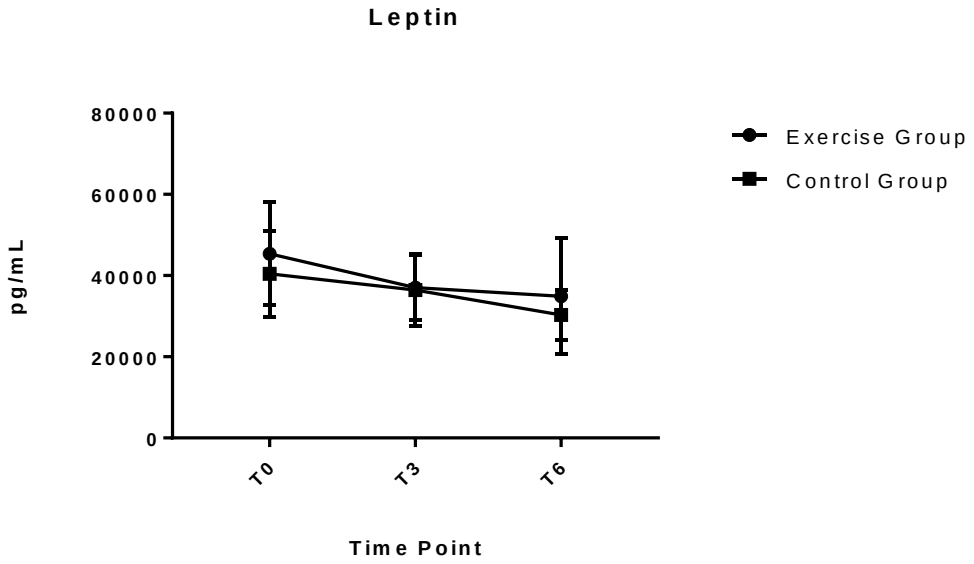


Figure 5.4: Leptin expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=26, exercise n=25.

(Data range; exercise T0 404-230427.25 pg/mL, control T0 1711.6-255958.1 pg/mL, exercise T3 311-160894.7 pg/mL, control T3 4069.3-1933521.1 pg/mL, exercise T6 474.1-339346 pg/mL and control T6 5138.4-113161.5 pg/mL).

Table 5.6: Changes in leptin expression over time, within and between, ExPeCT control and exercise groups.

	Estimate	SD	z Value	p Value
Exercise-Control	-0.0452	0.5042	-0.0897	0.9285
T3-T0	0.0222	0.1145	0.1942	0.8461
T6-T0	-0.1344	0.1207	-1.1134	0.2655
T6-T3	-0.1566	0.1218	-1.2854	0.1986

No significant differences were noted in CTC number or platelet cloaking when correlated with leptin expression, both within and between groups (Table 5.7).

Table 5.7: Correlation between CTC number, platelet cloaking and leptin expression.

	Estimate	SD	z Value	p Value
Control – Avg CTCs	-0.0146	0.0089	-1.6305	0.1030
Exercise – Avg CTCS	0.0125	0.0087	1.4330	0.1519
Platelet Cloaking	-0.1920	0.1705	-1.1256	0.2603

An increase in both weight and BMI were positively correlated with leptin expression (p=0.0001, p=0.0000 respectively) within each group. An inverse correlation between leptin and white cell count was also observed (p=0.0491). A significant difference was noted between the exercise and control groups over time. A positive correlation between leptin and haemoglobin was observed within the exercise group (p=0.0002), however no association was observed in the control group (Table 5.8).

Table 5.8: Clinical variables and leptin expression.

	Estimate	SD	z value	p value
BMI	0.8008	0.1996	4.0114	**0.0001**
Weight	0.8541	0.2021	4.2264	**0.0000**
WCC	-0.1624	0.0825	-1.9680	**0.0491**
PSA	-0.0726	0.1485	-0.4890	0.6249
Waist Circ	0.2279	0.1310	1.7398	0.0819
Control Height	-0.4427	0.3480	-1.2720	0.2034
Exercise Height	0.4612	0.3129	1.4742	0.1404
Control Hb	-0.1561	0.1999	-0.7811	0.4347
Exercise Hb	1.1981	0.3219	3.7215	**0.0002**

5.3.3 Resistin

Differences in resistin expression over time is illustrated in Figure 5.5. There was no significant difference in resistin expression over time between groups (p=0.9896). No significant differences in resistin expression were observed within groups (Table 5.9).

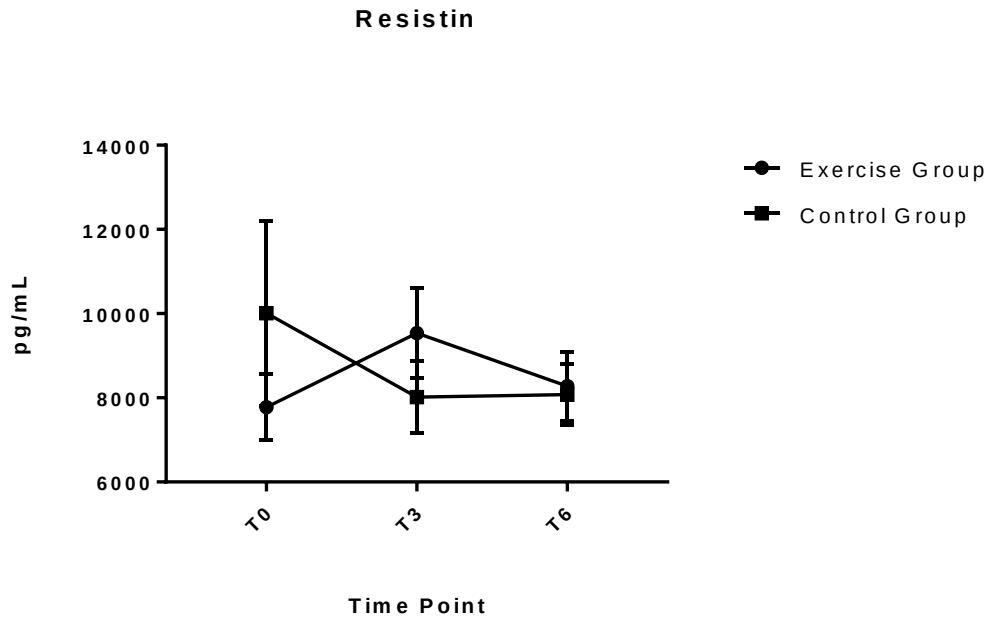


Figure 5.5: Resistin expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=27.

(Data range; exercise T0 2115.9-17074.6 pg/mL, control T0 2766.5-70615.5 pg/mL, exercise T3 1511.1-23809.5 pg/mL, control T3 2666.2-22816.3 pg/mL, exercise T6 1583.8-18918.2 pg/mL and control T6 2138.6-16295 pg/mL).

Table 5.9: Levels of resistin expression, within and between, ExPeCT exercise and control groups.

	Estimate	SD	z Value	p Value
Exercise-Control	-0.0452	0.5042	-0.0897	0.9285
T3-T0	0.0222	0.1145	0.1942	0.8461
T6-T0	-0.1344	0.1207	-1.1134	0.2655
T6-T3	-0.1566	0.1218	-1.2854	0.1986

Clinical variables were added to the model as previously. Within group, a positive relationship between CTCs and resistin expression was observed (p=0.0000). Furthermore, a negative correlation with platelet cloaking was observed (p=0.0004) (Table 5.10).

Table 5.10: Correlation between CTCs, platelet cloaking and resistin.

	Estimate	SD	z Value	p Value
Avg CTCs	0.0235	0.0049	4.7571	**0.0000**
Platelet Cloaking	-0.5526	0.1560	-3.5435	**0.0004**

Inverse correlations between resistin expression and height (p=0.0139), in addition to white cell count (p=0.0000) were established (Table 5.11).

Table 5.11: Relationship between clinical variables and resistin.

	Estimate	SD	z value	p value
BMI	-0.0034	0.1021	-0.0333	0.9734
Weight	0.1354	0.0975	1.3896	0.1646
Height	0.2436	0.0990	2.4609	**0.0139**
Hb	-0.1138	0.0981	-1.1599	0.2461
WCC	0.4508	0.0582	7.7528	**0.0000**
PSA	-0.0967	0.0717	-1.3481	0.1776
Waist Circ	-0.0038	0.0909	-0.0422	0.9663

5.4 C-peptide as a surrogate marker for insulin secretion

Levels of insulin secretion were analysed by C-peptide expression from ExPeCT participant plasma samples (Figure 5.6). There was no significant difference in C-peptide expression between groups. Within groups, a decrease in C-peptide expression between T0 and T3 (p=0.0317) was observed. Moreover, an increase in C-peptide expression was recorded within groups between T3-T6 (p=0.0596) (Table 5.12).

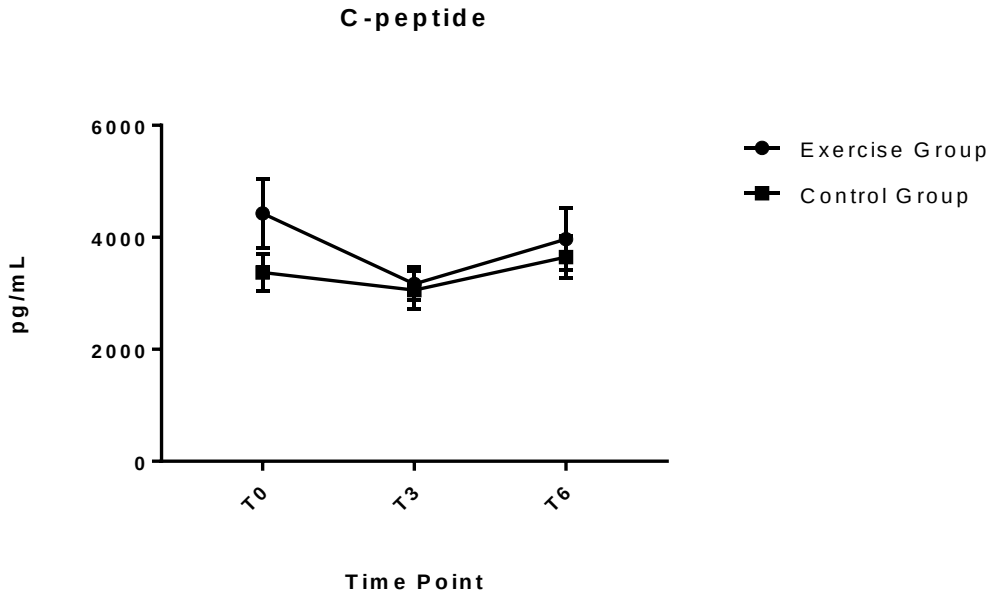


Figure 5.6: C-peptide expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=31, exercise n=26.

(Data range; exercise T0 779.3-16406.2 pg/mL, control T0 882.5-7179.9 pg/mL, exercise T3 1095.5-6143.9 pg/mL, control T3 273-8697.2 pg/mL, exercise T6 741.1-10355.3 pg/mL and control T6 7635.8-9492.7 pg/mL).

Table 5.12: Levels of C-peptide expression, within and between, ExPeCT exercise and control groups.

	Estimate	SD	z Value	p Value
Exercise-Control	0.1298	0.2070	0.6271	0.5306
T3-T0	-0.2091	0.0974	-2.1483	**0.0317**
T6-T0	-0.0151	0.1020	-0.1476	0.8827
T6-T3	0.1941	0.1030	1.8841	0.0596

All clinical variables were added to the model one at a time to determine their association with C-peptide expression. A positive association between CTC number and resistin expression was observed (p=0.0384). A negative correlation between platelet cloaking and C-peptide expression was also documented (p=0.0299) (Table 5.13).

Table 5.13: Correlation between C-peptide levels, CTCs and platelet cloaking.

	Estimate	SD	z Value	p Value
Avg CTCs	0.0102	0.0049	2.0706	**0.0384**
Platelet Cloaking	-0.3258	0.1432	-2.2754	**0.0229**

Positive associations between C-peptide expression and weight (p=0.0336) and C-peptide and waist circumference (p=0.0074) were present across both groups (Table 5.14).

Table 5.14: Relationship between C-peptide expression and clinical variables.

	Estimate	SD	z value	p value
BMI	0.1705	0.0993	1.7172	0.0859
Weight	0.2054	0.0966	2.1249	**0.0336**
Hb	-0.1506	0.0970	-1.5530	0.1204
WCC	0.0927	0.0657	1.4119	0.1580
PSA	0.0414	0.0725	0.5709	0.5681
Waist Circ	0.2204	0.0824	2.6764	**0.0074**
Control-Height	-0.1816	0.1536	-1.1822	0.2371
Exercise-Height	0.2488	0.1386	1.7957	0.0725

5.2 Discussion

Obesity, MetS and inflammation are associated with the progression of PrCa (Allott et al., 2013, Collier et al., 2012, Karan and Dubey, 2016). One goal of the ExPeCT trial was to elucidate the relationship between these variables and PrCa by examining adipokine and insulin secretion profiles in patients with metastatic PrCa, and to clarify any potential correlation between these profiles and CTCs, platelet cloaking of CTCs, and standard clinical variables. Furthermore, the impact of a structured exercise programme on adipokines and insulin secretion levels was also investigated.

Evaluation of an adipokine expression profile in patients with metastatic PrCa was performed, focussing on three common adipokines with known interactions in PrCa, adiponectin, leptin and resistin (Mistry et al., 2007, Kim et al., 2011). No significant changes in adipokine concentration were noted between the exercise and control groups over the six month time frame. However, adiponectin levels increased significantly within each group. A previous study, documented an increase in adiponectin, ranging from 18-48%, which was attributed to a reduction in the level of obesity of an individual (Silva et al., 2011). BMI, waist circumference, PSA, white cell count, haemoglobin, weight and height are all routine clinical measurements for patients with metastatic PrCa and were recorded in a clinical setting at each of the trial assessment time points. Changes in adiponectin and leptin expression were significantly correlated with BMI, weight and waist circumference. Adiponectin and leptin are understood to have biologically opposing relationships with adiposity, with this inverse affiliation having been previously documented in PrCa (Li et al., 2010). The increase in adiponectin levels in the ExPeCT exercise group, may be due to the aerobic programme undertaken, however this did not improve BMI and weight. Given that all patients were informed of the purpose of the trial, it may have lead participants to be more conscious of eating habits and daily exercise activity, substantiating the increase observed within the control group.

A positive correlation between leptin and haemoglobin was observed. The role between haemoglobin and leptin has not previously been documented in PrCa, and may signify dependence between these two variables in obesity and PrCa and further investigation into this finding is required. A significant interaction between white cell count and resistin expression was also recorded. High white blood cell count is classified as a predictor of MetS (Babio, 2013), which may provide an explanation for the trend observed in relation to white blood cell count. A significant correlation between resistin and white blood cell count in patients, independent of BMI, with diabetes mellitus has been previously published suggesting the mononuclear origin of resistin as a potential factor in this finding (Mojiminiyi and Abdella, 2007). The inverse correlation outlined in this study warrants increased participant numbers to further examine this outcome and the relationship between adipokines and clinical variables in metastatic PrCa.

In relation to CTCs and platelet cloaking, significant correlations were identified within groups. Positive correlations between adiponectin and resistin, were both independently significantly correlated with CTCs, in addition to a negative correlation between resistin and platelet cloaking. This finding indicates a potential interaction between adipokines and the metastatic cascade, providing an interplay between CTCs, platelet cloaking and adipokines. ExPeCT is the first trial of this nature comparing the impact of an exercise intervention on the relationship between adipokines, platelet cloaking and CTCs and has begun to improve the understanding of the influence of adipokine levels on CTC numbers and provides a solid foundation for future studies to further elucidate the underlying mechanisms.

Insulin secretion was measured using C-peptide expression (Van Cauter et al., 1992). No significant differences were noted between groups, however significant changes in C-peptide expression were detected within each group. C-peptide expression was observed to initially decrease and begin to increase once more over time. The range of C-peptide expression may provide an insight into the level of insulin resistance within this cohort of patients and hold relevance as a marker

of MetS (Gonzalez-Mejia et al., 2016). Significant positive correlations between C-peptide concentration and weight and waist circumference were present in ExPeCT participants. This finding is in accordance with the factors associated with MetS, such as central adiposity and high blood sugar (NIH, 2018a). A positive association between C-peptide expression and CTCs, in addition to a negative correlation with platelet cloaking, was determined. The role of C-peptide, platelet cloaking and CTCs in metastatic PrCa has not previously been established. The function of C-peptide as an indicator of MetS may be one factor aiding CTC number. Further investigation into this finding in a larger scale study, may determine the basis behind this association.

A limitation of this analysis into adipokine status and insulin secretion in patients with metastatic PrCa, is the potential for bias introduced in the control group. Exercise advice was provided to all participants at the end of the trial regardless of randomisation, however control participants were aware of the nature of the exercise component during their participation. This may have led to improved PA and dietary habits. Furthermore, analysis on this data was not compared in a site specific manner. Potential differences between the Irish and London cohorts, may have impacted the findings and future investigation into site specific effects may be warranted. This data has furthered the understanding of some of the mechanisms involved in obesity, MetS and metastatic PrCa and future larger scale studies would continue to determine the clinical relevance of this for patients living with PrCa.

Chapter 6:

Systemic Inflammation in ExPeCT Trial Participants

6.0 Introduction

The role that inflammation plays in cancer has been extensively documented (Korniluk et al., 2017, Grivennikov, 2010). Inflammatory cytokines may be involved in the initiation, promotion, progression and invasion of multiple cancer types (Landskron et al., 2014). In PrCa, multiple factors (e.g. hormonal imbalance, dietary factors) have been implicated as drivers in the development of chronic inflammation linked to carcinogenesis (De Marzo et al., 2007). Substantial crosstalk between stromal cells and the adjacent tumour cells have been documented in PrCa (Shiao et al., 2015). This relationship within the tumour microenvironment can lead to the repeated activation of inflammatory cells, generating a chronic systemic inflammatory state (Shiao et al., 2015). A study determining levels of systemic inflammation in PrCa compared the Glasgow Prognostic Score (GPR), a measurement of circulating levels of C-reactive protein and albumin, to the neutrophil lymphocyte ratio (NLR) which combines circulating neutrophil and lymphocyte counts (Shafique et al., 2012). The study demonstrated a significant correlation between high GPR and NLR, with poorer overall survival (Shafique et al., 2012). A further study, the Swedish AMORIS study, observed a significantly increased risk of developing advanced or metastatic PrCa with the presence of high levels of markers of systemic inflammation (e.g. C-reactive protein, albumin) (Arthur et al., 2018). Interestingly, some studies have demonstrated no significant changes in inflammatory marker levels (e.g. TNFα, IL-6) post exercise intervention (Jones, 2013, Galvao et al., 2008), suggesting varying effects of PA. Fluctuating levels of systemic inflammation in patients with metastatic PrCa may provide insight into disease progression, hence, a panel of cytokines associated with PrCa was compiled based on the current literature (Table 5.1).

Table 6.1: Panel of serum inflammatory markers utilised to assess systemic inflammation in ExPeCT trial participants.

	Analyte of Interest	Link to PrCa
Tissue Necrosis Factor	TNFα	High levels associated with progressive disease (Michalaki et al., 2004)
Vascular Endothelial Growth Factor	VEGF	Promotes cancer cell migration to the bone (Roberts et al., 2013)
Interleukin	IL-6	Elevated levels in CRPC (Nguyen et al., 2014)
	IL-1β	Aids skeletal metastatic progression (Liu et al., 2013)
	IL-17a	Enhances pathogenesis of PrCa (Liu et al., 2015)
	IL-4	Contributes to PrCa cell line proliferation (Roca et al., 2012)
	IL-10	Inhibits angiogenesis in PrCa primary cell lines (Stearns et al., 1999)
	IL-13	Negative regulator of tumour growth in several cancer types (Terabe et al., 2004)

Table 6.1 Continued: Panel of serum inflammatory markers utilised to assess systemic inflammation in ExPeCT trial participants.

	Analyte of Interest	**Link to PrCa**
Chemokine	MCP-1 (CCL2)	Release from adipocytes increases invasiveness in PrCa cells (Ito et al., 2015)
	RANTES (CCL5)	Expedites cancer progression in bone microenvironment (Roca et al., 2018)
	CXCL8 (IL-8)	Driver of PrCa progression (Neveu et al., 2014)
Metalloproteinase	MMP2	Highly expressed in PrCa (Xie et al., 2016)
	MMP9	Elevated expression linked to PrCa invasiveness (Aalinkeel et al., 2011)

Physical activity is thought to have multiple benefits for patients with cancer, including, reducing the effects of fatigue (Courneya et al., 2009), modifications in inflammatory marker levels (Rogers et al., 2013), improving physical fitness (Courneya et al., 2007) and increased overall survival (Kenfield et al., 2011). In line with the current literature, the ExPeCT trial aimed to determine the impact of inflammation in metastatic PrCa and the associated effects of a structured exercise intervention. The specific aims for this chapter were as follows; a) to assess

inflammatory mediator expression levels between exercise and control groups over time, b) to correlate clinical data to the ExPeCT inflammatory profile and c) to compare levels of inflammation mediators with CTCs and platelet cloaking.

6.1 Results

6.1.1 Inflammation in ExPeCT trial participants

The number of participant serum samples included in this analysis is outlined in Figure 5.2. Statistical analysis was performed in conjunction with Dr Bryan Stanfill (Study statistician). Data was transformed on the log base 2 scale and analysed using a linear mixed model with Gaussian response and graphed on the normal scale. Clinical variables were added to the model one at a time to test for any correlation with the analyte of interest. A summary of significant correlations is outlined in Table 6.2a and Table 6.2b.

Table 6.2a: Summary of significant correlations between inflammatory mediators and clinical variables within group

Analyte	Clinical Variable	p-value
MMP2	PSA	0.0186
MMP9	Platelet cloaking	0.0001
	Height	0.0045
	WCC	0.0000
	PSA	0.0006
VEGF	Platelet cloaking	0.0074
IL-6	Hb	0.0427
	WCC	0.0019
IL-8	CTCs	0.0337
	Platelet cloaking	0.0081
	Height	0.0170
	Hb	0.0450
IL-13	PSA	0.0002
TNFα	PSA	0.0426

Table 6.2b: Summary of significant correlations between inflammatory mediators and clinical variables between groups

Analyte	Clinical Variable	p-value
MMP2	Control-BMI	0.0418
	Control-Weight	0.0018
MMP9	Control-Weight	0.0207
VEGF	Exercise-WCC	0.0095
IL-17a	Exercise-WCC	0.0016
IL-1β	Exercise-hb	0.0242
IL-4	Exercise-CTCs	0.0067
	Control-Platelet Cloaking	0.0124
IL-10	Exercise-WCC	0.0244
IL-13	Exercise-Waist Circ	0.0123

6.1.1.1 CCL2 (MCP-1) expression in ExPeCT trial participants

CCL2 expression was determined in ExPeCT participants over time (Figure 6.1). No differences in expression levels were observed between groups over time (p=0.4222) (Table 6.3).

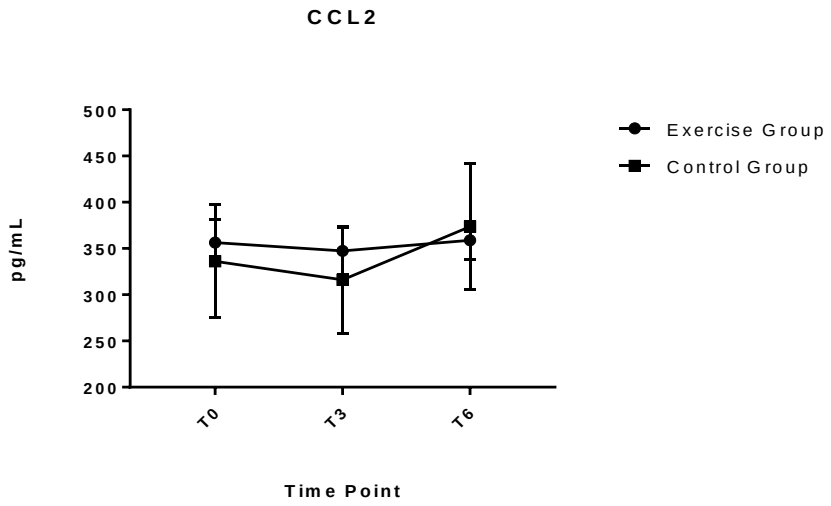


Figure 6.1: CCL2 expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=31, exercise n=26.

(Data range; exercise T0 61.4-606 pg/mL, control T0 178.7-883.8 pg/mL, exercise T3 154.5-567.9 pg/mL, control T3 172.6-484.4 pg/mL, exercise T6 177.5-645 pg/mL and control T6 171.8-1715.2 pg/mL).

Table 6.3: CCL2 expression, within and between, ExPeCT trial groups.

	Estimate	SD	z Value	p Value
Exercise-Control	0.1023	0.1275	0.8026	0.4222
T3-T0	-0.0492	0.0729	-0.6753	0.4995
T6-T0	0.0753	0.0755	0.9964	0.3191
T6-T3	0.1245	0.0766	1.6248	0.1042

No significant correlation between CTCs, platelet cloaking and CCL2 expression was identified (Table 6.4). Furthermore, no significant correlations between CCL2 and clinical variables were observed (Table 6.5).

Table 6.4: Correlation between CTCs, platelet cloaking and CCL2 expression.

	Estimate	SD	z Value	p Value
Avg CTCs	-0.0017	0.0035	-0.4825	0.6294
Platelet Cloaking	-0.0430	0.1080	-0.3980	0.6907

Table 6.5: Correlation between CCL2 and clinical variables.

	Estimate	SD	z value	p value
BMI	-0.0041	0.0645	-0.0634	0.9494
Weight	0.0325	0.0638	0.5098	0.6102
Height	0.0497	0.0661	0.7523	0.4518
Hb	0.0134	0.0636	0.2107	0.8331
WCC	0.0401	0.0457	0.8772	0.3804
PSA	-0.0344	0.0467	-0.7352	0.4622
Waist Circ	0.0100	0.0574	0.1738	0.8620

6.1.1.2 MMP2 expression in ExPeCT trial participants

The level of MMP2 was evaluated in ExPeCT participants (Figure 6.2). No significant differences in MMP2 expression were observed between groups (p=0.5186). There was a decrease in MMP2 expression registered within groups from T0-T3 (p=0.1063) (Table 6.6). There were no significant correlations between CTCs, platelet cloaking and MMP2 expression (Table 6.7).

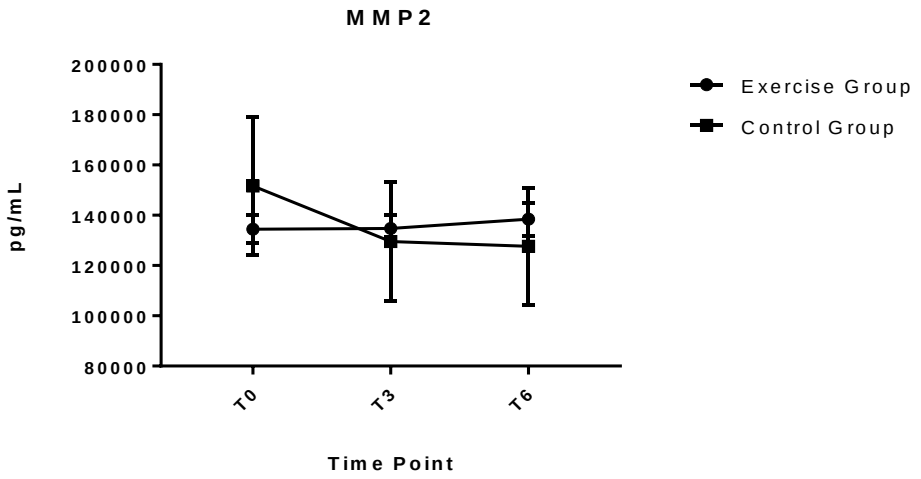


Figure 6.2: MMP2 expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 31126.4-244435.3 pg/mL, control T0 86080.5-503101.5 pg/mL, exercise T3 64699.4-203327.4 pg/mL, control T3 237.2-192346.9 pg/mL, exercise T6 69567.6-202265.4 pg/mL and control T6 94462.9-168854.5 pg/mL).

Table 6.6: MMP2 expression, within and between, ExPeCT trial groups.

	Estimate	SD	z Value	p Value
Exercise-Control	0.0978	0.1516	0.6456	0.5186
T3-T0	-0.2249	0.1392	-1.6150	0.1063
T6-T0	-0.1102	0.1446	-0.7620	0.4460
T6-T3	0.1147	0.1468	0.7813	0.4346

Table 6.7: Relationship between MMP2 expression, CTCs and platelet cloaking.

	Estimate	SD	z Value	p Value
Avg CTCs	0.0016	0.0054	0.3051	0.7603
Platelet Cloaking	0.1635	0.1833	0.8917	0.3726

A positive correlation between MMP2 expression was observed with PSA (p=0.0186). There was a significant difference between groups, in relation to BMI and weight. Within the control arm, MMP2 expression was positively correlated with both BMI and weight (p=0.0418 and p=0.0018, respectively). No relationship was observed between BMI and weight in the exercise group (Table 6.8).

Table 6.8: Correlation between MMP2 and clinical variables.

	Estimate	SD	z value	p value
Height	0.0657	0.0803	0.8173	0.4137
Hb	0.1335	0.0690	1.9341	0.0531
WCC	-0.0303	0.0242	-1.2549	0.2095
PSA	0.1591	0.0676	2.3542	**0.0186**
Waist Circ	0.0690	0.0767	0.8998	0.3682
Control BMI	0.2256	0.1108	2.0355	**0.0418**
Exercise BMI	-0.1015	0.1043	-0.9729	0.3306
Control Weight	0.3745	0.1203	3.1134	**0.0018**
Exercise Weight	-0.0946	0.0874	-1.0829	0.2789

6.1.1.3 MMP9 expression in ExPeCT trial participants

MMP9 expression was measured in ExPeCT participants and the range of expression is outlined in Figure 6.3. No significant difference in MMP9 expression was observed between groups (p=0.9043). Within groups, MMP9 expression increased over time from T0-T3 (p=0.0393) and T0-T6 (p=0.0547) (Table 6.9).

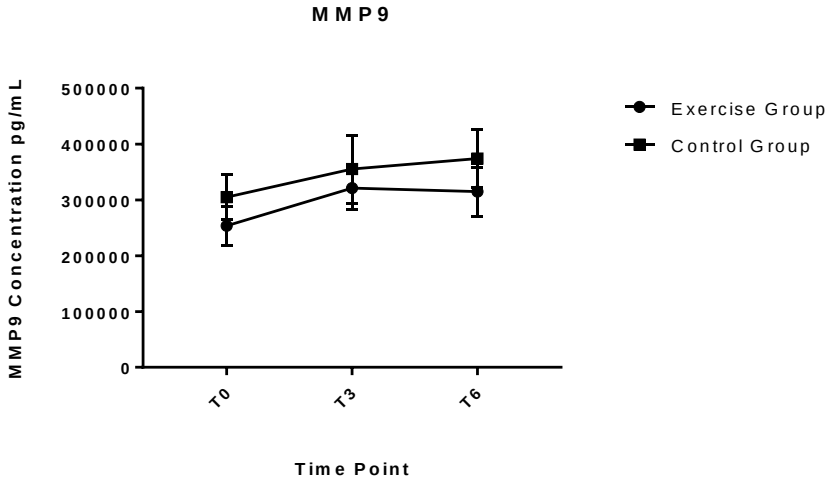


Figure 6.3: MMP9 expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 18343.6-731640.7 pg/mL, control T0 15886.2-904996.9 pg/mL, exercise T3 45630-855503.5 pg/mL, control T3 87116.9-1703781 pg/mL, exercise T6 87009.2-1123162 pg/mL and control T6 11561.8-1303303 pg/mL).

Table 6.9: MMP9 expression, between and within, ExPeCT participant groups.

	Estimate	SD	z Value	p Value
Exercise-Control	-0.0316	0.2627	-0.1202	0.9043
T3-T0	0.3329	0.1615	2.0612	**0.0393**
T6-T0	0.3194	0.1662	1.9214	**0.0547**
T6-T3	-0.0135	0.1698	-0.0794	0.9367

A positive correlation between CTCs and MMP9 expression was observed within groups (p=0.0540). A negative correlation between platelet cloaking and MMP9 expression was recorded within groups (p=0.0001) (Table 6.10).

Table 6.10: Correlation between MMP9 expression, CTCs and platelet cloaking.

	Estimate	SD	z Value	p Value
Avg CTCs	0.0142	0.0074	1.9269	0.0540
Platelet Cloaking	-0.9063	0.2254	-4.0206	**0.0001**

A significant correlation between MMP9 expression and several clinical variables was determined. MMP9 expression was positively correlated with height (p=0.0045) and white cell count (p=0.0000), while an inverse correlation with PSA (p=0.0006) was identified within groups. Between groups, a positive correlation between MMP9 expression and weight was

observed. In the control group, MMP9 expression was significantly associated with weight (p=0.0207). No relationship between weight and MMP9 expression was recorded in the exercise group (p=0.8021) (Table 6.11).

Table 6.11: Correlation between clinical variables and MMP9 expression.

	Estimate	SD	z value	p value
BMI	-0.0608	0.1353	-0.4494	0.6532
Height	0.3696	0.1303	2.8377	**0.0045**
Hb	0.2279	0.1238	1.8413	0.0656
WCC	0.4049	0.0906	4.4674	**0.0000**
PSA	-0.3228	0.0940	-3.4350	**0.0006**
Waist Circ	0.0276	0.1224	0.2252	0.8218
Control Weight	0.5048	0.2182	2.3130	**0.0207**
Exercise Weight	-0.0398	0.1588	-0.2506	0.8021

6.1.1.4 RANTES expression in ExPeCT trial participants

RANTES (CCL5) expression was determined in ExPeCT trial participants and is illustrated in Figure 6.4. No significant difference in RANTES expression was observed between groups over time (p=0.5431). Within groups, an increase in expression was determined (p=0.0554; Table 6.12).

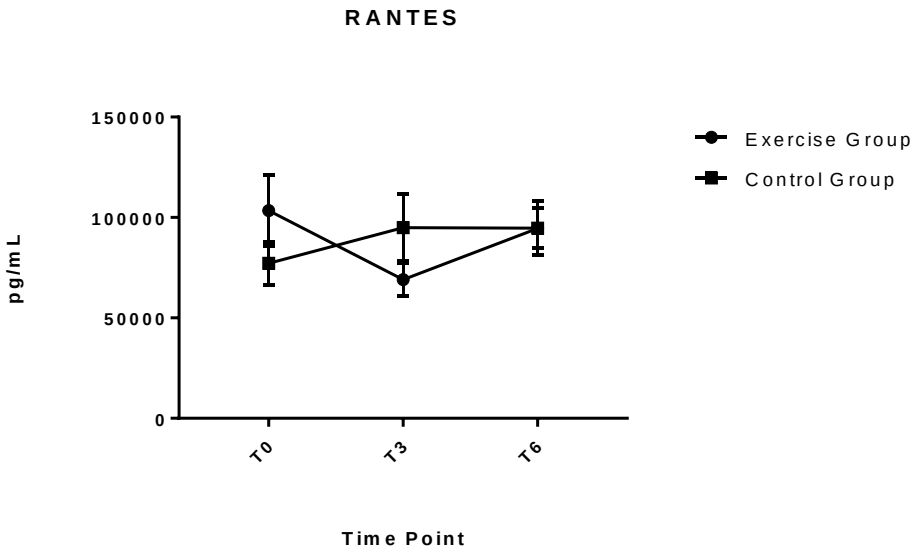


Figure 6.4: RANTES expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 13408.5-370259.3 pg/mL, control T0 19551.3-252098 pg/mL, exercise T3 2574.2-150675.9 pg/mL, control T3 6344.9-341543.2 pg/mL, exercise T6 24352.9-218103.4 pg/mL and control T6 4165.2-313550.1 pg/mL).

Table 6.12: RANTES expression, within and between, ExPeCT participant groups.

	Estimate	SD	z Value	p Value
Exercise-Control	0.1608	0.2645	0.6081	0.5431
T3-T0	-0.2830	0.1954	-1.4484	0.1475
T6-T0	0.1121	0.2022	0.5545	0.5792
T6-T3	0.3951	0.2063	1.9157	0.0554

The average number of CTCs was positively correlated with RANTES expression over time (p=0.1107). No significant association between platelet cloaking and RANTES expression was observed (p=0.9923; Table 6.13). No significant correlations were determined between RANTES expression and additional clinical variables (Table 6.14).

Table 6.13: Correlation between RANTES expression, CTCs and platelet cloaking.

	Estimate	SD	z Value	p Value
Avg CTCs	0.0132	0.0083	1.5949	0.1107
Platelet Cloaking	-0.0026	0.2732	-0.0097	0.9923

Table 6.14 Correlation between clinical variables and RANTES expression.

	Estimate	SD	z value	p value
BMI	0.2046	0.1338	1.5289	0.1263
Weight	0.0971	0.1350	0.7192	0.4720
Height	-0.1710	0.1373	-1.2457	0.2129
Hb	-0.2029	0.1304	-1.5563	0.1196
WCC	0.1396	0.1059	1.3179	0.1875
PSA	-0.0033	0.1033	-0.0320	0.9745
Waist Circ	0.0796	0.1279	0.6228	0.5334

6.1.1.5 VEGF expression in ExPeCT trial participants

The expression level of VEGF was determined in ExPeCT trial participants (Figure 6.5). No significant difference in VEGF expression was observed between groups (p=0.5509). Within groups, VEGF expression demonstrated no significant changes over time (Table 6.15).

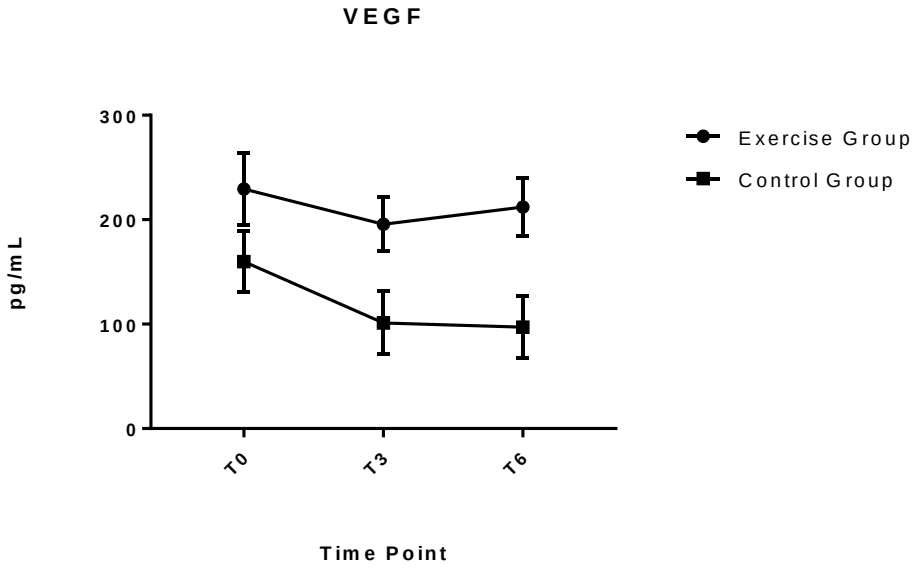


Figure 6.5: VEGF expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 57.3-675.9 pg/mL, control T0 49.4-444.3 pg/mL, exercise T3 22.6-518.4 pg/mL, control T3 57.1-526.8 pg/mL, exercise T6 35.4-539.7 pg/mL and control T6 100.6-388.2 pg/mL).

Table 6.15: VEGF expression, within and between, ExPeCT participant groups.

	Estimate	SD	z Value	p Value
Exercise-Control	0.1526	0.2559	0.5964	0.5509
T3-T0	-0.0098	0.0820	-0.1196	0.9048
T6-T0	-0.0371	0.0853	-0.4349	0.6636
T6-T3	-0.0273	0.0861	-0.3168	0.7514

Expression levels of VEGF were not significantly correlated with average CTC number, however a negative association between VEGF expression and platelet cloaking was observed (p=0.00740) (Table 6.16).

Table 6.16: Correlation between VEGF expression, CTCs and platelet cloaking.

	Estimate	SD	z Value	p Value
Avg CTCs	0.0042	0.0046	0.9268	0.3540
Platelet Cloaking	-0.3309	0.1235	-2.6796	**0.0074**

A significant correlation between white cell count and VEGF expression was observed between groups, with VEGF positively correlated with white cell count in the exercise group (p=0.0095). No significant relationship was identified in the control group (Table 6.17).

Table 6.17: Correlation between clinical variables and VEGF expression.

	Estimate	SD	z value	p value
Weight	-0.0019	0.1214	-0.0157	0.9874
Height	0.0099	0.1292	0.0768	0.9388
Hb	-0.0141	0.1107	-0.1272	0.8988
PSA	-0.0940	0.0813	-1.1561	0.2476
Waist Circ	-0.0803	0.0902	-0.8897	0.3736
Control BMI	0.2453	0.1685	1.4561	0.1454
Exercise BMI	-0.1999	0.1739	-1.1494	0.2504
Control WCC	0.1122	0.0600	1.8680	0.0618
Exercise WCC	0.4075	0.1571	2.5935	**0.0095**

6.1.1.6 IL-17a expression in ExPeCT trial participants

IL-17a expression was determined in ExPeCT trial participants (Figure 6.6). There was no significant difference in IL-17a expression either between or within ExPeCT groups over time (Table 6.18).

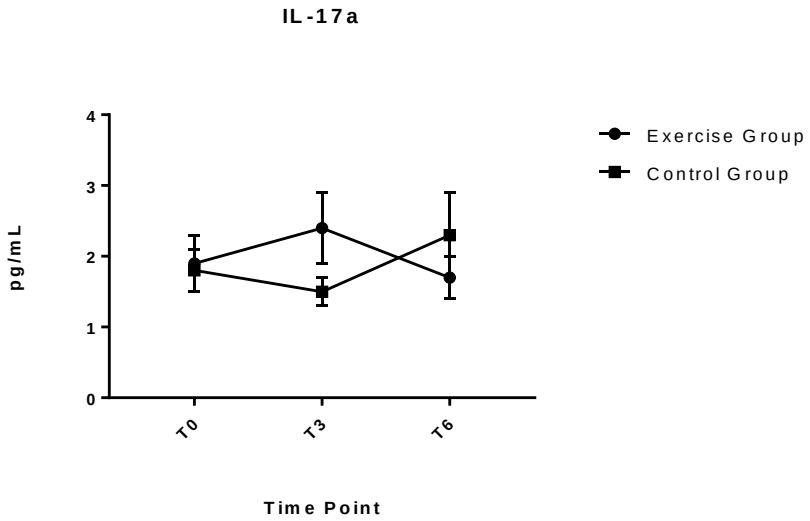


Figure 6.6: IL-17a expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 0.2-8 pg/mL, control T0 0.1-5.5 pg/mL, exercise T3 0.4-10.6 pg/mL, control T3 0.2-3.8 pg/mL, exercise T6 0.3-8.8 pg/mL and control T6 0.7-13.2 pg/mL).

Table 6.18: IL-17a expression, within and between, ExPeCT trial groups.

	Estimate	SD	z Value	p Value
Exercise-Control	-0.0496	0.3064	-0.1619	0.8714
T3-T0	0.1631	0.1685	0.9680	0.3330
T6-T0	0.0133	0.1730	0.0768	0.9388
T6-T3	-0.1499	0.1772	-0.8459	0.3976

No significant association between average CTC number or platelet cloaking with IL-17a expression were detected (Table 6.19).

Table 6.19: Correlation between IL-17a, CTCs and platelet cloaking.

	Estimate	SD	z Value	p Value
Avg CTCs	0.0054	0.0081	0.6630	0.5073
Platelet Cloaking	0.1621	0.2393	0.6775	0.4981

Most clinical variables added to the model, displayed no significant interactions with IL-17a expression. However, a significant association between IL-17a expression and white cell count was observed between groups. The exercise group demonstrated a significant positive correlation between IL-17a expression and white cell count (p=0.0016), with no significant relationship observed in the control group (Table 6.20).

Table 6.20: Correlation between clinical variables and IL-17a expression.

	Estimate	SD	z value	p value
BMI	0.2122	0.1537	1.3804	0.1675
Weight	0.1197	0.1552	0.7714	0.4405
Height	-0.1656	0.1577	-1.0501	0.2937
Hb	-0.2102	0.1455	-1.4441	0.1487
PSA	-0.0126	0.1100	-0.1142	0.9091
Waist Circ	0.0781	0.1368	0.5707	0.5682
Control WCC	0.0989	0.1080	0.9159	0.3597
Exercise WCC	0.8686	0.2749	3.1600	**0.0016**

6.1.1.7 IL-1β expression in ExPeCT trial participants

Levels of IL-1β expression in ExPeCT participants are illustrated in Figure 6.7. There was no significant difference in IL-1β expression between ExPeCT groups (p=0.4923). Within groups, a decrease in IL-1β expression was observed between T0 and T3 (p=0.0305). Contrastingly, an increase in IL-1β expression was recorded between T3 and T6 within groups (p=0.0190) (Table 6.21). No correlations were observed between IL-1β expression and CTC number or platelet cloaking (Table 6.22).

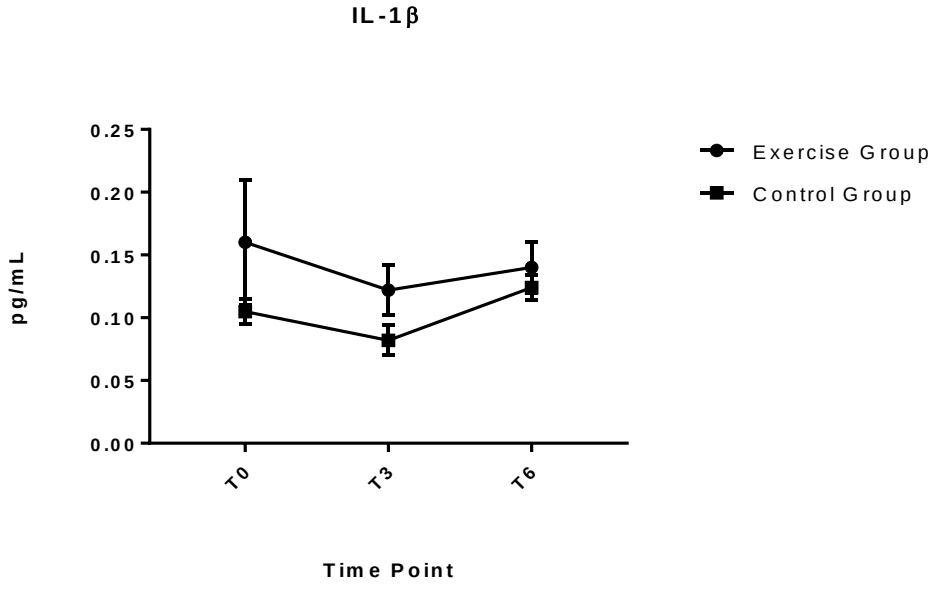


Figure 6.7: IL-1β expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 0.045-0.99 pg/mL, control T0 0.018-0.27 pg/mL, exercise T3 0.014-0.33 pg/mL, control T3 0.013-0.21 pg/mL, exercise T6 0.032-0.43 pg/mL and control T6 0.023-0.2 pg/mL).

Table 6.21: IL-1β expression, within and between, ExPeCT trial participants.

	Estimate	SD	z Value	p Value
Exercise-Control	0.2359	0.3434	0.6867	0.4923
T3-T0	-0.4587	0.2121	-2.1629	**0.0305**
T6-T0	0.1061	0.2394	0.4430	0.6577
T6-T3	0.5647	0.2409	2.3446	**0.0190**

Table 6.22: Correlation between IL-1β expression, CTCs and platelet cloaking.

	Estimate	SD	z Value	p Value
Avg CTCs	0.0117	0.0107	1.0917	0.2750
Platelet Cloaking	0.1528	0.3083	0.4956	0.6201

Between groups, a significant correlation was observed with IL-1β expression and haemoglobin. In the control group, an inverse correlation was reported (p=0.0242), with no significant association observed in the exercise group (Table 6.23).

Table 6.23: Correlation between clinical variables and IL-1β expression.

	Estimate	SD	z value	p value
BMI	0.2782	0.1803	1.5433	0.1228
Weight	0.1724	0.1596	1.0806	0.2799
Height	-0.1016	0.1868	-0.5437	0.5867
WCC	0.1846	0.1399	1.3194	0.1870
PSA	0.0184	0.1365	0.1351	0.8926
Waist Circ	0.1402	0.1522	0.9208	0.3572
Control Hb	-0.4811	0.2134	-2.2546	**0.0242**
Exercise Hb	0.1394	0.2508	0.5558	0.5784

6.1.1.8 IL-4 expression in ExPeCT trial participants

IL-4 expression was monitored in ExPeCT trial participants over time (Figure 6.8). No significant differences in IL-4 expression were determined between or within ExPeCT trial groups (Table 6.24).

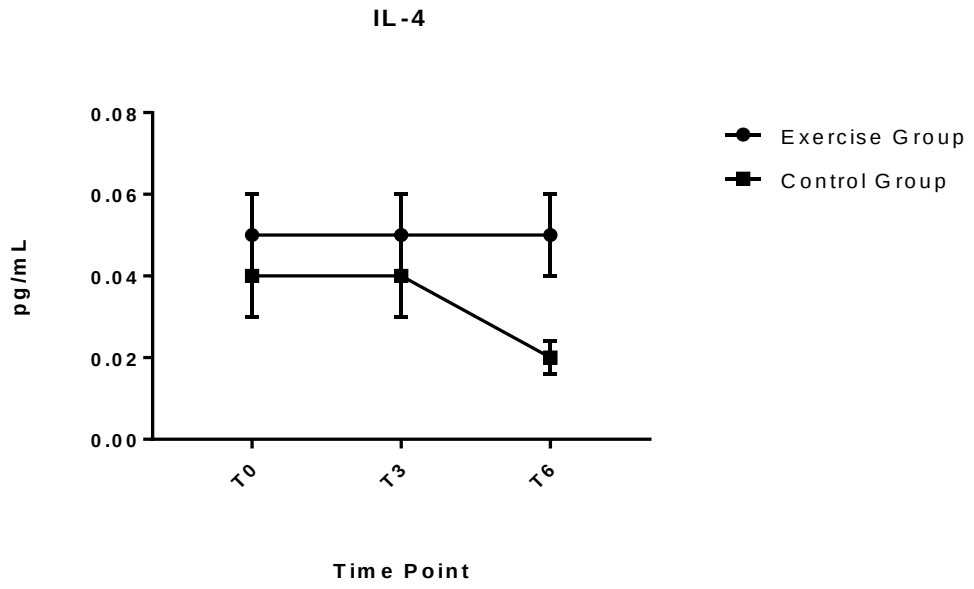


Figure 6.8: IL-4 expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 0.012-0.17 pg/mL, control T0 0.01-0.10 pg/mL, exercise T3 0.004-0.17 pg/mL, control T3 0.127-0.12 pg/mL, exercise T6 0.01-0.21 pg/mL and control T6 0.01-0.09 pg/mL).

Table 6.24: IL-4 expression, between and within, ExPeCT trial groups.

	Estimate	SD	z Value	p Value
Exercise-Control	0.1304	0.4202	0.3102	0.7564
T3-T0	0.0348	0.3390	0.1027	0.9182
T6-T0	-0.2854	0.3631	-0.7861	0.4318
T6-T3	-0.3202	0.3506	-0.9135	0.3610

Significant differences between groups were determined in terms of CTC number and platelet cloaking. A positive correlation between IL-4 expression and CTC number in the exercise group (p=0.0067) was determined, however no significant correlation was observed in the control group. A negative correlation was observed between IL-4 expression and platelet cloaking in the control group (p=0.0124) (Table 6.25).

Table 6.25: Correlation between IL-4 expression, CTC number and platelet cloaking.

	Estimate	SD	z Value	p Value
Control Avg CTCs	-0.0087	0.0276	-0.3157	0.7522
Exercise Avg CTCs	0.0540	0.0199	2.7090	**0.0067**
Control Platelet Cloaking	-1.1606	0.4639	-2.5018	**0.0124**
Exercise Platelet Cloaking	1.6142	1.1094	1.4550	0.1457

No further significant correlations between IL-4 expression and clinical variables were observed (Table 6.26).

Table 6.26: Correlation between IL-4 expression and clinical variables.

	Estimate	SD	z value	p value
BMI	-0.0161	0.2204	-0.0730	0.9418
Weight	0.0479	0.2102	0.2278	0.8198
Height	0.1287	0.2225	0.5782	0.5631
Hb	0.2727	0.2033	1.3413	0.1798
WCC	-0.0911	0.2086	-0.4365	0.6625
PSA	-0.0344	0.1959	-0.1757	0.8605
Waist Circ	-0.0073	0.2076	-0.0349	0.9721

6.1.1.9 IL-6 expression in ExPeCT trial participants

IL-6 expression was determined in ExPeCT trial participants at each time point (Figure 6.9). No significant differences, between or within, groups were observed over time (Table 6.27). No significant changes correlation between CTC number, platelet cloaking and IL-6 expression was determined (Table 6.28).

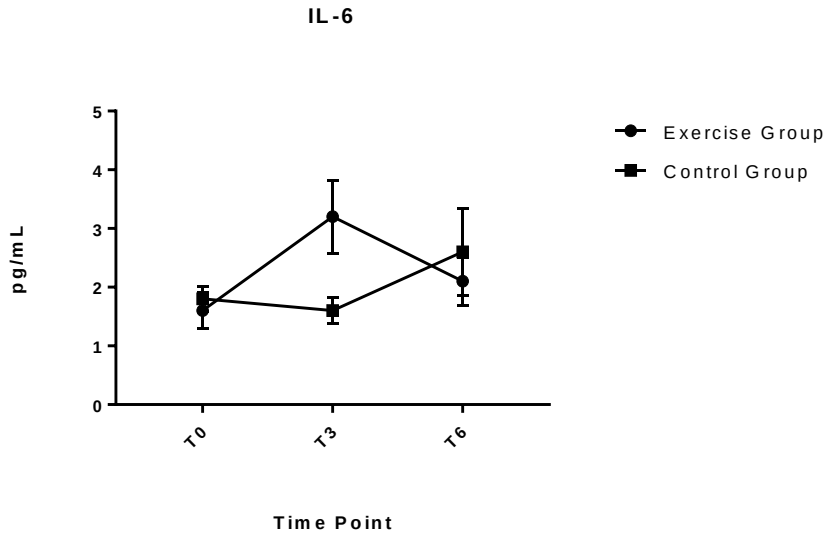


Figure 6.9: IL-6 expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 0.1-6.5 pg/mL, control T0 0.7-4.9 pg/mL, exercise T3 0.4-17.9 pg/mL, control T3 0.3-5 pg/mL, exercise T6 1.0-12.6 pg/mL and control T6 0.4-21 pg/mL).

Table 6.27: IL-6 expression, within and between, ExPeCT trial groups.

	Estimate	SD	z Value	p Value
Exercise-Control	-0.0647	0.2437	-0.2655	0.7906
T3-T0	0.1300	0.1861	0.6984	0.4849
T6-T0	0.1719	0.1895	0.9075	0.3642
T6-T3	0.0419	0.1940	0.2162	0.8288

Table 6.28: Correlation between IL-6 expression, CTC number and platelet cloaking.

	Estimate	SD	z Value	p Value
Avg CTCs	0.0097	0.0078	1.2474	0.2123
Platelet Cloaking	0.2479	0.2544	0.9745	0.3298

An inverse correlation between IL-6 expression and haemoglobin within groups was recorded (p=0.0427). Additionally, a positive correlation between IL-6 and white cell count was observed (p=0.0019) (Table 6.29).

Table 6.29: Correlation between IL-6 expression and clinical variables.

	Estimate	SD	z value	p value
BMI	0.0814	0.1257	0.6473	0.5174
Weight	0.0594	0.1237	0.4804	0.6310
Height	-0.0926	0.1279	-0.7240	0.4691
Hb	-0.2364	0.1167	-2.0266	**0.0427**
WCC	0.3014	0.0972	3.1008	**0.0019**
PSA	0.0964	0.0962	1.0029	0.3159
Waist Circ	0.0340	0.1184	0.2873	0.7739

6.1.1.10 IL-8 expression in ExPeCT trial participants

IL-8 expression was recorded in ExPeCT trial participants over time (Figure 6.10). There was no significant change in IL-8 expression between groups. A significant increase was observed within groups from T0-T6 (p=0.0257) and T3-T6 (p=0.0350) (Table 6.30).

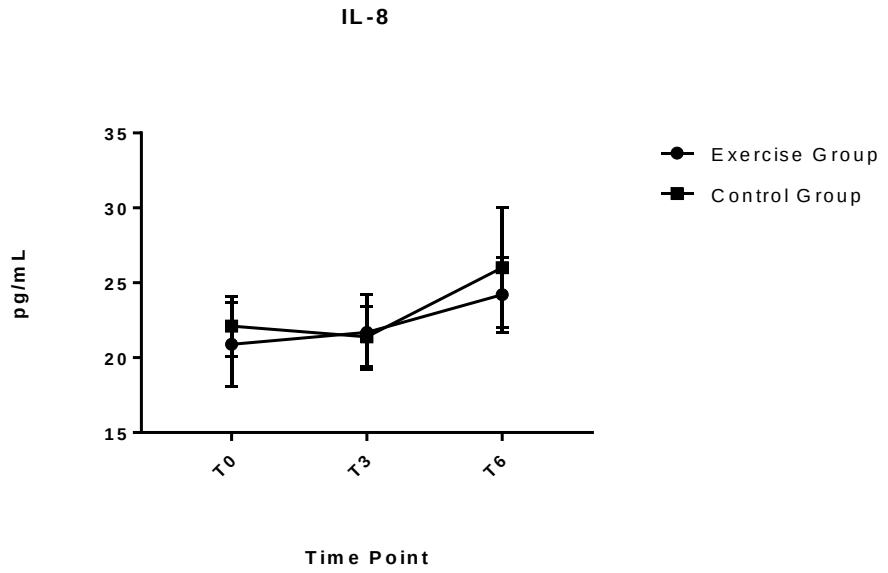


Figure 6.10: IL-8 expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 7.7-66.8 pg/mL, control T0 8.2-62.5 pg/mL, exercise T3 07.1-58.6 pg/mL, control T3 12.5-62.7 pg/mL, exercise T6 8.2-74.1 pg/mL and control T6 8.2-128 pg/mL).

Table 6.30: IL-8 expression, within and between, ExPeCT trial groups.

	Estimate	SD	z Value	p Value
Exercise-Control	-0.0622	0.1579	-0.3939	0.6937
T3-T0	0.0075	0.0964	0.0774	0.9383
T6-T0	0.2189	0.0981	2.2307	**0.0257**
T6-T3	0.2114	0.1003	2.1083	**0.0350**

IL-8 expression was significantly associated with CTC number and platelet cloaking (p=0.0337 and p=0.0081, respectively) (Table 6.31).

Table 6.31: Correlation between IL-8, CTC number and platelet cloaking.

	Estimate	SD	z Value	p Value
Avg CTCs	-0.0093	0.0044	-2.1241	**0.0337**
Platelet Cloaking	-0.3564	0.1346	-2.6470	**0.0081**

Significant correlations between additional clinical variables and IL-8 expression were observed within group. Positive correlations between IL-8 and haemoglobin (p=0.0450) and IL-8 and height were observed (p=0.0170) (Table 6.32).

Table 6.32: Correlation between IL-8 expression and clinical variables.

	Estimate	SD	z value	p value
BMI	-0.0825	0.0790	-1.0455	0.2958
Weight	0.0191	0.0784	0.2434	0.8077
Height	0.1857	0.0778	2.3867	**0.0170**
Hb	0.1541	0.0768	2.0051	**0.0450**
WCC	-0.0657	0.0592	-1.1085	0.2677
PSA	-0.0476	0.0597	-0.7967	0.4256
Waist Circ	-0.1006	0.0723	-1.3912	0.1642

6.1.1.11 IL-10 expression in ExPeCT trial participants

Differences in IL-10 expression were determined in ExPeCT trial participants over time (Figure 6.11). No significant difference in IL-10 expression was observed within or between ExPeCT trial groups (Table 6.33). No significant relationship between IL-10 expression, CTC number and platelet cloaking was determined (Table 6.34).

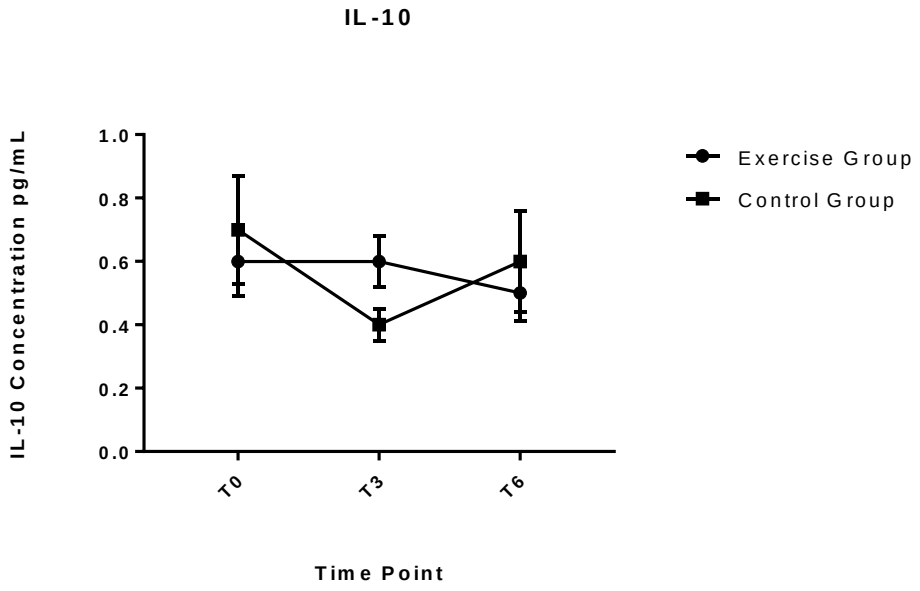


Figure 6.11: IL-10 expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 0.2-2.7 pg/mL, control T0 0.1-4.1 pg/mL, exercise T3 0.2-2.0 pg/mL, control T3 0.1-1.0 pg/mL, exercise T6 0.2-2.2 pg/mL and control T6 0.1-4.5 pg/mL).

Table 6.33: IL-10 expression, within or between, ExPeCT trial groups.

	Estimate	SD	z Value	p Value
Exercise-Control	0.1548	0.2225	0.6956	0.4867
T3-T0	-0.1688	0.1488	-1.1343	0.2567
T6-T0	-0.1495	0.1515	-0.9864	0.3239
T6-T3	0.0194	0.1550	0.1250	0.9005

.

Table 6.34: Correlation between IL-10 expression, CTC number and platelet cloaking.

	Estimate	SD	z Value	p Value
Avg CTCs	0.0055	0.0066	0.8348	0.4038
Platelet Cloaking	-0.1220	0.2102	-0.5802	0.5618

An association between white cell count and IL-10 expression was observed between groups. A positive correlation was identified in the exercise group (p=0.0244), with no significant association recorded in the control group (Table 6.35).

Table 6.35: Correlation between IL-10 expression and clinical variables.

	Estimate	SD	z value	p value
BMI	-0.0268	0.1157	-0.2313	0.8171
Weight	-0.0110	0.1111	-0.0994	0.9208
Height	0.0075	0.1180	0.0639	0.9491
Hb	0.0937	0.1117	0.8394	0.4013
PSA	0.0725	0.0867	0.8360	0.4031
Control WCC	-0.0549	0.0927	-0.5925	0.5535
Exercise WCC	0.5195	0.2308	2.2509	**0.0244**

6.1.1.12 IL-13 expression in ExPeCT trial participants

IL-13 expression measured in ExPeCT trial participants over time is highlighted in Figure 6.12. There were no significant changes in IL-13 expression between or within groups over time in ExPeCT participants (Table 6.36). There was no significant difference observed between CTC number, platelet cloaking and IL-13 expression (Table 6.37).

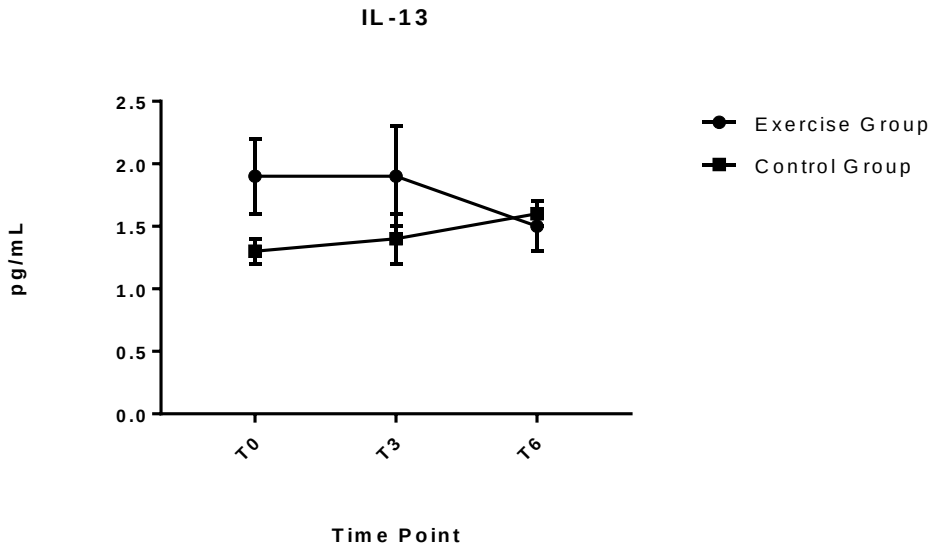


Figure 6.12: IL-13 expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 0.7-5.5 pg/mL, control T0 0.4-2.4 pg/mL, exercise T3 0.4-9.4 pg/mL, control T3 0.5-3.3 pg/mL, exercise T6 0.5-3.2 pg/mL and control T6 0.7-2.6 pg/mL).

Table 6.36: IL-13 expression, within or between, ExPeCT trial groups.

	Estimate	SD	z Value	p Value
Exercise-Control	0.2503	0.2408	1.0397	0.2985
T3-T0	-0.0687	0.1824	-0.3767	0.7064
T6-T0	0.0478	0.1816	0.2633	0.7923
T6-T3	0.1166	0.1887	0.6177	0.5368

Table 6.37: Correlation between CTCs, platelet cloaking and IL-13 expression.

	Estimate	SD	z Value	p Value
Avg CTCs	-0.0020	0.0068	-0.2864	0.7746
Control Platelet Cloaking	-0.4825	0.4206	-1.1471	0.2513
Exercise Platelet Cloaking	0.7579	0.5162	1.4683	0.1420

A significant inverse correlation within groups was observed between IL-13 expression and PSA (p=0.0002). A significant difference between groups was also determined in relation to waist circumference. An inverse correlation between IL-13 expression and waist circumference was observed in the exercise group (p=0.0123), no significant association was noted within the control group (Table 6.38).

Table 6.38: Correlation between IL-13 expression and clinical variables.

	Estimate	SD	z value	p value
BMI	-0.0857	0.1276	-0.6715	0.5019
Weight	-0.1465	0.1210	-1.2106	0.2260
Height	-0.0939	0.1274	-0.7373	0.4609
Hb	-0.0273	0.1223	-0.2235	0.8231
WCC	-0.0013	0.1030	-0.0128	0.9898
PSA	-0.4534	0.1216	-3.7299	**0.0002**
Waist Circ	-0.1967	0.1135	-1.7339	0.0829
Control – Waist Circ	0.0115	0.1595	0.0720	0.9426
Exercise – Waist Circ	-0.3910	0.1562	-2.5036	**0.0123**

6.1.1.13 TNFα expression in ExPeCT trial participants

TNFα expression levels were determined in ExPeCT trial participants (Figure 6.13). There was no significant difference in TNFα expression within or between ExPeCT trial groups (Table 6.39). No significant association between CTC number, platelet cloaking and TNFα expression was observed (Table 6.40).

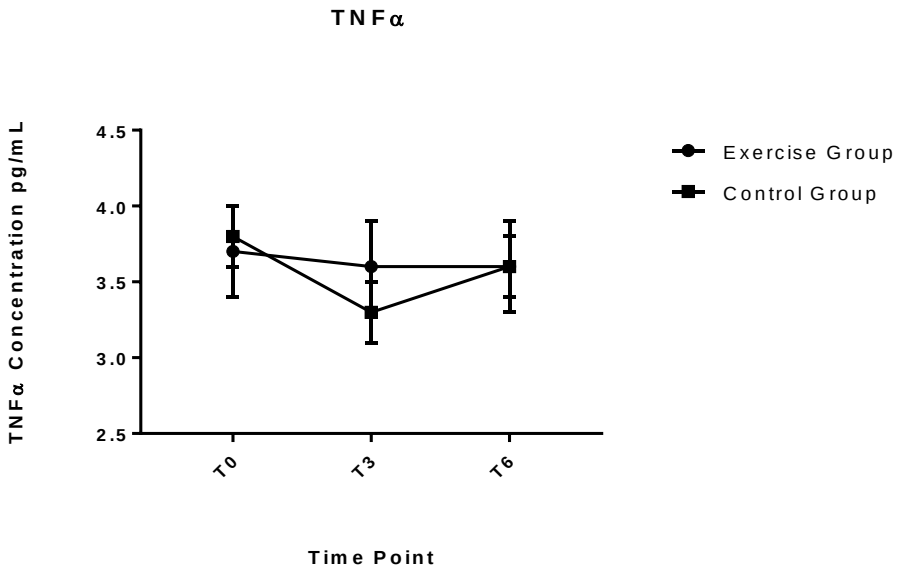


Figure 6.13: TNFα expression over time in ExPeCT trial participants.

Data graphed as mean ± SEM. Control n=30, exercise n=26.

(Data range; exercise T0 1.6-7.9 pg/mL, control T0 1.5-8 pg/mL, exercise T3 1.8-7.2 pg/mL, control T3 1.8-6.8 pg/mL, exercise T6 1.5-7.7 pg/mL and control T6 2.1-5.4 pg/mL).

Table 6.39: TNFα expression, within and between, ExPeCT participant groups.

	Estimate	SD	z Value	p Value
Exercise-Control	-0.0465	0.1290	-0.3606	0.7184
T3-T0	-0.0946	0.0618	-1.5321	0.1255
T6-T0	-0.0253	0.0628	-0.4031	0.6869
T6-T3	0.0693	0.0641	1.0808	0.2798

Table 6.40: Correlation between TNFα expression, CTCs and platelet cloaking.

	Estimate	SD	z Value	p Value
Avg CTCs	0.0009	0.0031	0.2762	0.7824
Platelet Cloaking	0.0796	0.0916	0.8682	0.3853

A significant positive correlation between PSA and TNFα expression was detected within group (p=0.0426) (Table 6.41).

Table 6.41: Correlation between TNFα expression and clinical variables.

	Estimate	SD	z value	p value
BMI	0.0626	0.0641	0.9776	0.3283
Weight	0.0250	0.0635	0.3936	0.6939
Height	-0.0994	0.0657	-1.5127	0.1303
Hb	0.0231	0.0615	0.3763	0.7067
WCC	0.0438	0.0409	1.0722	0.2836
PSA	0.0893	0.0440	2.0274	**0.0426**
Waist Circ	-0.0235	0.0548	-0.4298	0.6674

6.2 Discussion

Improved understanding of the role of inflammation in metastatic PrCa, may influence clinical outcomes and improve QoL for patients. The impact of a structured exercise intervention that is inclusive of skeletal metastasis, the primary site of metastasis in PrCa, may aid in these investigations. To this end, a panel of 13 inflammatory mediators was chosen based on peer reviewed publications on inflammation and PrCa. Each inflammatory mediator was investigated individually over time in ExPeCT participants.

No significant difference in mediator concentration was observed over time between groups. However, multiple changes were witnessed within groups. Significant increases in MMP9, RANTES, IL-1β and IL-8 expression occurred within group, as well as a significant decrease in MMP2 expression. A number of factors may have contributed to the lack of difference in the inflammatory profile between groups. The structured exercise intervention was aerobic based and for a period of 6 months. This length of time may not be sufficient to witness changes in systemic inflammation, especially in stable disease states. Additionally, as resistance training has been demonstrated to impact inflammatory mediators in PrCa previously (Galvao et al., 2008), introduction of a resistance component may illicit a greater response. The significant fluctuations in inflammatory mediator expression within each group may be reflective of the nature of systemic inflammation in metastatic PrCa. As discussed previously, inflammation is understood to be a hallmark of cancer and may contribute to disease progression (Hanahan and Weinberg, 2011). Inflammatory mediators are released within the tumour microenvironment and contribute to cell signalling within the metastatic cascade (Stark et al., 2015). Furthermore, multiple metastatic lesions within this population of patients, may contribute to the significant changes in expression. Crosstalk between immune and inflammatory mediators may also have contributed to the variable levels of expression observed.

Waist circumference, PSA, height, weight, BMI, white cell count and haemoglobin were assessed with each individual inflammatory

mediator. Significant correlations between PSA, haemoglobin and white cell count with inflammatory expression were recorded within both the exercise and control groups. Positive correlations were detected between haemoglobin and MMP9, IL-8 and IL-6 expression. Increased haemoglobin levels are associated with cardiac events (Chonchol and Nielson, 2008), and thus these results may be demonstrative of a relationship between cardiovascular issues or MetS and inflammation. Significant correlations with white cell count and IL-6 and MMP9 were identified. In the general non-cancer population, white cell count has been correlated with inflammatory mediators as an independent predictor of systemic inflammation (Compte et al., 2015), suggesting a similar role in this patient population. A positive correlation between PSA and expression of MMP2 and TNFα was confirmed, as well as an inverse correlation with expression of MMP9 and IL-13. These observations have not been extensively documented previously in metastatic PrCa, and may be indicative of disease phenotype. Taken together these results may be reflective of the inflammatory microenvironment in metastatic PrCa, and may provide useful tools for identifying future targeted therapies or immuno-oncology agents.

Between ExPeCT exercise and control groups, multiple correlations between expression levels and clinical variables were determined. Positive correlations between BMI, weight and waist circumference with MMP2, MMP9, VEGF and IL-13 were observed within the control group that differed to the exercise group. The differences between groups in this set of variables, commonly associated with obesity, infers a positive impact of the exercise intervention. While expression levels were not significantly altered within the exercise group, the lack of a structured exercise intervention in the control arm may have contributed to the significant changes witnessed. This result indicates that an aerobic exercise component may have considerable benefits on the modulation of the relationship between obesity and inflammation in metastatic PrCa. Additionally, significant changes in the exercise group in terms of interleukin expression and white cell count and haemoglobin were identified. These variations between clinical variables and

expression levels suggest a positive impact of exercise on low grade systemic inflammation and markers of advanced disease.

The relationship between CTC numbers, platelet cloaking and inflammatory mediators was assessed. A significant difference in IL-4 expression effecting CTC number between the exercise and control groups was observed. A number of mediators (IL-8, MMP9 and VEGF) exhibited significant changes in expression levels within group which were significantly correlated with CTCs and platelet cloaking. The relationship between inflammatory mediators and CTCs is one that has been documented previously. An increase in IL-4 expression has been associated with CTC number in patients with breast cancer, with the implication that it is linked to poorer outcomes (Konig et al., 2016). Furthermore, MMP expression and CTCs demonstrated the potential to predict the onset of metastases (Skerenova et al., 2017). These findings may hold clinical significance in terms of markers of disease progression and/or new therapeutic targets. The relationship between platelet cloaking of CTCs and inflammation in PrCa has not been documented previously. The negative correlations witnessed between some of the inflammatory mediators and levels of platelet cloaking, may be useful tools in elucidating the mechanisms associated with platelet cloaking in PrCa.

A limitation of this analysis is the number of participants involved. However, based on these findings from this study, a larger trial powered for these outcomes may aid in the improved understanding of the impact of exercise in metastatic PrCa. The results from this chapter aimed to elucidate the landscape of metastatic PrCa further, focussing on chronic systemic inflammation. The data reported provides information on inflammation in metastatic disease, for profiling aggressive disease subtypes, as well as highlighting mediators which could improve targeted treatment options in patients with PrCa.

Chapter 7:

Assessing the Link between Obesity and Enzalutamide Resistance

7.0 Introduction

ADT is currently the treatment option of choice in patients with disease progression. However, in spite of initial ADT success, all patients will eventually progress to CRPC. A range of treatment options, including cabazitaxel, abiraterone acetate, radium-223 and enzalutamide (Chrvala, 2012), have been developed to tackle disease progression in CRPC in recent years. One such agent, enzalutamide, an oral AR signalling inhibitor that binds to the ligand-binding domain of the AR and blocks translocation to the nucleus, significantly increased survival in patients with metastatic CRPC, both after treatment with chemotherapy and in chemotherapy naive patients (Scher et al., 2012, Beer et al., 2014).

The efficacy of enzalutamide in comparison to other single agents has also been documented. Bicalutamide, a second-line hormonal agent, has been utilised in the treatment of CRPC. A randomised trial of bicalutamide *vs.* enzalutamide, established a significant reduction in disease progression when treated with enzalutamide in comparison to bicalutamide. However, in spite of some success, acquired resistance is a clinical issue (Kregel et al., 2016). Additionally, a large portion of patients demonstrate intrinsic resistance (20-40%) from the outset (Antonarakis et al., 2014). Although not yet fully understood, resistance is thought to be due in part to the generation of AR variants, such as AR-V7, among other mechanisms. In CRPC cell lines, AR variants were determined to play a role in enzalutamide resistance by aiding AR transcription and activating AR associated genes, mediating cell proliferation (Li et al., 2013). AR variants may have undergone alternative splicing, resulting in the loss of the ligand binding domain, thus allowing specific variants to be unaffected by antagonists whose role it is to bind to the ligand-binding domain (Bubley and Balk, 2017). In a study examining AR-V7 expression on CTCs, it was established that in patients with CRPC with AR-V7 positive CTCs, intrinsic resistance to both enzalutamide and abiraterone acetate is commonplace (Antonarakis et al., 2014). Currently, no data is publicly available on the numbers of patients in Ireland undergoing treatment with Enzalutamide, highlighting

the novelty of these data and long term effects. Weight gain during ADT and the eventual resistance to anti-androgen treatment suggests the development of obesity may be linked to obesity related inflammation.

As outlined previously, obesity and MetS are associated with disease progression in PrCa (Hsing et al., 2007), yet the relationship between enzalutamide resistance, MetS, obesity and inflammation remains poorly understood. Conteduca et al. investigated the role between MetS, inflammation and enzalutamide/abiraterone treatment in patients with CRPC. The study identified significantly poorer overall survival and poorer progression free survival post treatment in patients with CRPC, who met the criteria for MetS and a pro-inflammatory state (Conteduca et al., 2018). These outcomes highlight the need for further investigation in this area. The ExPeCT trial aimed to further assess the role of MetS and obesity in metastatic PrCa by assessing mRNA expression levels of a panel of lethality-associated obesity and coagulation related genes (Table 7.1) in ExPeCT participant NCBs. This gene signature was derived from a dataset of genes known to be associated with lethal PrCa, generated by collaborator Professor Lorelei Mucci's research group using data from the HPFS (unpublished data) (Figure 7.1) (Harvard TH Chan School of Public Health). This signature was identified based on the role of the genes in obesity and coagulation.

Table 7.1: Summary of lethality-associated gene panel.

Gene of Interest	Full Name	Role
CXCR4	Chemokine Receptor 4	Aids bone metastasis (Conley-LaComb et al., 2016), mediates recruitment of adipose tissue-derived stromal cells by PrCa cells (Lin et al., 2010)
PLA2G7	Phospholipase A2, group VII	Increased oxidised phospholipids, inhibited by statin use, aids invasion in PrCa (Vainio et al., 2011)
PTGER1	Prostaglandin E Receptor 1	Role in COX2 pathway and inflammation, ERG activation cancer progression (Mohamed et al., 2011)
AVPR2	Arginine Vasopressin Receptor 2	Arginine vasopressin present in different cancers, may have effect on tumour growth and metabolism (North, 2000)
HTRB2	5-Hydroxytryptamine (serotonin) Receptor 2B	Binds serotonin, present in low and high grade PrCa tumours (Dizeyi et al., 2005)

The aims of this chapter were to a) determine the expression levels of a lethality associated gene signature in ExPeCT diagnostic NCBs and a panel of PrCa cell lines, b) to determine the expression of a lethality associated gene expression profile in enzalutamide resistance *in vitro,* c) examine the effect of adiponectin and leptin on enzalutamide resistant cell lines *in vitro*, d) to establish the effect of adipocyte conditioned media on enzalutamide resistant cell lines *in vitro*.

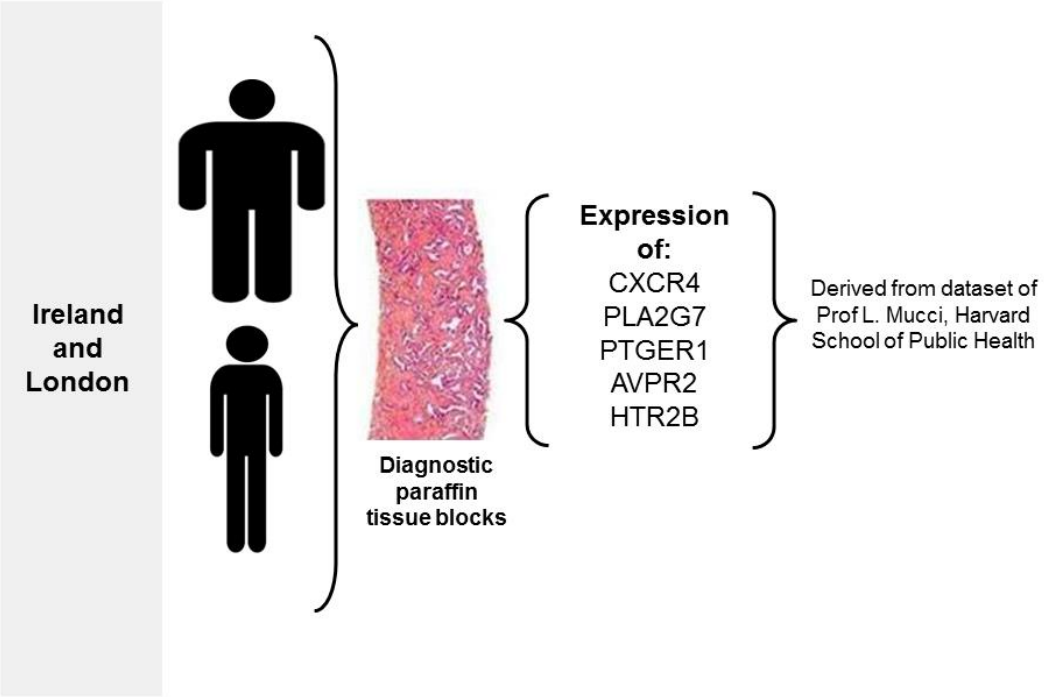


Figure 7.1: Outline of lethality gene signature analysis in ExPeCT NCBs.

7.1 Results

7.1.1 Expression of lethality associated gene signature in ExPeCT participant NCBs

Diagnostic NCBs were available for n=38 ExPeCT participants. Gene expression data was normalised based on an endogenous control (GAPDH) and quantified using $\Delta\Delta$CT method to calculate relative quantification (RQ) based on a reference sample of 1 (Figure 7.2). Statistical analysis of this data was performed in conjunction with Dr Bryan Stanfill (Study statistician).

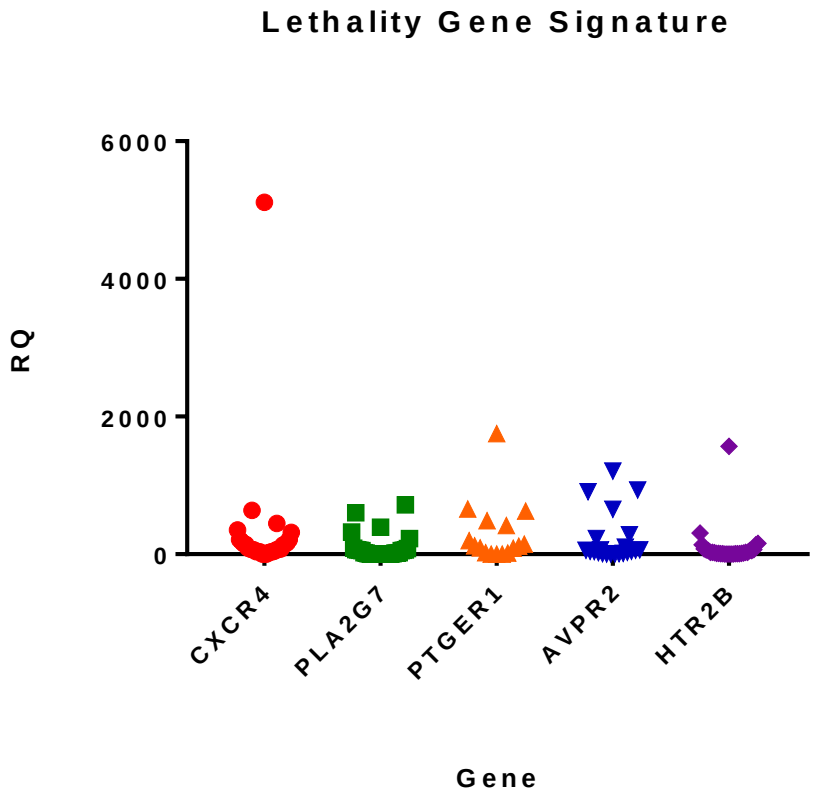


Figure 7.2: RQ expression of ExPeCT NCB (n=38).

Each gene was compared to the panel of inflammatory mediators assessed in ExPeCT participants at T0, in addition to clinical variables and CTCs, using a linear mixed model with a Gaussian response on the log base 2 scale. A Bonferroni correction was applied to eliminate false positives results, resulting in a new threshold for statistical significance (AVPR2 – p=0.0014, CXCR4 – p=0.0016, HTR2B – p=0.0017, PLA2G7 – p=0.0017 and PTGER1 – p=0.0017). A significant inverse correlation was observed between AVPR2 and resistin expression (p=0.0002) in ExPeCT participants (Figure 7.3, Table 7.2). Additionally, inverse correlations were observed between PTGER1 and IL-13 and IL4 between groups (p<0.0000) (Figure 7.4, Table 7.3). No significant associations were identified between CXCR4, HTR2B or PLA2G7 (Appendix VIII) inflammatory mediators (Table 6.1) or clinical variables.

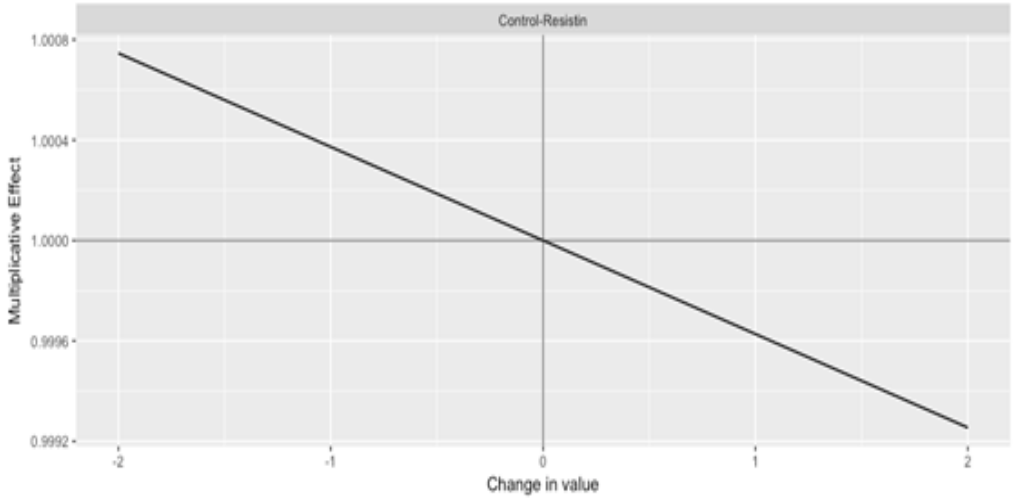


Figure 7.3: Correlation between AVPR2 gene expression and resistin expression in ExPeCT control group participants.

Table 7.2: Correlation between AVPR2 gene expression, inflammatory mediators and clinical variables.

	Estimate	SD	z_value	p_value
Exercise - Control	-2.7450	1.0531	-2.6065	0.0091
Weight	-0.0070	0.6212	-0.0113	0.9911
Height	0.4683	0.6123	0.7648	0.4533
Hb	0.2624	0.6185	0.4243	0.6759
WCC	0.4659	0.6124	0.7607	0.4557
PSA	-0.9104	0.5869	-1.5512	0.1365
WCir	0.2020	0.6491	0.3112	0.7590
AvgCTCs	0.0020	0.0629	0.0323	0.9746
Adiponectin	0.1212	0.6417	0.1889	0.8523
C_peptide	0.1972	0.6767	0.2913	0.7743
IL10	-1.2570	0.5699	-2.2056	0.0407
IL17A	0.1310	0.6416	0.2042	0.8405
IL4	-0.3676	0.9546	-0.3851	0.7134
IL8	0.7719	0.6160	1.2530	0.2263
Leptin	0.2910	0.7755	0.3753	0.7131
MMP2	0.0034	0.6423	0.0053	0.9958
RANTES	-0.8304	0.6118	-1.3572	0.1915
VEGF	0.7086	0.6203	1.1424	0.2683
Control-BMI	0.9038	0.7793	1.1599	0.2461
Exercise-BMI	-1.0110	0.7632	-1.3248	0.1852
Control-Cloaking	1.7890	1.8128	0.9869	0.3237
Exercise-Cloaking	-4.8121	2.3950	-2.0092	0.0445
Control-CCL2	-0.7696	0.5539	-1.3894	0.1647
Exercise-CCL2	1.8524	0.7943	2.3320	0.0197
Control-IL13	-8.8029	5.0554	-1.7413	0.0816
Exercise-IL13	0.3315	0.5980	0.5544	0.5793
Control-IL1β	-5.7672	2.7223	-2.1185	0.0341
Exercise-IL1β	-0.8504	0.5565	-1.5282	0.1265
Control-IL6	-1.0068	0.6171	-1.6314	0.1028
Exercise-IL6	1.7752	1.0526	1.6864	0.0917
Control-MMP9	-0.1855	0.5603	-0.3311	0.7406
Exercise-MMP9	2.4295	0.9704	2.5036	0.0123
Control-Resistin	-1.8880	0.5150	-3.6658	**0.0002**
Exercise-Resistin	1.3857	0.5495	2.5218	0.0117
Control-TNFa	-1.2645	0.9517	-1.3287	0.1839
Exercise-TNFa	0.6352	0.5952	1.0672	0.2859

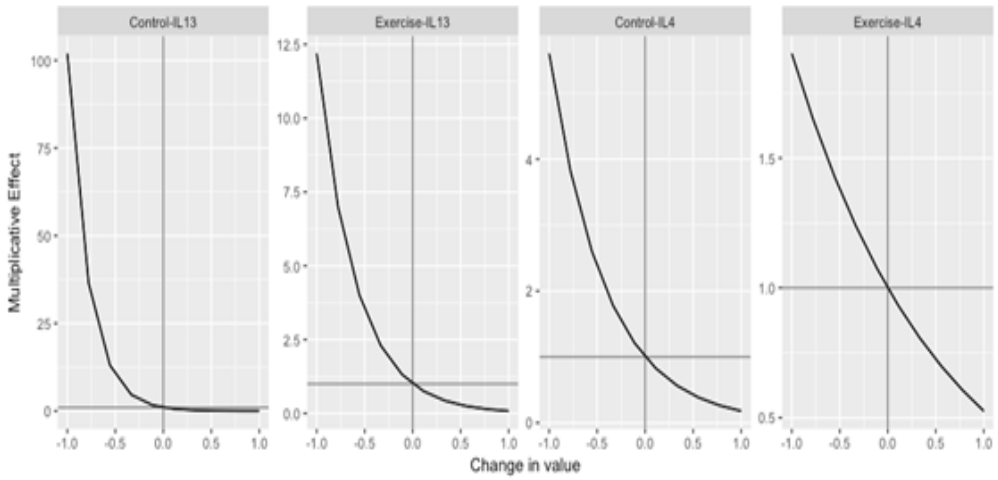


Figure 7.4: Correlation between PTGER1 gene expression, and IL-13, and IL-4 expression between ExPeCT groups.

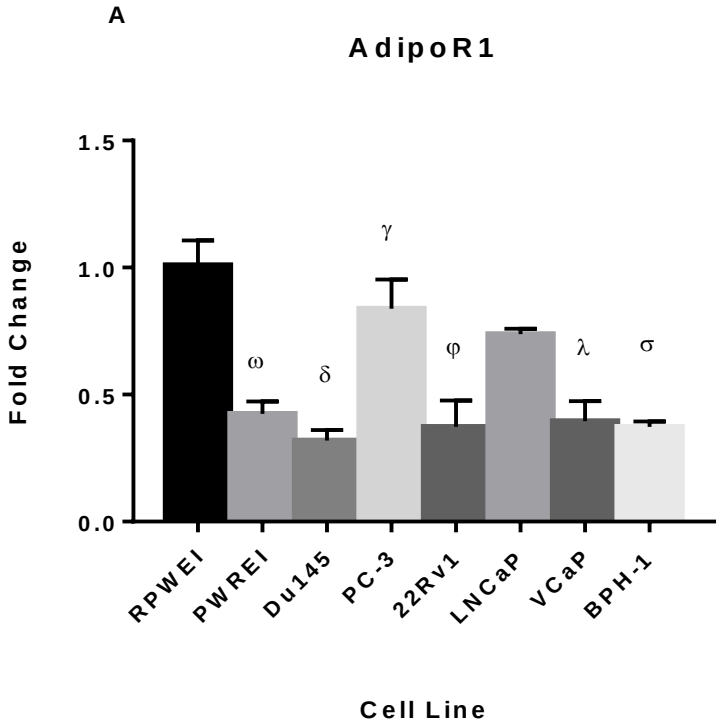
Table 7.3: Correlation between PTGER1, inflammatory mediators and clinical variables.

	Estimate	SD	z_value	p_value
Exercise - Control	-1.4210	1.7647	-0.8052	0.4207
BMI	0.8671	0.9029	0.9604	0.3531
Weight	0.8050	0.9070	0.8876	0.3898
Height	-0.2563	0.9296	-0.2757	0.7868
Hb	-0.5653	0.9198	-0.6146	0.5487
WCC	0.4930	0.9228	0.5343	0.6015
PSA	-1.3954	0.8543	-1.6334	0.1247
WCir	0.4947	0.9914	0.4989	0.6262
AvgCTCs	0.2140	0.1361	1.5719	0.1383
Cloaking	-0.2291	2.3116	-0.0991	0.9225
Adiponectin	-1.2596	0.8850	-1.4233	0.1801
C_peptide	-0.6681	0.8119	-0.8230	0.4280
CCL2	0.1995	0.9550	0.2089	0.8380
IL17A	0.3863	0.9502	0.4065	0.6915
IL1β	0.9741	1.2331	0.7900	0.4523
IL6	0.6332	0.9391	0.6742	0.5130
IL8	0.1768	0.9554	0.1851	0.8563
Leptin	-0.8358	1.2036	-0.6944	0.5049
MMP2	-0.2515	0.9540	-0.2636	0.7966
MMP9	-0.0964	0.9564	-0.1008	0.9214
RANTES	-0.6632	0.9374	-0.7075	0.4928
Resistin	-0.0303	0.9567	-0.0317	0.9753
TNFa	-0.0885	0.9564	-0.0925	0.9278
VEGF	0.3942	0.9499	0.4150	0.6861
Control-IL10	-0.1715	0.9049	-0.1895	0.8497
Exercise-IL10	-3.2618	1.4304	-2.2802	0.0226
Control-IL13	-5.6449	1.3070	-4.3191	**0.0000**
Exercise-IL13	-3.0523	0.1865	-16.3631	**0.0000**
Control-IL4	-2.1351	0.0507	-42.1430	**0.0000**
Exercise-IL4	-0.7969	0.1534	-5.1948	**0.0000**

7.1.2: Gene expression in PrCa cell lines

7.1.2.1 Adipokine associated gene expression profile

A gene expression profile, adiponectin, leptin and their associated receptors, was assessed in a panel of 8 prostate cell lines (5 malignant (DU145, PC-3, 22RV1, LNCaP and VCaP), 2 normal (PWREI and RPWEI) and one BPH). Data was normalised based on an endogenous control (GAPDH) and fold change calculated in reference to a calibrator sample, RPWEI. Significant differences in AdipoR1 were observed between cell lines (Figure 7.5). A lower expression level of AdipoR1 was demonstrated in all cancer cell lines and BPH *vs.* RPWE1. Significant differences between the prostate cell lines and AdipoR2 were also observed. As witnessed with AdipoR1, lower levels of AdipoR2 expression were evident in all cancers cell lines and BPH1 *vs*. RPWEI. A significant difference between the prostate cell lines and LepR expression was also identified. Expression of adiponectin was observed only in PC-3 cells (Appendix X). No expression of leptin was observed in any of the cell lines.



ω – p<0.05, PWREI *vs.* RPWEI

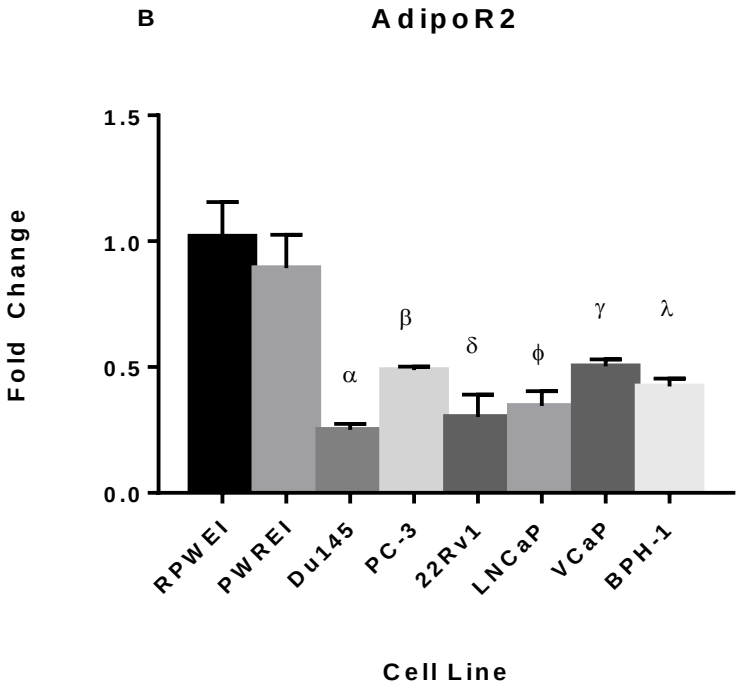
δ – p<0.05, DU145 *vs.* PC-3, DU145 *vs.* LNCaP, DU145 *vs*. RPWEI

γ – p<0.05, PC-3 *vs*. VCaP, PC-3 *vs*. BPH-1, PC-3 *vs.* PWREI

ψ - p>0.05, 22RV1 *vs*. RPWEI

λ – p<0.05, VCaP *vs.* RPWEI

σ – p<0.05, BPH-1 *vs.* RPWEI



α – p<0.05, DU145 *vs.* PWREI, DU145 *vs*. RPWEI

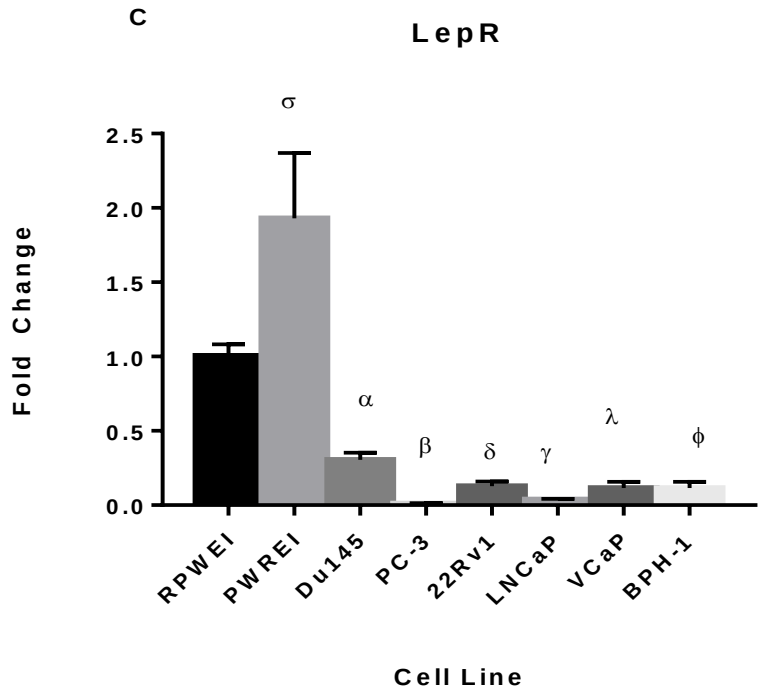
β – p<0.05, PC-3 *vs.* PWREI, PC-3 *vs.* RPWEI

δ – p<0.05, 22RV1 *vs*. PWREI, 22RV1 *vs*. PWREI

Φ – p<0.05, LNCaP *vs.* PWREI, LNCaP *vs*. RPWEI

γ – p<0.05, VCaP *vs.* PWREI, VCaP *vs*. RPWEI

λ – p<0.05, BPH-1 *vs*. PWREI, BPH-1 *vs*. RPWEI



σ – p<0.05, PWREI *vs.* RPWEI

α – p<0.05, DU145 *vs.* PWREI

β – p<0.05, PC-3 *vs.* PWREI, PC-3 *vs.* RPWEI

δ – p<0.05, 22RV1 *vs* PWREI, 22RV1 *vs.* RPWEI

γ – p<0.05, LNCap *vs*. PWREI, LNCaP *vs.* RPWEI

λ – p<0.05, VCaP *vs*. PWREI, VCaP *vs*. RPWEI

Φ – p<0.05, BPH-1 vs. PWREI, BPH-1 *vs.* RPWEI

Figure 7.5: Expression of adipokine associated gene profile in prostate cell lines.

A) Expression of AdipoR1.

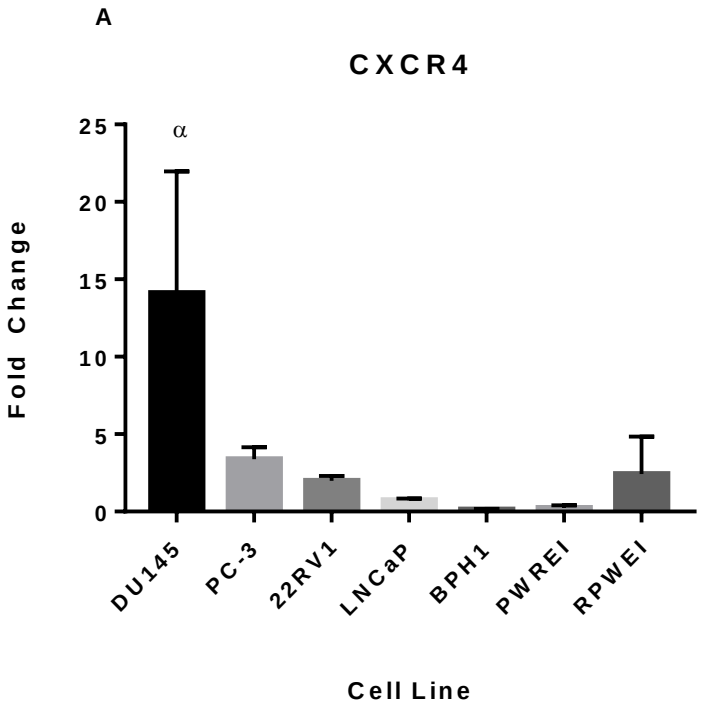
B) Expression of AdiopR2.

C) Expression of LepR.

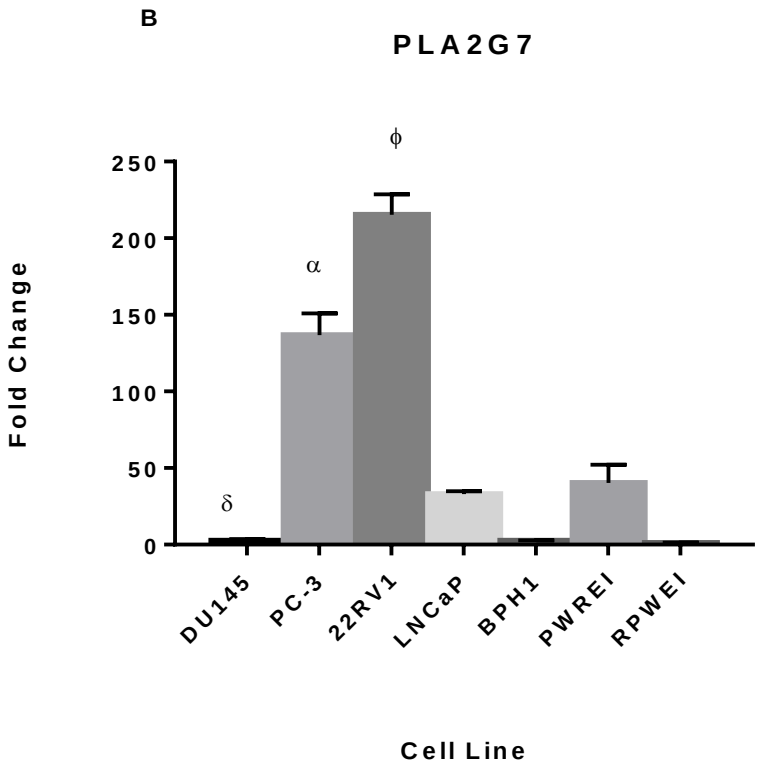
Data graphed as mean ± SEM (n=3). Data analysed using a one-way ANOVA followed by a Tukey post hoc test.

7.1.2.3 Lethality associated gene signature in prostate cell lines

The lethality associated gene signature was determined in a panel of prostate cell lines. Data was normalised based on an endogenous control (GAPDH) and fold change calculated in reference to a calibrator sample (RPWEI) (Figure 7.6). Expression of CXCR4 was significantly higher in DU145 compared with all other cell lines ($p<0.05$). Significant increases in PLA2G7 expression were observed across multiple cell lines. PC-3 and 22Rv1 displayed the largest increases in fold change *vs.* RPWE1. As per PLA2G7 expression, PC-3 and 22Rv1 displayed the largest increases in fold change *vs.* RPWE1 for PTGER1. No expression of HTR2B was observed across the panel of cell lines. Expression of AVPR2 was recorded in PC-3, BPH-1, PWREI and RPWEI cell lines only. A significant difference in expression was observed between PC-3 and each of the other cell lines.



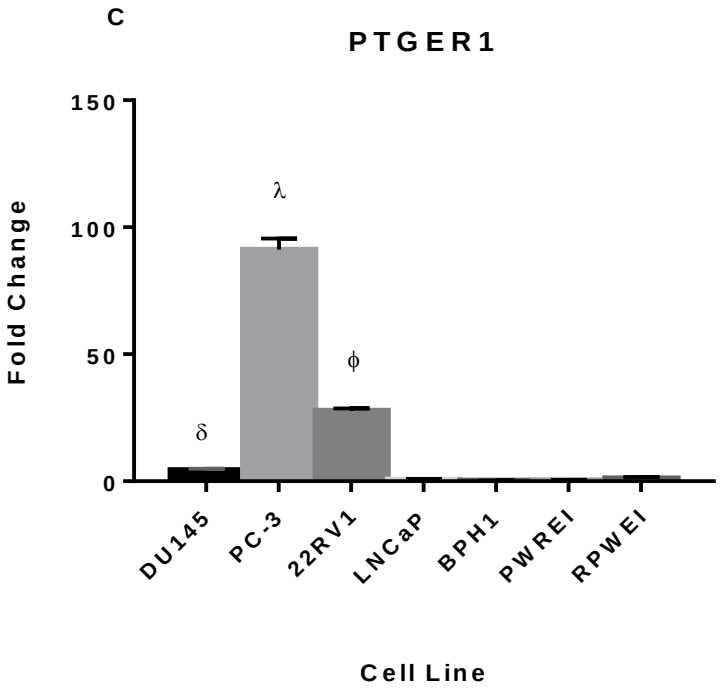
α - p>0.05, DU145 *vs.* PC-3, DU145 *vs.* 22RV1, DU145 *vs* .LNCaP, DU145 *vs.* BPH-1, DU145 *vs.* PWREI and DU145 *vs.* RPWEI.



δ – p<0.05, DU145 vs. PC-3, Du145 vs. 22RV1

α – p<0.05, PC-3 *vs*. 22RV1, PC-3 *vs*. LNCaP, 22RV1 *vs*. BPH-1, 22RV1 *vs*. PWREI and 22RV1 *vs*. RPWEI

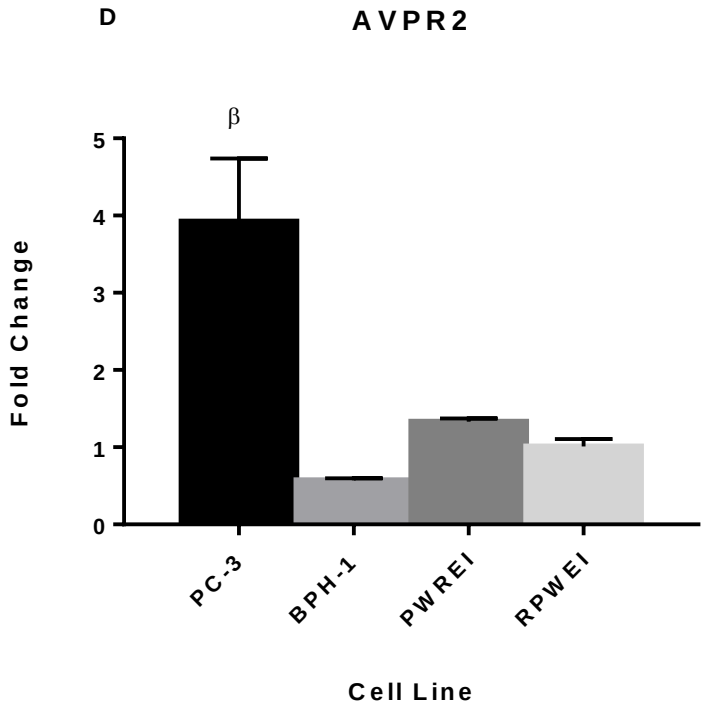
Φ – p<0.05, 22RV1 *vs*. LNCaP, 22RV1 *vs.* BPH-1, 22RV1 *vs*. PWREI and 22RV1 *vs.* RPWEI



δ – p<0.05, DU145 *vs.* PC-3, DU145 *vs*. 22RV1

λ - p<0.05, PC-3 *vs*. 22RV1, PC-3 *vs*. LNCaP, PC-3 *vs*. BPH-1, PC-3 *vs*. PWREI and PC-3 *vs.* RPWEI

Φ – p<0.05, 22RV1 *vs*. LNCaP, 22RV1 *vs*. BPH-1, 22RV1 *vs*. PWREI and 22RV1 *vs.* RPWEI



β - p<0.05, PC-3 *vs.* RPWEI, PC-3 *vs.* PWREI and PC-3 *vs.* BPH-1

Figure 7.6: Expression of lethality associated gene signature in prostate cell lines.

A) Expression of CXCR4.

B) Expression of PLA2G7.

C) Expression of PTGER1.

D) Expression of AVPR2.

Data graphed as mean ± SEM (n=3). Data analysed using a one-way ANOVA followed by a Tukey post hoc test.

7.1.3: SGBS cell line differentiation

SGBS cells were utilised for co-culture experiments with the Enzalutamide cell line panel. Differentiation of SGBS cells occurred over a period of 14 days (Wabitsch et al., 2001). Differentiation was confirmed by Oil red O staining at day 0, day 7 and day 14 (Figure 7.7). Pre-adipocytes were used as a control, marking the absence of lipid droplets.

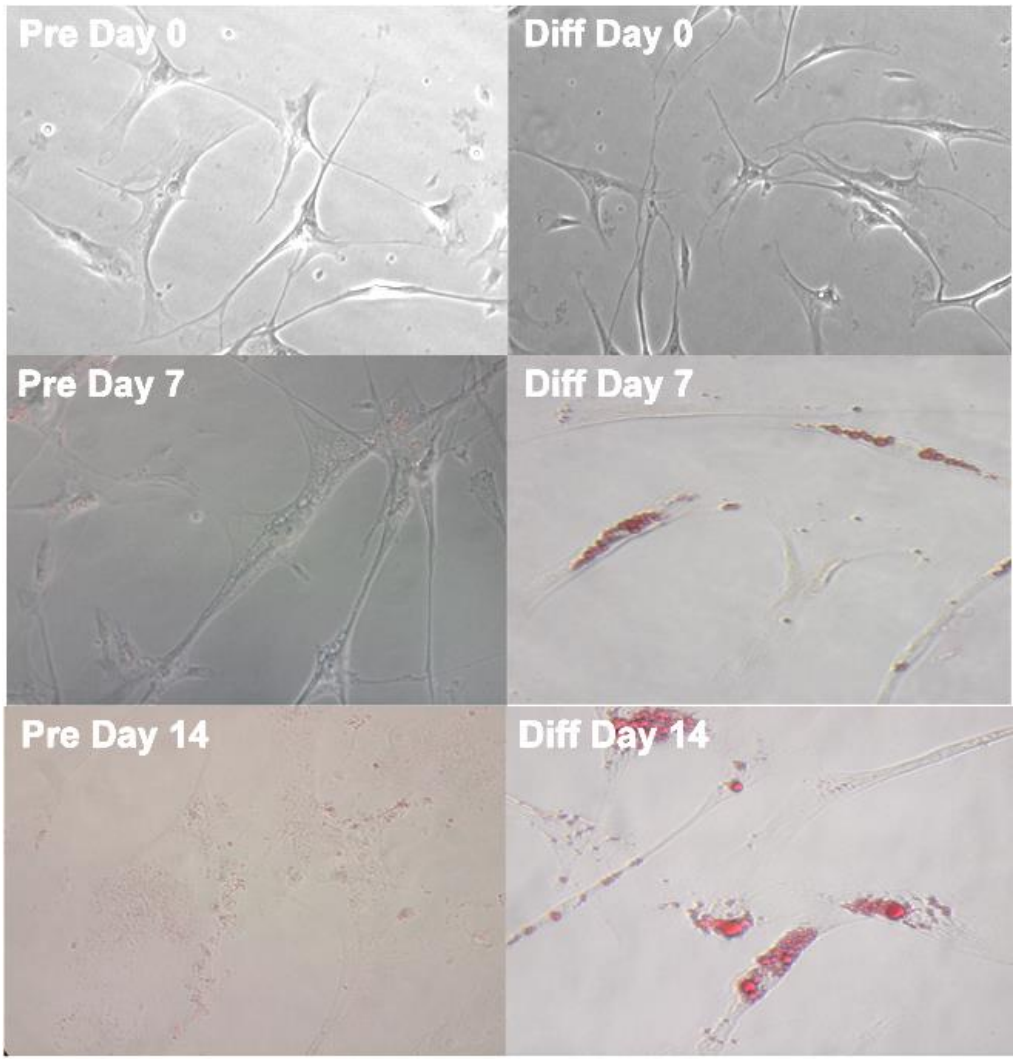
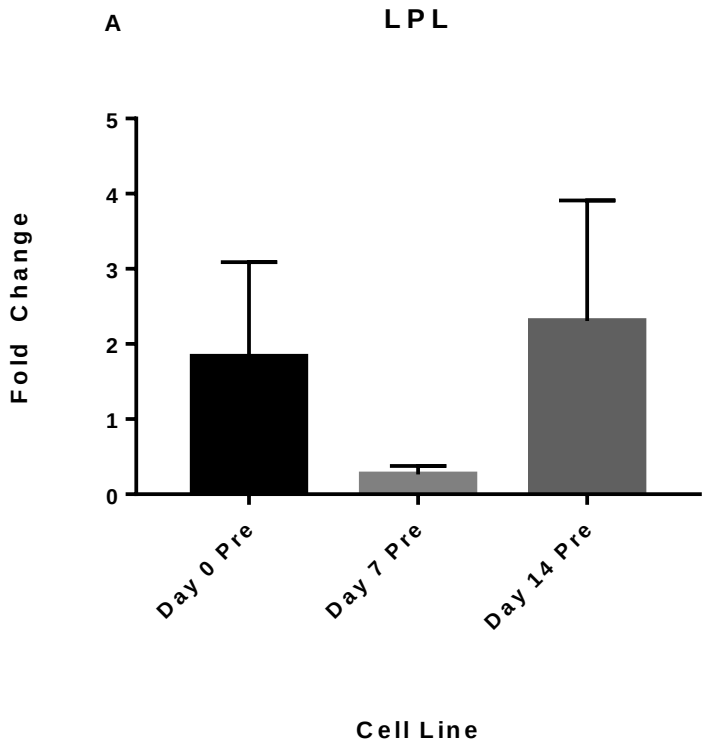


Figure 7.7: Differentiation of SGBS cell line, at day 0, day 7 and day 14. Oil red O depicts lipid droplet formation present on the cells.

Pre – pre differentiated adipocytes (SGBS)

Diff – differentiated adipocytes (SGBS)

Confirmation of the differentiation of the SGBS cells was characterised by a fat cell specific gene expression panel (Wabitsch et al., 2001, Allott et al., 2012). Pre-differentiated and differentiated SGBS cells were assessed for the expression of glucose transporter type 4 (GLUT4), lipoprotein lipase (LPL), adipocyte protein 2 (AP2) and peroxisome proliferator activated receptor gamma 2 (PPARg2). Samples were normalised to an endogenous control (18S) and analysed in reference to a calibrator sample (day 0). No significant difference in LPL or AP2 expression was observed over time in the pre-differentiated cells (Figure 7.8). Detection of GLUT4 and PPARg2 was beyond the limit of detection in the pre-differentiated cell line.



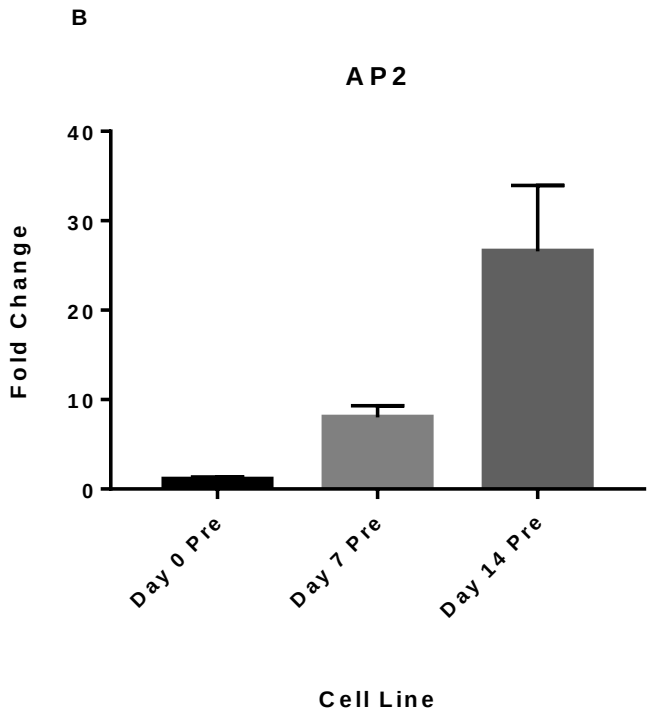


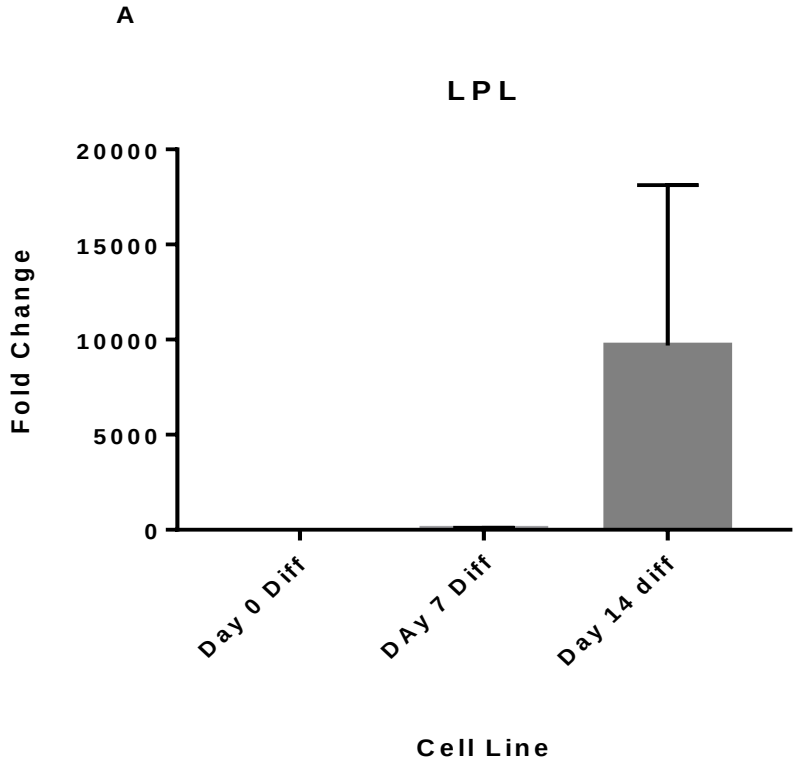
Figure 7.8: Gene expression in pre-differentiated SGBS cells.

A) Expression of LPL.

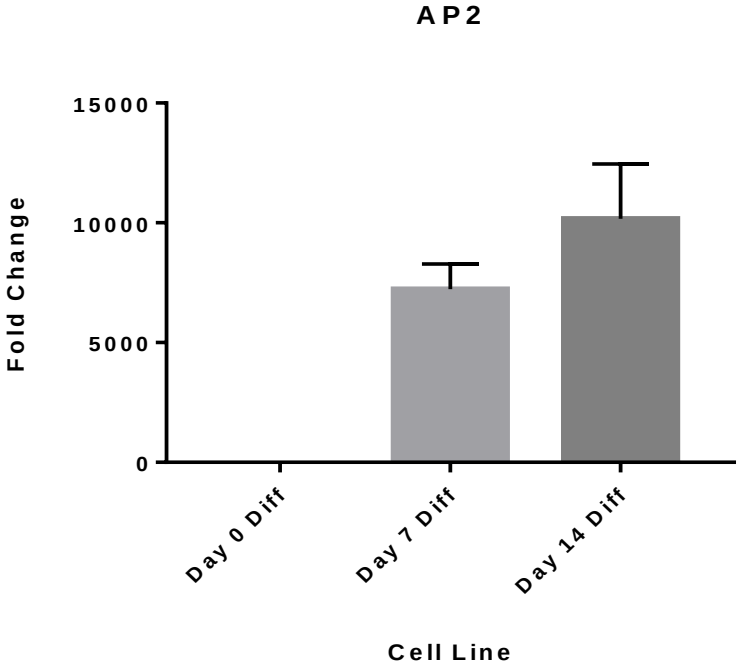
B) Expression of AP2.

Data graphed as mean ± SEM (n=3).

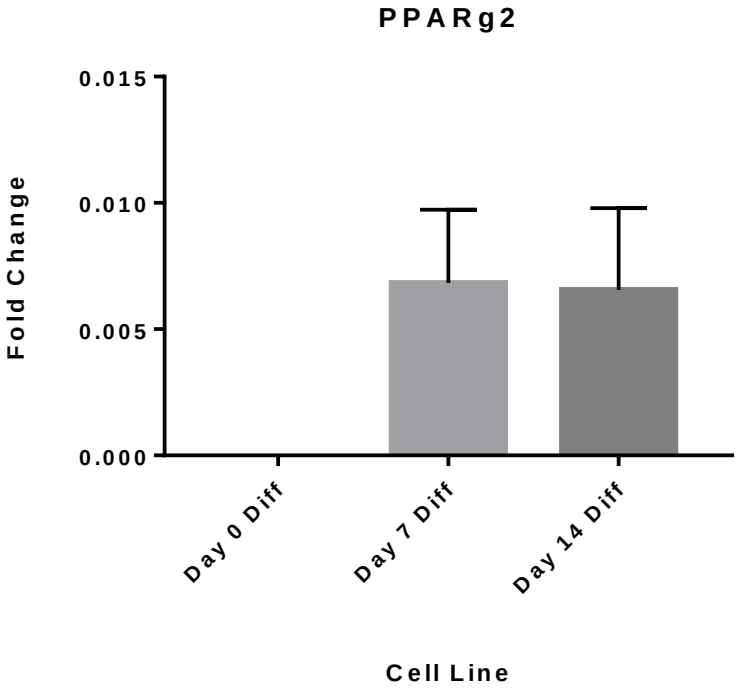
In the differentiated SGBS cells, no significant difference in LPL or AP2 expression was observed overt time (Figure 7.9). In contrast to the pre-differentiated cells, expression of GLUT4 and PPARg2 was determined at day 7 and day 14 in the differentiated SGBS cells. No significant difference in expression was observed over time.



B


AP2
15000
10000
5000
0
Fold Change
Day 0 Diff
Day 7 Diff
Day 14 Diff
Cell Line

C


PPARg2
0.015
0.010
0.005
0.000
Fold Change
Day 0 Diff
Day 7 Diff
Day 14 Diff
Cell Line

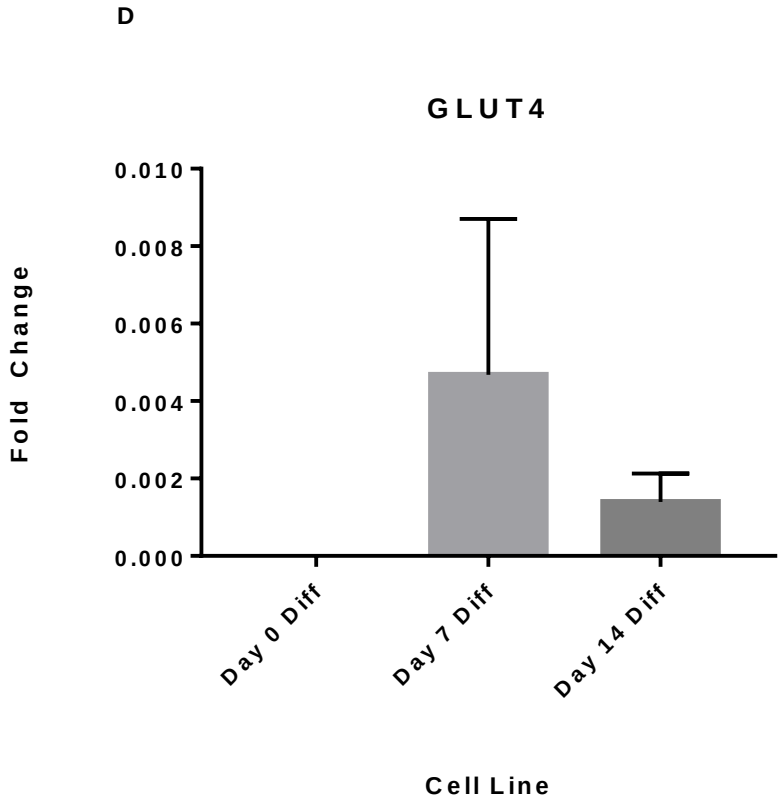


Figure 7.9: Gene expression in differentiated SGBS cells.

A) Expression of LPL.

B) Expression of AP2.

C) Expression of PPARg2.

D) Expression of GLUT4.

Data graphed as mean ± SEM (n=3).

7.1.4 Enzalutamide resistant PrCa cell line panel

An isogenic model of Enzalutamide resistance was used to examine the role of inflammation in drug resistance (Cells were a kind gift from Dr Manav Korpal, Novartis Institute for BioMedical Research (Korpal et al., 2013)). Briefly, short term culture of LNCaP cells with Enzalutamide generated resistant clones. These clones were isolated, cultured and characterised based on the levels of resistance observed (Korpal et al., 2013). The model in this study consisted of an age matched control cell line (Control) and two sub-lines with varying resistance to enzalutamide as outlined in Table 7.4.

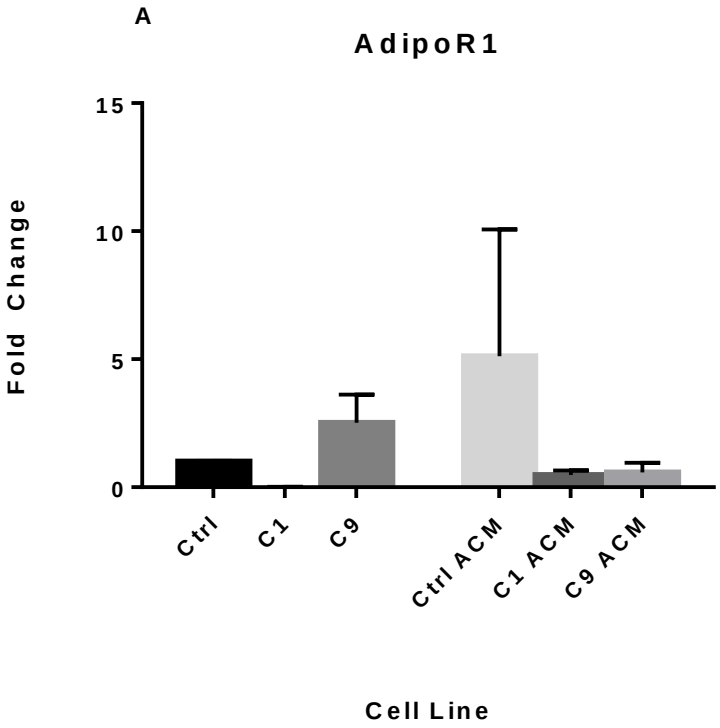
Table 7.4: Enzalutamide resistant cell lines.

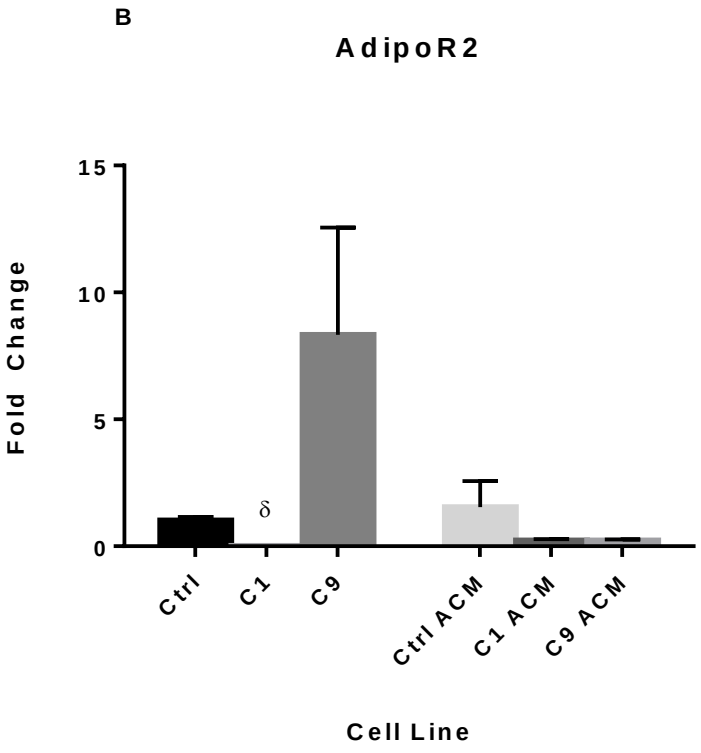
	Clone	Enzalutamide Resistance
Control	CTRL	Control cell line
Clone 1	C1	Enzalutamide Resistant
Clone 9	C9	Weakly Resistant to Enzalutamide

7.1.5: Gene expression analysis in Enzalutamide resistant cell line panel

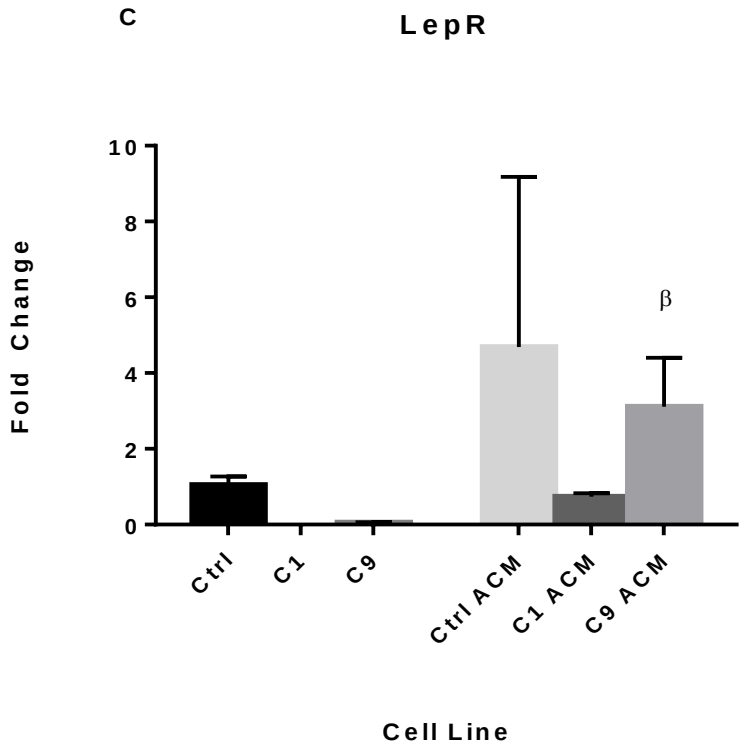
7.1.5.1 Adipokine associated gene expression profile in Enzalutamide resistant cell line panel

Basal gene expression was analysed on the Enzalutamide resistant cell lines. Cells from this model were also co-cultured in adipocyte conditioned media (ACM), generated from SGBS cells. All cells were examined for the expression of the adipokine associated gene expression profile. Samples were normalised against endogenous control, GAPDH, and fold change was calculated in reference to a calibrator sample (Appropriate untreated Ctrl line for both basal and co-cultured). No significant difference in AdipoR1 expression was observed under basal or ACM conditions (Figure 7.10). A significant increase in AdipoR2 expression was observed between the C1 and C9 cell lines ($p=0.0486$). A significant difference in LepR expression was observed between C1 and C9 ACM cell lines ($p=0.0242$).





δ – p<0.05, C1 *vs.* C9



β – p<0.05, C9 ACM *vs.* C1

Figure 7.10: Adipokine associated gene expression in Enzalutamide resistant panel.

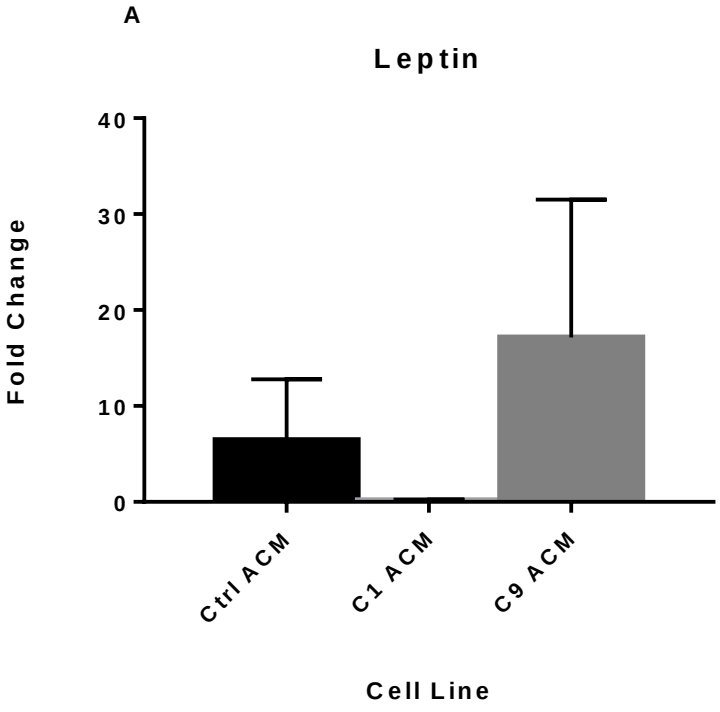
A) Expression of AdipoR1.

B) Expression of AdipoR2.

C) Expression of LepR.

Data graphed as mean ± SEM. Data analysed using a non-parametric one-way ANOVA Kruskal-Wallis.*p<0.05

Basally, no mRNA expression of adiponectin and leptin was observed in the cell line panel. However, both were detected in cells co-cultured in ACM. No significant difference in leptin or adiponectin expression was determined between the cell lines (Figure 7.11).



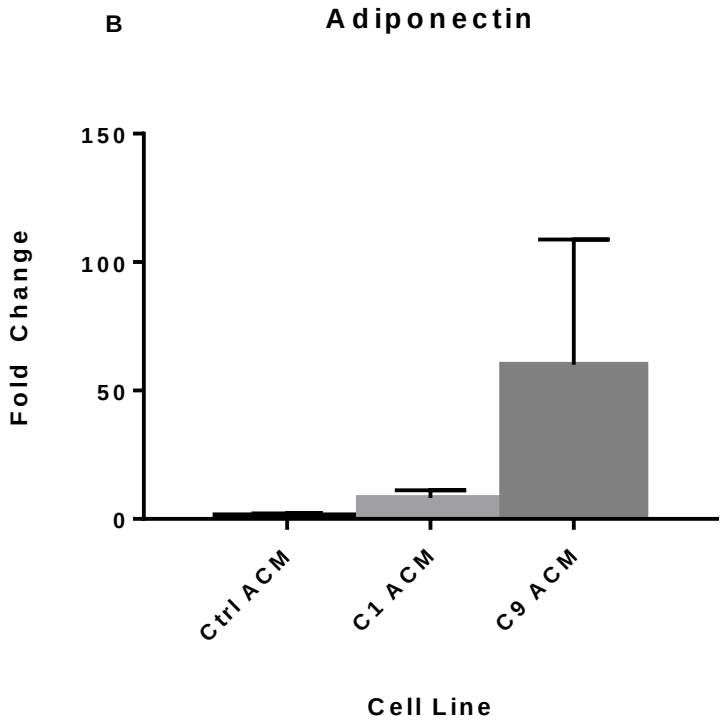
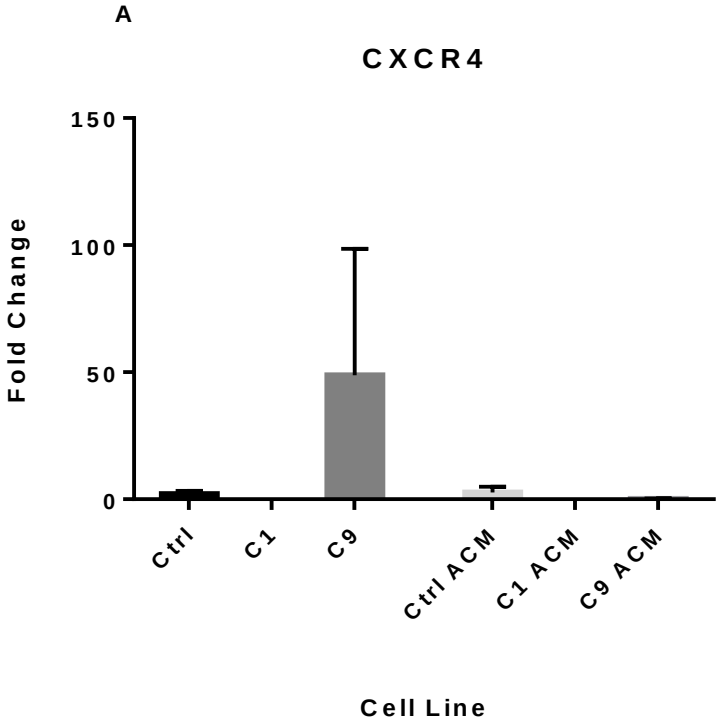


Figure 7.11: Expression of leptin and adiponectin in Enzalutamide resistant adipocyte conditioned panel.

Data graphed as mean ± SEM.

7.1.5.2 Lethality associated gene signature in Enzalutamide resistant cell line panel

Expression of the lethality associated gene signature was determined in the Enzalutamide cell line panel. Samples were normalised against endogenous control, GAPDH, and fold change was calculated in reference to a calibrator sample (Ctrl line, basal and ACM). No significant difference in CXCR4 expression was observed across all cell lines in panel (Figure 7.12). Analysis of PTGER1 data was not possible as detection was beyond the sensitivity of the assay. Expression of HTR2B was not detected in any of the cell lines in this panel under basal or ACM conditions. AVPR2 expression was detected in the C1, C9 and C1 ACM cell lines only (Appendix X). No significant difference in PLA2G7 expression was identified between the cell lines.



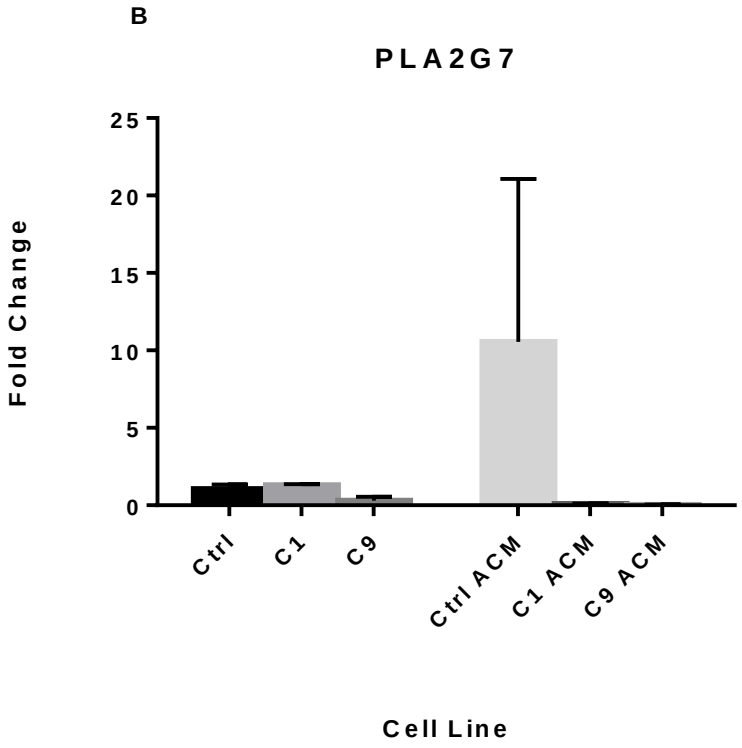


Figure 7.12: Expression of CXCR4 and PLA2G7 in Enzalutamide cell line panel.

Data graphed as mean ± SEM.

7.1.6 Effect of adiponectin and leptin on Enzalutamide resistant cell line panel

The effect of adipokines, adiponectin and leptin, on cell proliferation was assessed by BrdU assays. A time-course, utilising 200 ng/mL leptin and 50 mg/mL adiponectin, was completed at 24 hr, 48 hr and 72 hr in each of the cell lines. Proliferation was calculated compared with the UTcontrol, which was set at 100%. No significant changes in proliferation were observed over time (Figure 7.13).

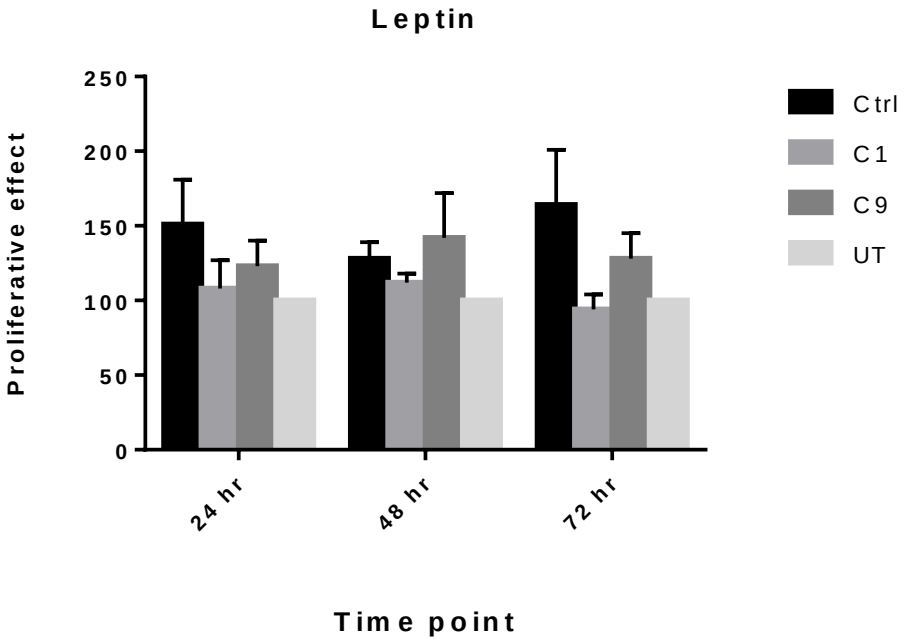


Figure 7.13: Time-course of the proliferative effect of 200 ng of recombinant leptin on Enzalutamide resistant PrCa cell line panel.

Data graphed as mean ± SEM (n=3)

Treatment with 50 mg of adiponectin generated no significant change in proliferation rate on the cell lines at 24 hr, 48 hr and 72 hr (Figure 7.13).

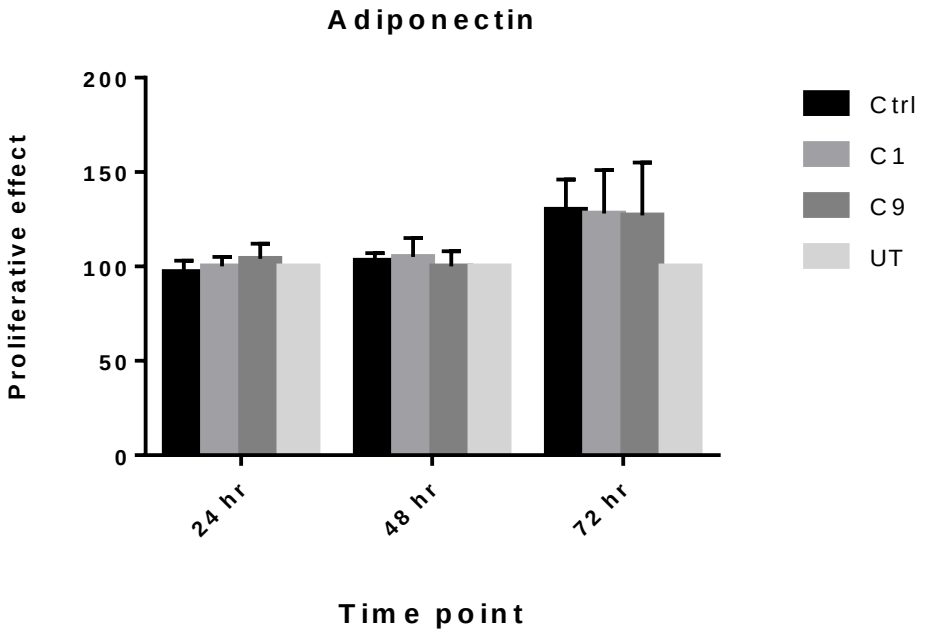


Figure 7.14: Time-course of the proliferative effect of 50 mg of recombinant adiponectin on Enzalutamide resistant PrCa cell line panel.

Date graphed as mean ± SEM (n=3),

7.1.7: Effect of adipocyte conditioned media on Enzalutamide resistant cell line panel

The Enzalutamide resistant panel was treated for 24 hr with adipocyte conditioned media from pre and differentiated SGBS cells at day 0, 7 and 14. Proliferation was calculated as a percentage increase/decrease in comparison with the UT (untreated control, which was untreated pre and differentiated media) set at 100%. No significant difference in proliferation was detected across all cell lines (p=0.7729) (Figure 7.15).

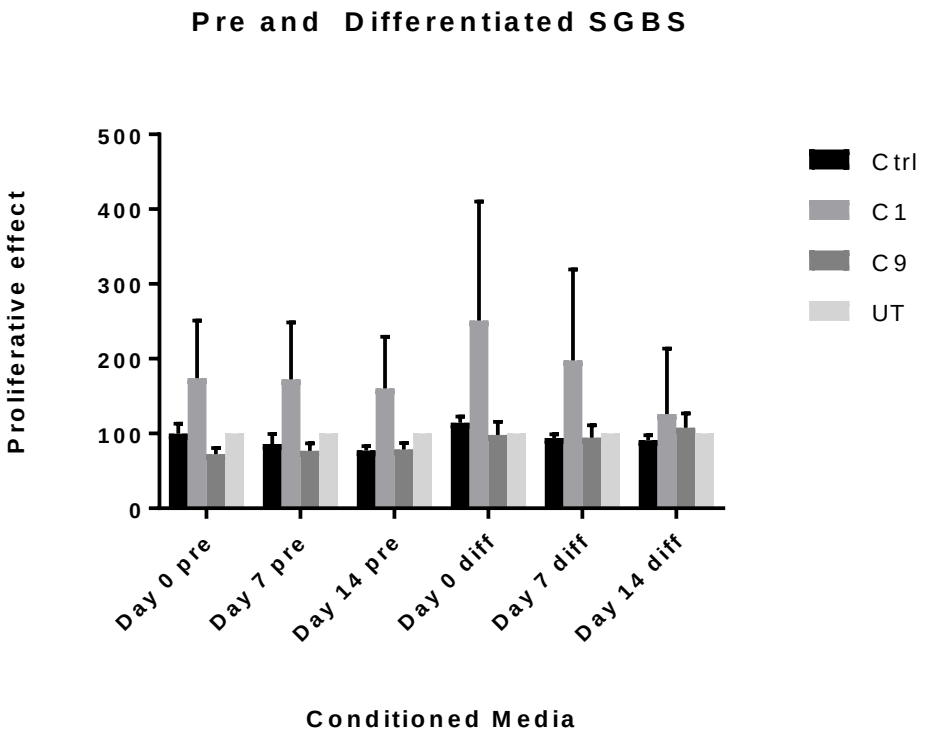


Figure 7.15: Proliferative effect of pre and differentiated SGBS conditioned media on Enzalutamide resistant cell line.

Data graphed as mean ± SEM (n=3).

ACM from co-culture experiments at day 14, was used to culture the Enzalutamide resistant panel and the rate of proliferation determined. Significant differences were observed between Enzalutamide cell lines ($p<0.05$) (Figure 7.16).

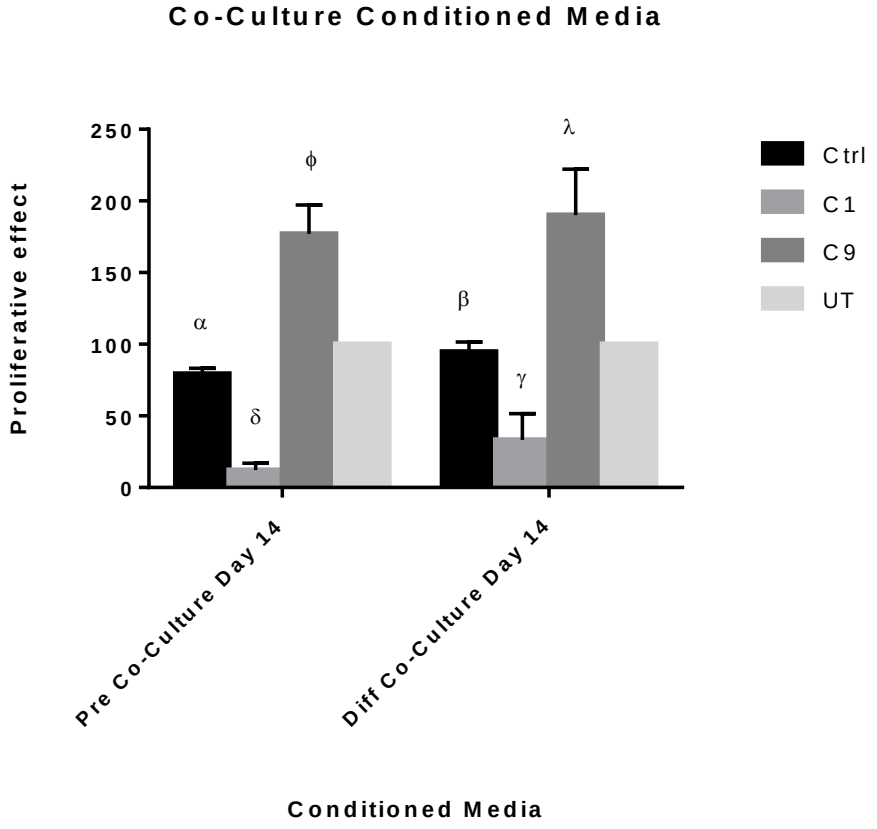


Figure 7.16: Proliferative effect of co-culture conditioned media on Enzalutamide resistant cell line panel.

Date graphed as mean ± SEM (n=3). Data analysed using a one-way ANOVA followed by a Tukey post hoc test.

α - p<0.05, Ctrl pre day 14 *vs.* C9 pre day 14, Ctrl pre day 14 *vs.* C9 diff day 14

β – p<0.05, Ctrl diff day 14 *vs.* C1 pre 14, ctrl diff 14 *vs*. c9 pre day 14, Ctrl diff 14 *vs.* C9 diff 14

δ – p<0.05, C1 pre day 14 *vs*. C9 pre day 14, C1 pre day 14 *vs.* C9 diff 14, c1 diff 14 *vs*. c9 pre day 14, C1 diff day 14 *vs*. c9 diff day 14

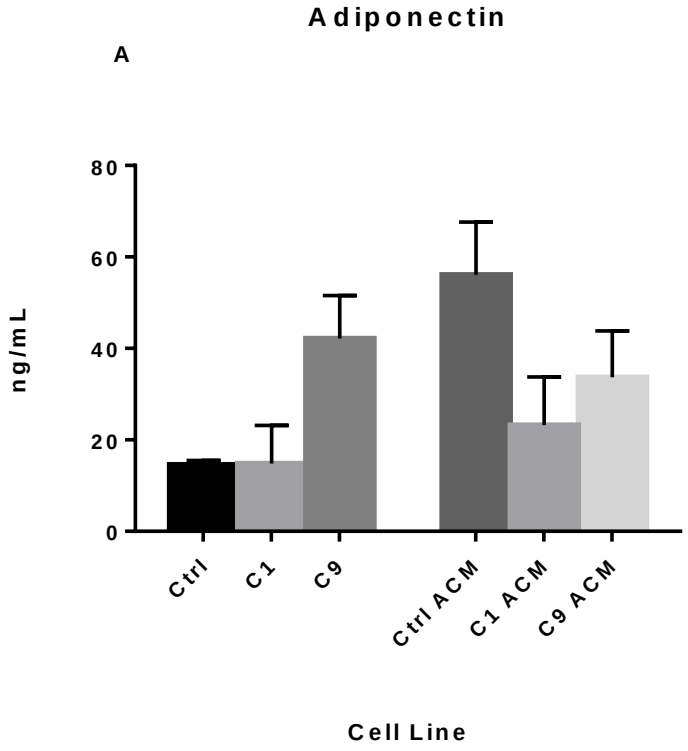
γ – p<0.05, C1 diff day 14 *vs.* C9 pre day 14, C1 diff day 14 *vs*. C9 diff day 14

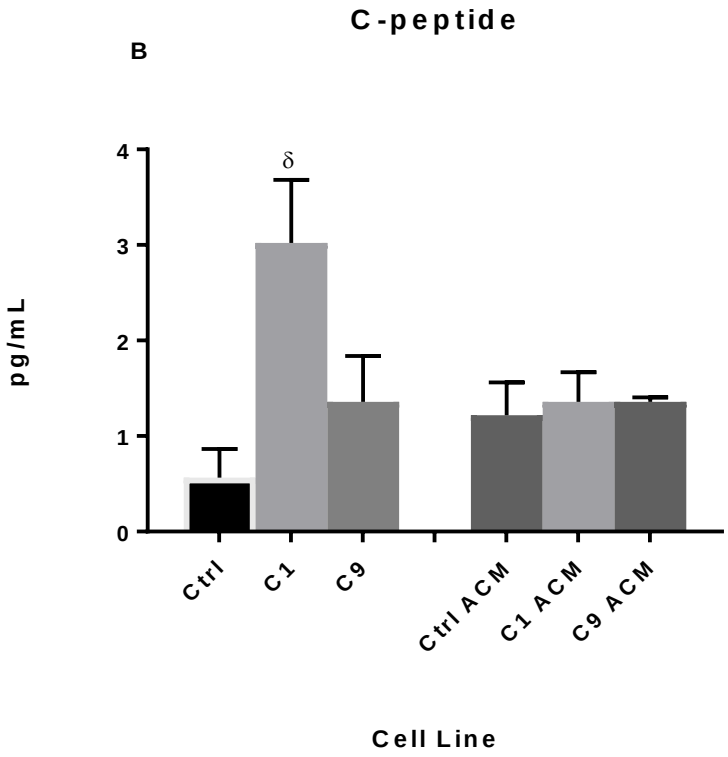
Φ – p<0.05, C9 pre day 14 *vs.* UT pre, C9 pre day 14 *vs.* UT diff

λ – p<0.05, C9 diff day 14 *vs.* UT pre, C9 diff day 14 *vs*. UT diff

7.1.8: Expression of adiponectin and C-Peptide in Enzalutamide resistance cell lines

Secreted levels of adiponectin, leptin, resistin and C-peptide were determined in cell supernatant from the Enzalutamide cells under standard cell culture conditions and in the Enzalutamide cells exposed to ACM. Levels of leptin and resistin were below the level of sensitivity of the assay. No significant differences in adiponectin were observed between cell lines (Figure 7.17). A significant change in C-peptide expression between the basal C1 and C9 cell lines was observed (p=0.0088).





δ – p<0.05, C1 *vs.* C9

Figure 7.17: Secreted levels of adiponectin and C-peptide in the Enzalutamide resistant cell line panel.

A) Expression of adiponectin.

B) Expression of C-peptide.

Date graphed as mean ± SEM (n=3). Data analysed using a one-way ANOVA followed by a Tukey post hoc test.

7.2 Discussion

The treatment landscape of metastatic PrCa is hampered by the eventual onset of treatment resistance, in both ADT and second-generation anti-androgens. Additional factors, such as obesity and MetS, may influence disease progression in the tumour microenvironment by promoting a pro-inflammatory effect (Himbert et al., 2017). Targeting the obesity related inflammatory pathways and mechanisms of resistance may provide improved treatment strategies with the overall aim of improving the lives of patients living with metastatic PrCa. Gene expression analysis of diagnostic NCBs may provide insight into these pathways and the onset of disease progression. The overall aim of this chapter was to utilise NCBs to examine expression of a lethality associated gene signature and to further understand the interaction between obesity and Enzalutamide resistance in metastatic PrCa.

The ExPeCT trial aimed to investigate the role of a lethality associated gene signature, with links to obesity and coagulation, in diagnostic NCBs associated with the trial participants. Three significant correlations were observed between genes in this panel and inflammatory mediator data, previously established in ExPeCT participants. An inverse correlation between AVPR2 and resistin expression was observed. AVPR2 has been implicated as having a role to play in carcinogenesis in breast and lung cancers (North, 2000). However in spite of having been previously identified as a gene associated with lethality (unpublished data), the role of AVPR2 has not been fully elucidated in PrCa. As resistin has been linked to proliferation in PrCa (Kim et al., 2011), the inverse correlation observed in this study, may be indicative of a role between AVPR2 and obesity. Significant inverse correlations were also observed between PTGER1 and IL-4 and IL-13 expression levels. IL-4 and IL-13 are typically associated with a pro-inflammatory environment in PrCa (Terabe et al., 2004, Goldstein et al., 2011) and this finding may demonstrate a relationship between lethality gene expression and chronic inflammation. The role of PTGER1 in PrCa has been associated with the ERG fusions, which are present in approximately 50% of PrCa cases (Mohamed et al., 2011). Identification

of genomic alterations present in ExPeCT participants NCBs may enable further clarification on the role between PTGER1 and inflammation in patients with metastatic PrCa. Based on the novel nature of these findings and the potential role of exercise as a successful adjunct to treatment in PrCa, investigation into a correlation between the lethality associated gene expression signature, biological outcomes and physical activity is necessitated in future studies.

To further the investigation into gene expression and biological outcomes, *in vitro* cell line experiments were performed utilising a panel of prostate cell lines, that incorporated malignant, normal and BPH phenotypes. Of this panel, 3 cell lines, LNCaP, 22RV1 and VCaP, express the AR-V7 variant, associated with treatment resistance (Antonarakis et al., 2014), suggesting potential of this panel to further understanding into treatment resistance and obesity. An adipokine expression profile, consisting of adiponectin and leptin and their associated receptors, was determined within the panel of cells. No expression of the adiponectin and leptin ligands was detected throughout the panel. In contrast to the ligands, significant differences in expression of each receptor were observed between cell lines. Generally, expression of AdipoR1 was significantly lower in the malignant cells when compared to the normal cells. Significant differences in expression were also observed between BPH-1 and normal prostate cells. These data indicate reduced expression of AdipoR1 in disease progression. This finding was mirrored in the comparison of AdipoR2 and LepR expression across each of the cell lines. Each of the malignant lines exhibited significantly reduced expression in comparison to the normal cells, including reduced expression of the BPH-1 cell line. Decreased expression of adiponectin receptors has been linked to insulin resistance and MetS (Kadowaki, 2006), thus the findings in this study support evidence of the relationship between obesity and progression in PrCa. The significant difference in expression between BPH-1 cells and normal prostate cells, may infer the initial alterations in adiponectin expression in changing prostate cell phenotypes. Conflicting reports exist in the literature with respect to AdipoR1 and AdipoR1 in PrCa. A study by Michalakis et al., reported

reduced expression of both receptors in men with PrCa (Michalakis et al., 2007), however a subsequent study by Rider et al. reported a positive correlation between AdipoR2 expression and aggressive disease in PrCa (Rider et al., 2015). These data highlight the need for further investigation into the role of AdipoR1 and AdipoR2 in PrCa and this *in vitro* analysis may aid in the elucidation of the correlation between adiponectin and PrCa. Based on the documented inverse correlation between adiponectin and leptin (Lubkowska et al., 2015), low expression of the adiponectin receptors may be linked to the high level of LepR observed in the normal prostate cells (PWREI). A previous study has shown that low expression of LepR in tumours, demonstrating positive surgical margins and seminal vesical invasion, is suggestive of a negative correlation between LepR and disease progression (Osorio et al., 2014), which may provide an explanation for the low levels observed in malignant cells in this panel. This *in vitro* data, signifies a potential role of LepR as a marker of advanced disease and may hold potential as a therapeutic target.

Expression of the lethality associated gene signature was also determined in this panel of prostate cell lines. A significant increase in CXCR4 expression was observed in the DU145 cell line when compared to each of the other lines, irrespective of origin. DU145 cell line was derived from PrCa brain metastasis and demonstrated aggressive properties. CXCR4 has been strongly implicated in the metastatic cascade (Xing et al., 2008), and the results determined in this study are in agreement with the literature. High expression of PLA2G7, AVPR2 and PTGER1 was identified in 22RV1 and PC-3 cell lines. As a lethality signature, up-regulation of these genes are indicative of a worse prognosis, hence these findings imply a correlation between high expression and advanced disease in PrCa. 22Rv1 cells express AR and ARV-7, while PC-3 cells are androgen independent. The contrasting profiles of these cells, highlights the heterogeneous nature of aggressive PrCa and warrants further investigation into the impact of these phenotypes on treatment resistance.

To further underpin the relationship between treatment resistance and obesity in PrCa, an Enzalutamide resistant panel of cells was acquired. Each of the cell lines in the panel had positive expression of AR, with C1 (resistant to Enzalutamide) demonstrating increased expression of AR-V7 in comparison to C9 (weakly resistant to Enzalutamide) (Greene et al., manuscript in preparation). Co-culture of these cells with pre and differentiated SGBS fat cells, allowed for the modelling of an obesity related environment. The adipokine and lethality associated gene expression profiles were determined in this panel of cells. The lethality gene signature, identified no significant correlations between expression and Enzalutamide resistance, both at a basal level and in ACM. This may indicate that these genes are not implicated in the mechanisms involved in Enzalutamide resistance. Larger scale gene expression analysis could identify a gene signature of interest in this panel.

Examination of the adipokine associated expression profile, produced mixed results. In the basal cell panel, no significant differences in expression were observed in relation to AdipoR1, however significant differences were observed in AdipoR2 expression between the C1 and C9 cell lines. The increased levels of AdipoR2 in C9, may suggest a role between weak levels of resistance and obesity. A correlation between the ACM and basal cell panel was observed in terms of LepR expression. A significant difference in expression as observed between C1 and C9 ACM, with increased expression observed in C9 ACM. This change in expression, implies an effect of the ACM on LepR expression and could signify differences between levels of Enzalutamide resistance. Interestingly, expression of adiponectin and leptin was observed in the ACM panel, despite not have being detected in any of the other prostate cell lines. The co-culture conditions may have stimulated the secretion of adiponectin and leptin in this cohort of cells, altering the expression profile, which may have implications in resistance to treatment. To further assess the level of secretion of adipokines, cell supernatant was analysed on the Meso-Scale Discovery platform. Although secreted levels of leptin and resistin were below the sensitivity of the assay,

adiponectin secretion was detected across the basal and ACM panels. However, no significant differences were observed. C-peptide secretion was also assessed, as a surrogate marker for insulin resistance, and a significant difference in expression was observed between basal C1 and C9. The high expression of C-peptide in the C1 line, suggests a possible connection between insulin resistance and the development of Enzalutamide resistance. IGF, which is related to insulin resistance, has been implicated in the advancement of PrCa (Yanase, 2017, Friedrich et al., 2012).

The proliferative effect of adiponectin and leptin on the Enzalutamide resistant panel was determined. No significant differences in proliferation were observed, however a trend towards increased proliferation was observed over time. Noda et al., demonstrated an increase in proliferation with treatment of leptin over a period of 28 days (Noda et al., 2015), which suggests that over an increased time-frame, these adipokines may begin to demonstrate an effect on proliferation similar to effects observed *in vivo*. The proliferative effect of ACM from co-culture was also examined. Significant differences in proliferation were observed across the cell lines. Ctrl and C9 proliferation rates were significantly higher in comparison to the C1 cell line. Based on the level of Enzalutamide resistance in the C1 cell line, this finding may highlight a role between resistance, obesity and proliferation in PrCa.

The results from this chapter indicate a possible role between Enzalutamide resistance and obesity in PrCa. From a clinical perspective, elucidating the association between resistance mechanisms and obesity, may aid in the improvement of new therapeutic targets.

Chapter 8:

Concluding Discussion

8.0 Discussion

Globally, PrCa is the most common type of cancer for males. While survival rates are high for primary or localised PrCa, the onset of metastasis and eventual resistance to treatment, causes an increase in mortality (NCRI, 2017). The need for new targeted therapeutic strategies and increased understanding of biological pathways involved in the metastatic cascade is warranted. The overall aim of this thesis was to examine the relationship between inflammation, obesity, CTCs and exercise in a prospective cohort of patients with advanced PrCa.

Personalised medicine, an individualised treatment based on biological analysis, has been an area of interest for a number of years but has not yet been fully realised. Patient to patient heterogeneity is extensively documented in localised PrCa (Shoag and Barbieri, 2016), thus profiling molecularly distinct subtypes may aid in targeted treatment options. Profiling of common genomic alterations and distinct molecular subtypes of PrCa was undertaken in a cohort of patients who met various criteria for disease progression. The aim was to characterise progression of PrCa in terms of ERG, PTEN and SPOP mutation status. While no SPOP mutations were observed across groups within this cohort, within the group of patients classed as *immediate progression* after surgery, low levels of ERG expression were reported. These levels were determined to be significant when compared to patients who did not progress (based on BCR criteria). The model was adjusted for PTEN, age and Gleason grade, and although no other correlations were reported, ERG expression in the *immediate progression* group after surgery remained significant. The documented mutual exclusivity witnessed between SPOP mutations and ERG expression (Mani, 2014), is not discernible in this cohort based on the lack of SPOP mutations. However, the lack of expression of both ERG and SPOP mutations, may infer the presence of an additional molecularly distinct subtype of PrCa that may promote disease progression. As the landscape of PrCa biology evolves over time, subsequent follow up analysis, such as genomic analysis of metastatic lesions, may provide insight into any further alterations at a genomic level. A limitation of this analysis is the small number of patients

included in the group classed as *immediate progression* after surgery. The data generated from this pilot study hold promise for furthering knowledge of the role of genomic alterations, such as ERG, in PrCa and their implication in disease progression. Future studies are merited in larger cohorts of patients.

The ExPeCT trial was the first trial of its kind in Ireland for patients with metastatic PrCa, with the aim of better understanding the impact of exercise on the metastatic cascade. The primary endpoint of ExPeCT was to examine the impact of exercise on CTC number and platelet cloaking in patients with metastatic PrCa. No distinct difference was observed between the exercise and control groups enrolled in ExPeCT, with respect to CTC number over time. The impact of exercise on CTCs has not been documented previously. The absence of differences between groups may be due in part to the type of exercise intervention, in this case aerobic. Other exercise trials in PrCa have incorporated a resistance element, and demonstrated significant decreases in levels of CRP and improvements in QoL measures (Galvao et al., 2010). Galvao's study focused on men without bone metastasis, however safe resistance exercise tailored for patients with bone metastasis has also been described (Cormie et al., 2013). It is possible that a trial incorporating resistance elements may affect CTC numbers compared with aerobic exercise only. The INTERVAL-GAP4 trial is actively recruiting and consists of aerobic and resistance components for patients with metastatic PrCa (Newton et al., 2018) and may further elucidate these findings from the ExPeCT trial. CTCs hold potential to track the evolution of disease progression over time and may hold clinical relevance in terms of predictive markers (Maas et al., 2017). The observation of significant alterations in CTC number within each group, may be attributed to disease progression, systemic therapy or the eventual resistance to treatment that can occur naturally over time (Nakazawa, 2017). These factors may have impacted both the exercise and control groups. Recent advances in cfDNA may be more beneficial than CTCs in terms of molecular characterisation. Analysis of cfDNA *vs.* matched metastatic tumour tissue from patients with metastatic PrCa noted similar genomic

characteristics and somatic attributes (Wyatt et al., 2017), hence analysis of cfDNA may provide increased information over that of CTCs in this cohort. The presence of CTC clusters was observed in ExPeCT participants and was positively correlated with PSA. CTC clusters are associated with aggressive disease sub types in other cancers (Mu et al., 2015), and PSA is considered an independent marker for aggressive PrCa (Larsen et al., 2013). Taken together, the presence of CTC clusters and high PSA, may hold potential as a robust screening tool for more aggressive disease. CTCs have been profiled in patients with high risk non-metastatic PrCa, resulting in a high levels of detection and promoting the use of CTCs as a screening tool (Kuske et al., 2016). These data highlight the potential of CTCs to be used as an indicator of high-risk disease and as a liquid biopsy marker in metastatic PrCa. Acquisition of survival data relating to participants involved in ExPeCT, would allow for further analysis into disease progression, which could be linked to CTCs. Continued research is required to molecularly profile CTCs and to determine the clinical use of CTC number as a surrogate marker of disease progression and treatment response.

Interestingly, a positive correlation between CTC number and white cell count was recorded within group, for the first time in PrCa. Investigation into the relationship between white blood cells and tumour cells, has previously determined possible crosstalk between tumour cells and the immune response, with the potential for an immune evasion mechanism. In breast cancer, it was established that tumour specific glycoprotein, MUC1-ST, binds to myeloid cells, allowing the cancer cells to manipulate the myeloid population (Beatson et al., 2016). This novel data sets the foundation for future studies examining the communication between white blood cells and CTCs. In addition to the role of white blood cells in inflammation, platelets are also implicated in inflammatory response and many inflammatory mediators are derived from platelet granules (Morrell et al., 2014). Platelet cloaking of CTCs has been suggested as an important mechanism aiding the extravasation of CTCs to distant sites (Egan et al., 2011). A trend towards an increased presence of platelet cloaking was observed in the control group, which

may infer reduced platelet cloaking as a result of an exercise intervention. Structured physical activity may have an impact on immune cells, such as platelet derived inflammatory mediators and NK cells, and biological systems within the body. Physical activity is thought to alter levels of cells present in both the innate and adaptive immune system (Koelwyn et al., 2015), and may account for the variation in platelet cloaking observed between groups. An example of this, is a potential increase in NK cells post exercise (Timmons and Cieslak, 2008), which increase clearance of CTCs from the bloodstream. Expanding the intervention over an increased period of time or the previously discussed inclusion of a resistance component, may help to expand this concept and provide insight into the potential positive effects of exercise in metastatic disease. The presence of platelet cloaking in PrCa has not been documented previously and targeting platelets may hold clinical relevance in the prevention of disease progression. A clinical trial utilising anti-coagulants (e.g. aspirin) in patients with PrCa demonstrated reduced risk of prostate specific mortality (Choe et al., 2012), which may suggest an interaction between platelets and PrCa progression. *In vitro* cell line models were utilised to further examine the impact of platelet cloaking on prostate cells. A trend towards increased platelet cloaking in malignant cells when compared to normal prostate cells was observed. Tumour-cell induced platelet aggregation occurs through tumour cell signalling inducing the release of platelet granules and may be useful in identifying pathways that could be potential targets for interrupting this cloaking occurrence. Further *in vitro* analysis, using an anti-coagulant such as aspirin, may help to better inform these novel findings. A limitation of this research was the shearing of the cytoplasm in CTCs present in patient samples. Platelet cloaking was exclusively identified as present adhered to CTC cytoplasm, and the loss may be attributed to reduced levels of cloaking observed. Optimisation of isolation techniques to ensure the cytoplasm remains intact would be an important step in future platelet cloaking research.

In addition to CTCs and platelet cloaking, the ExPeCT trial aimed to broaden the understanding of the role of obesity and inflammation in

PrCa. Significant correlations were observed between adipokines and clinical variables, (e.g. adiponectin and BMI), which was reflective of their biological roles in obesity and disease progression. CTCs and platelet cloaking were compared to the markers of obesity and insulin secretion. Positive correlations between adipokines, adiponectin and resistin, and C-peptide were observed with CTC number and platelet cloaking. These data are suggestive of a role between obesity, insulin secretion and metastatic spread. The role of obesity in PrCa has been extensively documented, although obesity is not thought to be predictive of diagnosis, it is associated with a more aggressive disease phenotype (Allott et al., 2013). Further investigation into the regulatory pathways involved in the expression of these adipokines and surrogate insulin secretion markers, may provide insight into the positive correlations observed between them and CTCs. Thus providing increased evidence on the complex role which obesity plays in metastasis. Furthermore, the relationship between platelet cloaking and adipokine status has not been documented previously and may indicate a promising avenue of research into the role between platelets and obesity in patients with metastatic PrCa.

Inflammation was examined in ExPeCT trial participants by monitoring expression of a panel of 13 inflammatory mediators over time. Significant changes in inflammatory mediators were observed within groups over time, with no significant difference observed between groups. As discussed previously, this may be due to the nature of systemic inflammation and reflect the natural course of disease progression. The most common site of metastasis in PrCa is the bone, and the bone microenvironment consists of myeloid cell precursors and immune cells which are linked to the metastatic cascade (Roca and McCauley, 2015). Hence, this population of patients is likely to have a more inflamed profile. The role between inflammatory mediators and CTCs has been documented previously in other cancers (Mego et al., 2017, Li et al., 2017). The findings from the ExPeCT study indicated positive correlations between inflammatory mediators such as IL-6 and IL-8, with clinical variables such as haemoglobin, PSA and white cell

count. These data suggest the possibility that an inflamed environment promotes progression or vice versa. Monoclonal antibodies targeting pro-inflammatory mediators such as IL-6 have been analysed in PrCa with some success (Culig, 2014a). These biological therapies could provide alternative treatment strategies for patients with metastatic PrCa. As with obesity, the relationship between platelet cloaking and inflammatory mediators was assessed in this research for the first time in metastatic PrCa. Multiple negative correlations were observed, demonstrating a relationship between the inflammatory environment and platelet cloaking. A limitation associated with the ExPeCT trial, refers to the number of participants. Future clinical trials, including increased numbers of patients with different types of PrCa, which examined platelet cloaking and inflammatory mechanisms, would allow for direct comparisons between cancer types and could determine the makeup of the pro-inflammatory environment surrounding platelet cloaking.

A lethality gene signature associated with obesity and coagulation was determined in ExPeCT NCBs, with significant correlations between gene expression and inflammatory mediator levels, such as Il-13 and IL-4, observed. The analysis of diagnostic biopsies could provide insight into the course of disease progression. Further analysis, investigating this signature with reference to response to exercise, may allow for increased understanding of the impact of structured aerobic exercise on biological outcomes in patients with metastatic PrCa or which patients would benefit most. In conjunction with the ExPeCT trial analysis, gene expression was performed on *in vitro* cell line models, to compare *in vivo* data to an *in vitro* model. The original cell line model, consisting of a range of prostate cell lines, demonstrated significant differences between malignant and normal prostate cells, demonstrating changes in levels from normal through to progression, which could be linked to the onset of disease advancement. An Enzalutamide panel was subsequently included to perform additional analysis, to develop an understanding of the role of obesity in Enzalutamide resistance. Although the role of inflammation and Enzalutamide resistance has not been documented in PrCa previously, studies in lung and ovarian cancers have established a

link between drug resistance and inflammatory mechanisms (Yang et al., 2011, Godwin et al., 2013). Increased rates of proliferation were witnessed in the Enzalutamide resistant panel, when exposed to ACM, between cell lines expressing varying levels of resistance, inferring an interplay with obesity. Improved understanding of the role of obesity in PrCa is important, based on the documented role of obesity in PrCa aggressiveness. Additional experiments, examining the sensitivity of second generation anti-androgens, such as Enzalutamide and abiraterone acetate, when exposed to ACM, may aid in the understanding of the obesity/resistance relationship and provide a translational perspective.

The results from this project have improved the understanding of the relationships between exercise, metastasis, inflammation and obesity in advanced PrCa. The data generated will help to improve the outcomes for patients living with metastatic PrCa by furthering the understanding of biological relationships involved in disease progression, utilising physical activity to improve overall QoL and highlighting potential for personalised, targeted treatment strategies. Personalised medicine, an individualised treatment based on biological analysis, has been an area of interest for several years but has not yet been fully realised. The use of minimally invasive methods to inform treatment strategies is paramount for patients with metastatic disease and the results from ExPeCT, in particular from the CTC and platelet cloaking data, may hold potential as screening tools in a clinical setting in the long-term. While further investigation into these findings in larger cohorts is require in the short-term, data generated from this trial is promising and has further increased the understanding of the PrCa paradigm. ExPeCT has provided the foundation for larger scale studies, such as INTERVAL-GAP 4, with increased numbers in multiple locations, to further elucidate the role of exercise, both aerobic and resistance, as an effective adjunct to treatment for patients with metastatic PrCa. This research has powerful implications for aggressive disease, which may be translatable into a clinical setting and has potential to improve the lives of patients living with metastatic PrCa.

References

AALINKEEL, R., NAIR, B. B., REYNOLDS, J. L., SYKES, D. E., MAHAJAN, S. D., CHADHA, K. C. & SCHWARTZ, S. A. 2011. Overexpression of MMP-9 contributes to invasiveness of prostate cancer cell line LNCaP. *Immunol Invest,* 40**,** 447-64.

ABATE-SHEN, C. & SHEN, M. M. 2000. Molecular genetics of prostate cancer. *Genes Dev,* 14**,** 2410-34.

ABRAMSON, J. L. & VACCARINO, V. 2002. Relationship between physical activity and inflammation among apparently healthy middle-aged and older US adults. *Arch Intern Med,* 162**,** 1286-92.

ACS. 2018. *Hormone therapy for Prostate Cancer* [Online]. Available: https://www.cancer.org/cancer/prostate-cancer/treating/hormone-therapy.html [Accessed 02-07-2018 2018].

ADAMO, P. & LADOMERY, M. R. 2016. The oncogene ERG: a key factor in prostate cancer. *Oncogene,* 35**,** 403-14.

ADAMS, D. L., STEFANSSON, S., HAUDENSCHILD, C., MARTIN, S. S., CHARPENTIER, M., CHUMSRI, S., CRISTOFANILLI, M., TANG, C. M. & ALPAUGH, R. K. 2015. Cytometric characterization of circulating tumor cells captured by microfiltration and their correlation to the CellSearch((R)) CTC test. *Cytometry A,* 87**,** 137-44.

ADLER, H. L., MCCURDY, M. A., KATTAN, M. W., TIMME, T. L., SCARDINO, P. T. & THOMPSON, T. C. 1999. Elevated levels of circulating interleukin-6 and transforming growth factor-beta1 in patients with metastatic prostatic carcinoma. *J Urol,* 161**,** 182-7.

AICR, W. A. 2007. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective.

ALCOVER, J., FILELLA, X., LUQUE, P., MOLINA, R., IZQUIERDO, L., AUGE, J. M. & ALCARAZ, A. 2010. Prognostic value of IL-6 in localized prostatic cancer. *Anticancer Res,* 30**,** 4369-72.

ALDINUCCI, D. & COLOMBATTI, A. 2014. The inflammatory chemokine CCL5 and cancer progression. *Mediators Inflamm,* 2014**,** 292376.

ALLARD, W. J., MATERA, J., MILLER, M. C., REPOLLET, M., CONNELLY, M. C., RAO, C., TIBBE, A. G., UHR, J. W. & TERSTAPPEN, L. W. 2004. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res,* 10**,** 6897-904.

ALLOTT, E. H., MASKO, E. M. & FREEDLAND, S. J. 2013. Obesity and prostate cancer: weighing the evidence. *Eur Urol,* 63**,** 800-9.

ALLOTT, E. H., OLIVER, E., LYSAGHT, J., GRAY, S. G., REYNOLDS, J. V., ROCHE, H. M. & PIDGEON, G. P. 2012. The SGBS cell strain as a model for the in vitro study of obesity and cancer. *Clin Transl Oncol,* 14**,** 774-82.

AN, J., REN, S., MURPHY, S. J., DALANGOOD, S., CHANG, C., PANG, X., CUI, Y., WANG, L., PAN, Y., ZHANG, X., ZHU, Y., WANG, C., HALLING, G. C., CHENG, L., SUKOV, W. R., KARNES, R. J., VASMATZIS, G., ZHANG, Q., ZHANG, J., CHEVILLE, J. C., YAN, J., SUN, Y. & HUANG, H. 2015. Truncated ERG Oncoproteins from TMPRSS2-ERG Fusions Are Resistant to SPOP-Mediated Proteasome Degradation. *Mol Cell,* 59**,** 904-16.

ANTONARAKIS, E. S., LU, C., WANG, H., LUBER, B., NAKAZAWA, M., ROESER, J. C., CHEN, Y., MOHAMMAD, T. A., CHEN, Y., FEDOR, H. L., LOTAN, T. L., ZHENG, Q., DE MARZO, A. M., ISAACS, J. T., ISAACS, W. B., NADAL, R., PALLER, C. J., DENMEADE, S. R., CARDUCCI, M. A., EISENBERGER, M. A. & LUO, J. 2014. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. *N Engl J Med,* 371**,** 1028-38.

ARITA, Y., KIHARA, S., OUCHI, N., TAKAHASHI, M., MAEDA, K., MIYAGAWA, J., HOTTA, K., SHIMOMURA, I., NAKAMURA, T., MIYAOKA, K., KURIYAMA, H., NISHIDA, M., YAMASHITA, S., OKUBO, K., MATSUBARA, K., MURAGUCHI, M., OHMOTO, Y., FUNAHASHI, T. & MATSUZAWA, Y. 1999. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun,* 257**,** 79-83.

ARMSTRONG, A. J., EISENBERGER M.A., HALABI S., OUDARD S., NANUS D.M., PETRYLAK D.P., SARTOR A.O. AND SCHER H.I. 2012. Biomarkers in the Management and Treatment of Men with Metastatic Castration-Resistant Prostate Cancer. *European Urology,* 61**,** 549-559.

ARNOLD, M., PANDEYA, N., BYRNES, G., RENEHAN, A. G., STEVENS, G. A., EZZATI, M., FERLAY, J., MIRANDA, J. J., ROMIEU, I., DIKSHIT, R., FORMAN, D. & SOERJOMATARAM, I. 2015. Global burden of cancer attributable to high body-mass index in 2012: a population-based study. *Lancet Oncol,* 16**,** 36-46.

ARTHUR, R., WILLIAMS, R., GARMO, H., HOLMBERG, L., STATTIN, P., MALMSTROM, H., LAMBE, M., HAMMAR, N., WALLDIUS, G., ROBINSSON, D., JUNGNER, I. & VAN HEMELRIJCK, M. 2018. Serum inflammatory markers in relation to prostate cancer severity and death in the Swedish AMORIS study. *Int J Cancer*.

AWICK, E. A., EHLERS, D. K., AGUINAGA, S., DAUGHERTY, A. M., KRAMER, A. F. & MCAULEY, E. 2017. Effects of a randomized exercise trial on physical activity, psychological distress and quality of life in older adults. *Gen Hosp Psychiatry*.

AWICK, E. A., WOJCICKI, T. R., OLSON, E. A., FANNING, J., CHUNG, H. D., ZUNIGA, K., MACKENZIE, M., KRAMER, A. F. & MCAULEY, E. 2015. Differential exercise effects on quality of life and health-related quality of life in older adults: a randomized controlled trial. *Qual Life Res,* 24**,** 455-62.

AYALA, G., FROLOV, A., CHATTERJEE, D., HE, D., HILSENBECK, S. & ITTMANN, M. 2015. Expression of ERG protein in prostate cancer: variability and biological correlates. *Endocr Relat Cancer,* 22**,** 277-87.

BABIO, N. 2013. White Blood Cell Counts as Risk Markers of Developing Metabolic Syndrome and Its Components in the Predimed Study. 8.

BALDUCCI, S., ZANUSO, S., NICOLUCCI, A., FERNANDO, F., CAVALLO, S., CARDELLI, P., FALLUCCA, S., ALESSI, E., LETIZIA, C., JIMENEZ, A., FALLUCCA, F. & PUGLIESE, G. 2010. Anti-inflammatory effect of exercise training in subjects with type 2 diabetes and the metabolic syndrome is dependent on exercise modalities and independent of weight loss. *Nutr Metab Cardiovasc Dis,* 20**,** 608-17.

BALKWILL, F. 2004. Cancer and the chemokine network. *Nat Rev Cancer,* 4**,** 540-50.

BALKWILL, F. 2006. TNF-alpha in promotion and progression of cancer. *Cancer Metastasis Rev,* 25**,** 409-16.

BALKWILL, F. 2009. Tumour necrosis factor and cancer. *Nature Reviews Cancer,* 9**,** 361.

BALKWILL, F. & MANTOVANI, A. 2001. Inflammation and cancer: back to Virchow? *Lancet,* 357**,** 539-45.

BAMBACE, N. M. & HOLMES, C. E. 2011. The platelet contribution to cancer progression. *J Thromb Haemost,* 9**,** 237-49.

BARBIERI, C. E., BACA, S. C., LAWRENCE, M. S., DEMICHELIS, F., BLATTNER, M., THEURILLAT, J.-P., WHITE, T. A., STOJANOV, P., VAN ALLEN, E., STRANSKY, N., NICKERSON, E., CHAE, S.-S., BOYSEN, G., AUCLAIR, D., ONOFRIO, R., PARK, K., KITABAYASHI, N., MACDONALD, T. Y., SHEIKH, K., VUONG, T., GUIDUCCI, C., CIBULSKIS, K., SIVACHENKO, A., CARTER, S. L., SAKSENA, G., VOET, D., HUSSAIN, W. M., RAMOS, A. H., WINCKLER, W., REDMAN, M. C., ARDLIE, K., TEWARI, A. K., MOSQUERA, J. M., RUPP, N., WILD, P. J., MOCH, H., MORRISSEY, C., NELSON, P. S., KANTOFF, P. W., GABRIEL, S. B., GOLUB, T. R.,

MEYERSON, M., LANDER, E. S., GETZ, G., RUBIN, M. A. & GARRAWAY, L. A. 2012. Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. *Nature genetics,* 44**,** 685-689.

BATTAGLINI, C. L., HACKNEY, A. C., GARCIA, R., GROFF, D., EVANS, E. & SHEA, T. 2009. The effects of an exercise program in leukemia patients. *Integr Cancer Ther,* 8**,** 130-8.

BEATSON, R., TAJADURA-ORTEGA, V., ACHKOVA, D., PICCO, G., TSOUROUKTSOGLOU, T. D., KLAUSING, S., HILLIER, M., MAHER, J., NOLL, T., CROCKER, P. R., TAYLOR-PAPADIMITRIOU, J. & BURCHELL, J. M. 2016. The mucin MUC1 modulates the tumor immunological microenvironment through engagement of the lectin Siglec-9. *Nat Immunol,* 17**,** 1273-1281.

BEER, T. M., ARMSTRONG, A. J., RATHKOPF, D. E., LORIOT, Y., STERNBERG, C. N., HIGANO, C. S., IVERSEN, P., BHATTACHARYA, S., CARLES, J., CHOWDHURY, S., DAVIS, I. D., DE BONO, J. S., EVANS, C. P., FIZAZI, K., JOSHUA, A. M., KIM, C. S., KIMURA, G., MAINWARING, P., MANSBACH, H., MILLER, K., NOONBERG, S. B., PERABO, F., PHUNG, D., SAAD, F., SCHER, H. I., TAPLIN, M. E., VENNER, P. M. & TOMBAL, B. 2014. Enzalutamide in metastatic prostate cancer before chemotherapy. *N Engl J Med,* 371**,** 424-33.

BLATTNER, M., LEE, D. J., O'REILLY, C., PARK, K., MACDONALD, T. Y., KHANI, F., TURNER, K. R., CHIU, Y. L., WILD, P. J., DOLGALEV, I., HEGUY, A., SBONER, A., RAMAZANGOLU, S., HIERONYMUS, H., SAWYERS, C., TEWARI, A. K., MOCH, H., YOON, G. S., KNOWN, Y. C., ANDREN, O., FALL, K., DEMICHELIS, F., MOSQUERA, J. M., ROBINSON, B. D., BARBIERI, C. E. & RUBIN, M. A. 2014. SPOP mutations in prostate cancer across demographically diverse patient cohorts. *Neoplasia,* 16**,** 14-20.

BLATTNER, M., LIU, D., ROBINSON, B. D., HUANG, D., POLIAKOV, A., GAO, D., NATARAJ, S., DEONARINE, L. D., AUGELLO, M. A., SAILER, V., PONNALA, L., ITTMANN, M., CHINNAIYAN, A. M., SBONER, A., CHEN, Y., RUBIN, M. A. & BARBIERI, C. E. 2017. SPOP Mutation Drives Prostate Tumorigenesis In Vivo through Coordinate Regulation of PI3K/mTOR and AR Signaling. *Cancer Cell,* 31**,** 436-451.

BONILLA, F. A. & OETTGEN, H. C. 2010. Adaptive immunity. *J Allergy Clin Immunol,* 125.

BORASCHI, D., LUCCHESI, D., HAINZL, S., LEITNER, M., MAIER, E., MANGELBERGER, D., OOSTINGH, G. J., PFALLER, T., PIXNER, C., POSSELT, G., ITALIANI, P., NOLD, M. F., NOLD-PETRY, C. A., BUFLER, P. & DINARELLO, C. A. 2011. IL-37: a new anti-inflammatory cytokine of the IL-1 family. *Eur Cytokine Netw,* 22**,** 127-47.

BOSTWICK, D. G. & QIAN, J. 2004. High-grade prostatic intraepithelial neoplasia. *Mod Pathol,* 17**,** 360-79.

BOURKE, L., SMITH, D., STEED, L., HOOPER, R., CARTER, A., CATTO, J., ALBERTSEN, P. C., TOMBAL, B., PAYNE, H. A. & ROSARIO, D. J. 2016. Exercise for Men with Prostate Cancer: A Systematic Review and Meta-analysis. *Eur Urol,* 69**,** 693-703.

BUB, J. D., MIYAZAKI, T. & IWAMOTO, Y. 2006. Adiponectin as a growth inhibitor in prostate cancer cells. *Biochem Biophys Res Commun,* 340**,** 1158-66.

BUBLEY, G. J. & BALK, S. P. 2017. Association Between Androgen Receptor Splice Variants and Prostate Cancer Resistance to Abiraterone and Enzalutamide. *J Clin Oncol,* 35**,** 2103-2105.

CABARKAPA, S., PERERA, M., MCGRATH, S. & LAWRENTSCHUK, N. 2016. Prostate cancer screening with prostate-specific antigen: A guide to the guidelines. *Prostate Int,* 4**,** 125-129.

CAMPBELL, P. T., NEWTON, C. C., FREEDMAN, N. D., KOSHIOL, J., ALAVANJA, M. C., BEANE FREEMAN, L. E., BURING, J. E., CHAN, A. T., CHONG, D. Q., DATTA, M.,

GAUDET, M. M., GAZIANO, J. M., GIOVANNUCCI, E. L., GRAUBARD, B. I., HOLLENBECK, A. R., KING, L., LEE, I. M., LINET, M. S., PALMER, J. R., PETRICK, J. L., POYNTER, J. N., PURDUE, M. P., ROBIEN, K., ROSENBERG, L., SAHASRABUDDHE, V. V., SCHAIRER, C., SESSO, H. D., SIGURDSON, A. J., STEVENS, V. L., WACTAWSKI-WENDE, J., ZELENIUCH-JACQUOTTE, A., RENEHAN, A. G. & MCGLYNN, K. A. 2016. Body Mass Index, Waist Circumference, Diabetes, and Risk of Liver Cancer for U.S. Adults. *Cancer Res,* 76**,** 6076-6083.

CANCERRESEARCHUK. 2018. *Prostate Cancer: Types and Grades* [Online]. Available: https://about-cancer.cancerresearchuk.org/about-cancer/prostate-cancer/types-grades [Accessed 02-07-2018 2018].

CARY, K. C. & COOPERBERG, M. R. 2013. Biomarkers in prostate cancer surveillance and screening: past, present, and future. *Ther Adv Urol,* 5**,** 318-29.

CHALFIN, H. J., VERDONE, J. E., VAN DER TOOM, E. E., GLAVARIS, S., GORIN, M. A. & PIENTA, K. J. 2017. Nucleolin Staining May Aid in the Identification of Circulating Prostate Cancer Cells. *Clin Genitourin Cancer,* 15**,** e477-e481.

CHANG, C. H., HSIAO, C. F., YEH, Y. M., CHANG, G. C., TSAI, Y. H., CHEN, Y. M., HUANG, M. S., CHEN, H. L., LI, Y. J., YANG, P. C., CHEN, C. J., HSIUNG, C. A. & SU, W. C. 2013. Circulating interleukin-6 level is a prognostic marker for survival in advanced nonsmall cell lung cancer patients treated with chemotherapy. *Int J Cancer,* 132**,** 1977-85.

CHEN, J., DE, S., BRAINARD, J. & BYZOVA, T. V. 2004. Metastatic properties of prostate cancer cells are controlled by VEGF. *Cell Commun Adhes,* 11**,** 1-11.

CHEN, R. C., RUMBLE, R. B., LOBLAW, D. A., FINELLI, A., EHDAIE, B., COOPERBERG, M. R., MORGAN, S. C., TYLDESLEY, S., HALUSCHAK, J. J., TAN, W., JUSTMAN, S. & JAIN, S. 2016. Active Surveillance for the Management of Localized Prostate Cancer (Cancer Care Ontario Guideline): American Society of Clinical Oncology Clinical Practice Guideline Endorsement. *J Clin Oncol,* 34**,** 2182-90.

CHEN, Y., LIU, L., WANG, X., WANG, J., YAN, Z., CHENG, J., GONG, G. & LI, G. 2013. Body mass index and risk of gastric cancer: a meta-analysis of a population with more than ten million from 24 prospective studies. *Cancer Epidemiol Biomarkers Prev,* 22**,** 1395-408.

CHIAM, K., RICCIARDELLI, C. & BIANCO-MIOTTO, T. 2014. Epigenetic biomarkers in prostate cancer: Current and future uses. *Cancer Lett,* 342**,** 248-56.

CHO, E. H., WENDEL, M., LUTTGEN, M., YOSHIOKA, C., MARRINUCCI, D., LAZAR, D., SCHRAM, E., NIEVA, J., BAZHENOVA, L., MORGAN, A., KO, A. H., KORN, W. M., KOLATKAR, A., BETHEL, K. & KUHN, P. 2012. Characterization of circulating tumor cell aggregates identified in patients with epithelial tumors. *Phys Biol,* 9**,** 016001.

CHOE, K. S., COWAN, J. E., CHAN, J. M., CARROLL, P. R., D'AMICO, A. V. & LIAUW, S. L. 2012. Aspirin Use and the Risk of Prostate Cancer Mortality in Men Treated With Prostatectomy or Radiotherapy. *J Clin Oncol,* 30**,** 3540-4.

CHONCHOL, M. & NIELSON, C. 2008. Hemoglobin levels and coronary artery disease. *Am Heart J,* 155**,** 494-8.

CHRVALA, C. A. 2012. The Changing Landscape of Treatment Options For Metastatic Castrate-Resistant Prostate Cancer: Challenges and Solutions for Physicians and Patients. *P t,* 37**,** 453-63.

CLINTON, T. N., BAGRODIA, A., LOTAN, Y., MARGULIS, V., RAJ, G. V. & WOLDU, S. L. 2017. Tissue-based biomarkers in prostate cancer. *Expert Rev Precis Med Drug Dev,* 2**,** 249-60.

COCO, S., ALAMA, A., VANNI, I., FONTANA, V., GENOVA, C., DAL BELLO, M. G., TRUINI, A., RIJAVEC, E., BIELLO, F., SINI, C., BURRAFATO, G., MAGGIONI, C., BARLETTA, G. & GROSSI, F. 2017. Circulating Cell-Free DNA and Circulating

Tumor Cells as Prognostic and Predictive Biomarkers in Advanced Non-Small Cell Lung Cancer Patients Treated with First-Line Chemotherapy. *Int J Mol Sci,* 18.

COLLIER, A., GHOSH, S., MCGLYNN, B. & HOLLINS, G. 2012. Prostate cancer, androgen deprivation therapy, obesity, the metabolic syndrome, type 2 diabetes, and cardiovascular disease: a review. *Am J Clin Oncol,* 35**,** 504-9.

COLOTTA, F., ALLAVENA, P., SICA, A., GARLANDA, C. & MANTOVANI, A. 2009. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis,* 30**,** 1073-81.

COMPTE, N., BAILLY, B., DE BREUCKER, S., GORIELY, S. & PEPERSACK, T. 2015. Study of the association of total and differential white blood cell counts with geriatric conditions, cardio-vascular diseases, seric IL-6 levels and telomere length. *Exp Gerontol,* 61**,** 105-12.

CONLEY-LACOMB, M. K., SEMAAN, L., SINGAREDDY, R., LI, Y., HEATH, E. I., KIM, S., CHER, M. L. & CHINNI, S. R. 2016. Pharmacological targeting of CXCL12/CXCR4 signaling in prostate cancer bone metastasis. *Mol Cancer,* 15**,** 68.

CONTEDUCA, V., CAFFO, O., GALLI, L., MAUGERI, A., SCARPI, E., MAINES, F., CHIURI, V. E., LOLLI, C., KINSPERGHER, S., SCHEPISI, G., SANTONI, M., SANTINI, D., FRATINO, L., BURGIO, S. L., SALVI, S., MENNA, C. & GIORGI, U. 2018. Association among metabolic syndrome, inflammation, and survival in prostate cancer. *Urol Oncol*.

COOKE, N. M., SPILLANE, C. D., SHEILS, O., O'LEARY, J. & KENNY, D. 2015. Aspirin and P2Y12 inhibition attenuate platelet-induced ovarian cancer cell invasion. *BMC Cancer,* 15**,** 627.

CORMIE, P., GALVAO, D. A., SPRY, N., JOSEPH, D., CHEE, R., TAAFFE, D. R., CHAMBERS, S. K. & NEWTON, R. U. 2015. Can supervised exercise prevent treatment toxicity in patients with prostate cancer initiating androgen-deprivation therapy: a randomised controlled trial. *BJU Int,* 115**,** 256-66.

CORMIE, P., NEWTON, R. U., SPRY, N., JOSEPH, D., TAAFFE, D. R. & GALVAO, D. A. 2013. Safety and efficacy of resistance exercise in prostate cancer patients with bone metastases. *Prostate Cancer Prostatic Dis,* 16**,** 328-35.

COURNEYA, K. S. 2017. Exercise guidelines for cancer survivors: are fitness and quality-of-life benefits enough to change practice? *Curr Oncol,* 24**,** 8-9.

COURNEYA, K. S., SEGAL, R. J., MACKEY, J. R., GELMON, K., FRIEDENREICH, C. M., YASUI, Y., REID, R. D., JESPERSEN, D., COOK, D., PROULX, C., TRINH, L., DOLAN, L. B., WOODING, E., FORBES, C. C. & MCKENZIE, D. C. 2014. Effects of exercise dose and type on sleep quality in breast cancer patients receiving chemotherapy: a multicenter randomized trial. *Breast Cancer Res Treat,* 144**,** 361-9.

COURNEYA, K. S., SEGAL, R. J., MACKEY, J. R., GELMON, K., REID, R. D., FRIEDENREICH, C. M., LADHA, A. B., PROULX, C., VALLANCE, J. K., LANE, K., YASUI, Y. & MCKENZIE, D. C. 2007. Effects of aerobic and resistance exercise in breast cancer patients receiving adjuvant chemotherapy: a multicenter randomized controlled trial. *J Clin Oncol,* 25**,** 4396-404.

COURNEYA, K. S., SELLAR, C. M., STEVINSON, C., MCNEELY, M. L., PEDDLE, C. J., FRIEDENREICH, C. M., TANKEL, K., BASI, S., CHUA, N., MAZUREK, A. & REIMAN, T. 2009. Randomized controlled trial of the effects of aerobic exercise on physical functioning and quality of life in lymphoma patients. *J Clin Oncol,* 27**,** 4605-12.

COUSSENS, L. M. & WERB, Z. 2002. Inflammation and cancer. *Nature,* 420**,** 860-7.

CRAFT, L. L., VANITERSON, E. H., HELENOWSKI, I. B., RADEMAKER, A. W. & COURNEYA, K. S. 2012. Exercise effects on depressive symptoms in cancer

survivors: a systematic review and meta-analysis. *Cancer Epidemiol Biomarkers Prev,* 21**,** 3-19.
CRAWFORD, E. D. & MOUL, J. W. 2015. ADT risks and side effects in advanced prostate cancer: cardiovascular and acute renal injury. *Oncology (Williston Park),* 29**,** 55-8, 65-6.
CULIG, Z. 2014a. Proinflammatory cytokine interleukin-6 in prostate carcinogenesis. *Am J Clin Exp Urol,* 2**,** 231-8.
CULIG, Z. 2014b. Targeting the androgen receptor in prostate cancer. *Expert Opin Pharmacother,* 15**,** 1427-37.
DAI, X., GAN, W., LI, X., WANG, S., ZHANG, W., HUANG, L., LIU, S., ZHONG, Q., GUO, J., ZHANG, J., CHEN, T., SHIMIZU, K., BECA, F., BLATTNER, M., VASUDEVAN, D., BUCKLEY, D. L., QI, J., BUSER, L., LIU, P., INUZUKA, H., BECK, A. H., WANG, L., WILD, P. J., GARRAWAY, L. A., RUBIN, M. A., BARBIERI, C. E., WONG, K. K., MUTHUSWAMY, S. K., HUANG, J., CHEN, Y., BRADNER, J. E. & WEI, W. 2017. Prostate cancer-associated SPOP mutations confer resistance to BET inhibitors through stabilization of BRD4. *Nat Med,* 23**,** 1063-1071.
DANILA, D. C., ANAND, A., SUNG, C. C., HELLER, G., LEVERSHA, M. A., CAO, L., LILJA, H., MOLINA, A., SAWYERS, C. L., FLEISHER, M. & SCHER, H. I. 2011. TMPRSS2-ERG status in circulating tumor cells as a predictive biomarker of sensitivity in castration-resistant prostate cancer patients treated with abiraterone acetate. *Eur Urol,* 60**,** 897-904.
DE BONO, J. S., SCHER, H. I., MONTGOMERY, R. B., PARKER, C., MILLER, M. C., TISSING, H., DOYLE, G. V., TERSTAPPEN, L. W., PIENTA, K. J. & RAGHAVAN, D. 2008. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res,* 14**,** 6302-9.
DE LAERE, B., OEYEN, S., VAN OYEN, P., GHYSEL, C., AMPE, J., OST, P., DEMEY, W., HOEKX, L., SCHRIJVERS, D., BROUWERS, B., LYBAERT, W., EVERAERT, E., VAN KERCKHOVE, P., DE MAESENEER, D., STRIJBOS, M., BOLS, A., FRANSIS, K., BEIJE, N., DE KRUIJFF, I., VAN DAM, V., BROUWER, A., VAN DAM, P. J., VAN DEN EYNDEN, G., RUTTEN, A., SLEIJFER, S., VANDEBROEK, J., VAN LAERE, S. & DIRIX, L. 2018. Circulating tumor cells and survival in abiraterone- and enzalutamide-treated patients with castration-resistant prostate cancer. *Prostate,* 78**,** 435-445.
DE MARZO, A. M., PLATZ, E. A., SUTCLIFFE, S., XU, J., GRONBERG, H., DRAKE, C. G., NAKAI, Y., ISAACS, W. B. & NELSON, W. G. 2007. Inflammation in prostate carcinogenesis. *Nat Rev Cancer,* 7**,** 256-69.
DE NUNZIO, C., PASTORE, A. L., LOMBARDO, R., SIMONE, G., LEONARDO, C., MASTROIANNI, R., COLLURA, D., MUTO, G., GALLUCCI, M., CARBONE, A., FUSCHI, A., DUTTO, L., WITT, J. H., DE DOMINICIS, C. & TUBARO, A. 2018. The new Epstein gleason score classification significantly reduces upgrading in prostate cancer patients. *Eur J Surg Oncol,* 44**,** 835-839.
DEGAWA-YAMAUCHI, M., BOVENKERK, J. E., JULIAR, B. E., WATSON, W., KERR, K., JONES, R., ZHU, Q. & CONSIDINE, R. V. 2003. Serum resistin (FIZZ3) protein is increased in obese humans. *J Clin Endocrinol Metab,* 88**,** 5452-5.
DESITTER, I., GUERROUAHEN, B. S., BENALI-FURET, N., WECHSLER, J., JANNE, P. A., KUANG, Y., YANAGITA, M., WANG, L., BERKOWITZ, J. A., DISTEL, R. J. & CAYRE, Y. E. 2011. A new device for rapid isolation by size and characterization of rare circulating tumor cells. *Anticancer Res,* 31**,** 427-41.
DETRE, S., SACLANI JOTTI, G. & DOWSETT, M. 1995. A "quickscore" method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas. *J Clin Pathol,* 48**,** 876-8.

DI MEO, A., BARTLETT, J., CHENG, Y., PASIC, M. D. & YOUSEF, G. M. 2017. Liquid biopsy: a step forward towards precision medicine in urologic malignancies. *Mol Cancer,* 16**,** 80.

DI SEBASTIANO, K. M., PINTHUS, J. H., DUIVENVOORDEN, W. C., PATTERSON, L., DUBIN, J. A. & MOURTZAKIS, M. 2017. Elevated C-Peptides, Abdominal Obesity, and Abnormal Adipokine Profile are Associated With Higher Gleason Scores in Prostate Cancer. *Prostate,* 77**,** 211-221.

DIAKOS, C. I., CHARLES, K. A., MCMILLAN, D. C. & CLARKE, S. J. 2014. Cancer-related inflammation and treatment effectiveness. *Lancet Oncol,* 15**,** e493-503.

DIZEYI, N., BJARTELL, A., HEDLUND, P., TASKEN, K. A., GADALEANU, V. & ABRAHAMSSON, P. A. 2005. Expression of serotonin receptors 2B and 4 in human prostate cancer tissue and effects of their antagonists on prostate cancer cell lines. *Eur Urol,* 47**,** 895-900.

DOH. 2016. *Obesity Policy and Action Plan* [Online]. Available: www.health.gov.ie/wp-content/uploads/2016/09/A-Healthy-Weight-For-Ireland-Obesity-Policy-and-Action-Plan-2016-2025.pdf [Accessed 02-04-2018].

DOUGAN, M. M., HANKINSON, S. E., VIVO, I. D., TWOROGER, S. S., GLYNN, R. J. & MICHELS, K. B. 2015. Prospective study of body size throughout the life-course and the incidence of endometrial cancer among premenopausal and postmenopausal women. *Int J Cancer,* 137**,** 625-37.

DRANOFF, G. 2004. Cytokines in cancer pathogenesis and cancer therapy. *Nat Rev Cancer,* 4**,** 11-22.

DREGAN, A., CHOWIENCZYK, P. & MOLOKHIA, M. 2017. Cardiovascular and type 2 diabetes morbidity and all-cause mortality among diverse chronic inflammatory disorders. *Heart*.

EFSTATHIOU, J. A., BAE, K., SHIPLEY, W. U., HANKS, G. E., PILEPICH, M. V., SANDLER, H. M. & SMITH, M. R. 2007. Obesity and mortality in men with locally advanced prostate cancer: analysis of RTOG 85-31. *Cancer,* 110**,** 2691-9.

EGAN, K., CROWLEY, D., SMYTH, P., O'TOOLE, S., SPILLANE, C., MARTIN, C., GALLAGHER, M., CANNEY, A., NORRIS, L., CONLON, N., MCEVOY, L., FFRENCH, B., STORDAL, B., KEEGAN, H., FINN, S., MCENEANEY, V., LAIOS, A., DUCREE, J., DUNNE, E., SMITH, L., BERNDT, M., SHEILS, O., KENNY, D. & O'LEARY, J. 2011. Platelet adhesion and degranulation induce pro-survival and pro-angiogenic signalling in ovarian cancer cells. *PLoS One,* 6**,** e26125.

EIRÓ, N. & VIZOSO, F. J. 2012. Inflammation and cancer. *World J Gastrointest Surg,* 4**,** 62-72.

EL-HELIEBI, A., KRONEIS, T., ZOHRER, E., HAYBAECK, J., FISCHEREDER, K., KAMPEL-KETTNER, K., ZIGEUNER, R., POCK, H., RIEDL, R., STAUBER, R., GEIGL, J. B., HUPPERTZ, B., SEDLMAYR, P. & LACKNER, C. 2013. Are morphological criteria sufficient for the identification of circulating tumor cells in renal cancer? *J Transl Med,* 11**,** 214.

EMANUELA, F., GRAZIA, M., MARCO DE, R., MARIA PAOLA, L., GIORGIO, F. & MARCO, B. 2012. Inflammation as a Link between Obesity and Metabolic Syndrome. *J Nutr Metab,* 2012**,** 476380.

ENSARI, I., GREENLEE, T. A., MOTL, R. W. & PETRUZZELLO, S. J. 2015. META-ANALYSIS OF ACUTE EXERCISE EFFECTS ON STATE ANXIETY: AN UPDATE OF RANDOMIZED CONTROLLED TRIALS OVER THE PAST 25 YEARS. *Depress Anxiety,* 32**,** 624-34.

EUCAN. 2018. *Cancer Factsheets* [Online]. Available: http://eco.iarc.fr/eucan [Accessed 05-03-2018 2018].

FARACE, F., MASSARD, C., VIMOND, N., DRUSCH, F., JACQUES, N., BILLIOT, F., LAPLANCHE, A., CHAUCHEREAU, A., LACROIX, L., PLANCHARD, D., LE

MOULEC, S., ANDRÉ, F., FIZAZI, K., SORIA, J. C. & VIELH, P. 2011. A direct comparison of CellSearch and ISET for circulating tumour-cell detection in patients with metastatic carcinomas. *Br J Cancer,* 105**,** 847-53.
FAYANJU, O. M. 2017. Body Mass Index Mediates the Prognostic Significance of Circulating Tumor Cells in Inflammatory Breast Cancer. 214**,** 666-71.
FELDMAN, B. J. & FELDMAN, D. 2001. The development of androgen-independent prostate cancer. *Nat Rev Cancer,* 1**,** 34-45.
FERLAY, J., SOERJOMATARAM, I., DIKSHIT, R., ESER, S., MATHERS, C., REBELO, M., PARKIN, D. M., FORMAN, D. & BRAY, F. 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer,* 136**,** E359-86.
FERRAJOLI, A., KEATING, M. J., MANSHOURI, T., GILES, F. J., DEY, A., ESTROV, Z., KOLLER, C. A., KURZROCK, R., THOMAS, D. A., FADERL, S., LERNER, S., O'BRIEN, S. & ALBITAR, M. 2002. The clinical significance of tumor necrosis factor-alpha plasma level in patients having chronic lymphocytic leukemia. *Blood,* 100**,** 1215-9.
FILKOVA, M., HALUZIK, M., GAY, S. & SENOLT, L. 2009. The role of resistin as a regulator of inflammation: Implications for various human pathologies. *Clin Immunol,* 133**,** 157-70.
FORD, N. A., LASHINGER, L. M., ALLOTT, E. H. & HURSTING, S. D. 2013. Mechanistic Targets and Phytochemical Strategies for Breaking the Obesity-Cancer Link. *Front Oncol,* 3.
FREGNI, G., PERIER, A., AVRIL, M. F. & CAIGNARD, A. 2012. NK cells sense tumors, course of disease and treatments: Consequences for NK-based therapies. *Oncoimmunology,* 1**,** 38-47.
FRIEDLANDER, T. W., ROY, R., TOMLINS, S. A., NGO, V. T., KOBAYASHI, Y., AZAMEERA, A., RUBIN, M. A., PIENTA, K. J., CHINNAIYAN, A., ITTMANN, M. M., RYAN, C. J. & PARIS, P. L. 2012. Common structural and epigenetic changes in the genome of castration-resistant prostate cancer. *Cancer Res,* 72**,** 616-25.
FRIEDRICH, N., THUESEN, B., JORGENSEN, T., JUUL, A., SPIELHAGEN, C., WALLASCHOFKSI, H. & LINNEBERG, A. 2012. The association between IGF-I and insulin resistance: a general population study in Danish adults. *Diabetes Care,* 35**,** 768-73.
GABAY, C. 2006. Interleukin-6 and chronic inflammation. *Arthritis Res Ther,* 8**,** S3.
GALVAO, D. A., NOSAKA, K., TAAFFE, D. R., PEAKE, J., SPRY, N., SUZUKI, K., YAMAYA, K., MCGUIGAN, M. R., KRISTJANSON, L. J. & NEWTON, R. U. 2008. Endocrine and immune responses to resistance training in prostate cancer patients. *Prostate Cancer Prostatic Dis,* 11**,** 160-5.
GALVAO, D. A., TAAFFE, D. R., SPRY, N., JOSEPH, D. & NEWTON, R. U. 2010. Combined resistance and aerobic exercise program reverses muscle loss in men undergoing androgen suppression therapy for prostate cancer without bone metastases: a randomized controlled trial. *J Clin Oncol,* 28**,** 340-7.
GAO, Q. & ZHENG, J. 2014. Adiponectin-induced antitumor activity on prostatic cancers through inhibiting proliferation. *Cell Biochem Biophys,* 70**,** 461-5.
GARCIA-FLORES, M., CASANOVA-SALAS, I., RUBIO-BRIONES, J., CALATRAVA, A., DOMINGUEZ-ESCRIG, J., RUBIO, L., RAMIREZ-BACKHAUS, M., FERNANDEZ-SERRA, A., GARCIA-CASADO, Z. & LOPEZ-GUERRERO, J. A. 2014. Clinico-pathological significance of the molecular alterations of the SPOP gene in prostate cancer. *Eur J Cancer,* 50**,** 2994-3002.
GASI TANDEFELT, D., BOORMANS, J., HERMANS, K. & TRAPMAN, J. 2014. ETS fusion genes in prostate cancer. *Endocr Relat Cancer,* 21**,** R143-52.
GENG, C., HE, B., XU, L., BARBIERI, C. E., EEDUNURI, V. K., CHEW, S. A., ZIMMERMANN, M., BOND, R., SHOU, J., LI, C., BLATTNER, M., LONARD, D. M.,

DEMICHELIS, F., COARFA, C., RUBIN, M. A., ZHOU, P., O'MALLEY, B. W. & MITSIADES, N. 2013. Prostate cancer-associated mutations in speckle-type POZ protein (SPOP) regulate steroid receptor coactivator 3 protein turnover. *Proc Natl Acad Sci U S A,* 110**,** 6997-7002.

GIALELI, C., THEOCHARIS, A. D. & KARAMANOS, N. K. 2011. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. *Febs j,* 278**,** 16-27.

GODWIN, P., BAIRD, A. M., HEAVEY, S., BARR, M. P., O'BYRNE, K. J. & GATELY, K. 2013. Targeting nuclear factor-kappa B to overcome resistance to chemotherapy. *Front Oncol,* 3**,** 120.

GOLDKORN, A., ELY, B., QUINN, D. I., TANGEN, C. M., FINK, L. M., XU, T., TWARDOWSKI, P., VAN VELDHUIZEN, P. J., AGARWAL, N., CARDUCCI, M. A., MONK, J. P., 3RD, DATAR, R. H., GARZOTTO, M., MACK, P. C., LARA, P., JR., HIGANO, C. S., HUSSAIN, M., THOMPSON, I. M., JR., COTE, R. J. & VOGELZANG, N. J. 2014. Circulating tumor cell counts are prognostic of overall survival in SWOG S0421: a phase III trial of docetaxel with or without atrasentan for metastatic castration-resistant prostate cancer. *J Clin Oncol,* 32**,** 1136-42.

GOLDSTEIN, R., HANLEY, C., MORRIS, J., CAHILL, D., CHANDRA, A., HARPER, P., CHOWDHURY, S., MAHER, J. & BURBRIDGE, S. 2011. Clinical Investigation of the Role of Interleukin-4 and Interleukin-13 in the Evolution of Prostate Cancer. *Cancers (Basel),* 3**,** 4281-93.

GOMES, M., TEIXEIRA, A. L., COELHO, A., ARAUJO, A. & MEDEIROS, R. 2014. The role of inflammation in lung cancer. *Adv Exp Med Biol,* 816**,** 1-23.

GONG, W. J., ZHENG, W., XIAO, L., TAN, L. M., SONG, J., LI, X. P., XIAO, D., CUI, J. J., LI, X., ZHOU, H. H., YIN, J. Y. & LIU, Z. Q. 2016. Circulating resistin levels and obesity-related cancer risk: A meta-analysis. *Oncotarget,* 7**,** 57694-704.

GONZALEZ-MEJIA, M. E., PORCHIA, L. M., TORRES-RASGADO, E., RUIZ-VIVANCO, G., PULIDO-PEREZ, P., BAEZ-DUARTE, B. G. & PEREZ-FUENTES, R. 2016. C-Peptide Is a Sensitive Indicator for the Diagnosis of Metabolic Syndrome in Subjects from Central Mexico. *Metab Syndr Relat Disord,* 14**,** 210-6.

GORDETSKY, J. & EPSTEIN, J. 2016. Grading of prostatic adenocarcinoma: current state and prognostic implications. *Diagn Pathol,* 11**,** 25.

GORGES, T. M. & PANTEL, K. 2013. Circulating tumor cells as therapy-related biomarkers in cancer patients. *Cancer Immunol Immunother,* 62**,** 931-9.

GREENE, G. F., KITADAI, Y., PETTAWAY, C. A., VON ESCHENBACH, A. C., BUCANA, C. D. & FIDLER, I. J. 1997. Correlation of metastasis-related gene expression with metastatic potential in human prostate carcinoma cells implanted in nude mice using an in situ messenger RNA hybridization technique. *Am J Pathol,* 150**,** 1571-82.

GREENE, K. L., ALBERTSEN, P. C., BABAIAN, R. J., CARTER, H. B., GANN, P. H., HAN, M., KUBAN, D. A., SARTOR, A. O., STANFORD, J. L., ZIETMAN, A. & CARROLL, P. 2013. Prostate specific antigen best practice statement: 2009 update. *J Urol,* 189**,** S2-s11.

GRIVENNIKOV, S., KARIN, E., TERZIC, J., MUCIDA, D., YU, G. Y., VALLABHAPURAPU, S., SCHELLER, J., ROSE-JOHN, S., CHEROUTRE, H., ECKMANN, L. & KARIN, M. 2009. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell,* 15**,** 103-13.

GRIVENNIKOV, S. I. 2010. Immunity, Inflammation, and Cancer. 140**,** 883-99.

GROTTA, A., BOTTAI, M., ADAMI, H. O., ADAMS, S. A., AKRE, O., BLAIR, S. N., MARIOSA, D., NYREN, O., YE, W., STATTIN, P., BELLOCCO, R. & TROLLE LAGERROS, Y. 2015. Physical activity and body mass index as predictors of prostate cancer risk. *World J Urol,* 33**,** 1495-502.

GURBEL, P. A., BLIDEN, K. P., DICHIARA, J., NEWCOMER, J., WENG, W., NEERCHAL, N. K., GESHEFF, T., CHAGANTI, S. K., ETHERINGTON, A. & TANTRY, U. S. 2007. Evaluation of dose-related effects of aspirin on platelet function: results from the Aspirin-Induced Platelet Effect (ASPECT) study. *Circulation,* 115**,** 3156-64.

HÄGGLÖF, C., HAMMARSTEN, P., STRÖMVALL, K., EGEVAD, L., JOSEFSSON, A., STATTIN, P., GRANFORS, T. & BERGH, A. 2014. TMPRSS2-ERG Expression Predicts Prostate Cancer Survival and Associates with Stromal Biomarkers. *PLoS ONE,* 9**,** e86824.

HAN, T. S., WILLIAMS, K., SATTAR, N., HUNT, K. J., LEAN, M. E. & HAFFNER, S. M. 2002. Analysis of obesity and hyperinsulinemia in the development of metabolic syndrome: San Antonio Heart Study. *Obes Res,* 10**,** 923-31.

HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of cancer: the next generation. *Cell,* 144**,** 646-74.

HAUNER, H. 2005. Secretory factors from human adipose tissue and their functional role. *Proc Nutr Soc,* 64**,** 163-9.

HAYES, D. F. & PAOLETTI, C. 2013. Circulating tumour cells: insights into tumour heterogeneity. *J Intern Med,* 274**,** 137-43.

HIMBERT, C., DELPHAN, M., SCHERER, D., BOWERS, L. W., HURSTING, S. & ULRICH, C. M. 2017. Signals from the Adipose Microenvironment and the Obesity-Cancer Link-A Systematic Review. *Cancer Prev Res (Phila),* 10**,** 494-506.

HO, S. S., DHALIWAL, S. S., HILLS, A. P. & PAL, S. 2013. Effects of chronic exercise training on inflammatory markers in Australian overweight and obese individuals in a randomized controlled trial. *Inflammation,* 36**,** 625-32.

HODA, M. R., THEIL, G., MOHAMMED, N., FISCHER, K. & FORNARA, P. 2012. The adipocyte-derived hormone leptin has proliferative actions on androgen-resistant prostate cancer cells linking obesity to advanced stages of prostate cancer. *J Oncol,* 2012**,** 280386.

HOON KIM, J., LEE, S. Y., MYUNG, S. C., KIM, Y. S., KIM, T. H. & KIM, M. K. 2008. Clinical significance of the leptin and leptin receptor expressions in prostate tissues. *Asian J Androl,* 10**,** 923-8.

HPFS. 2016. *Health Professionals Follow up Study. About the Study* [Online]. Available: https://www.hsph.harvard.edu/hpfs/hpfs_about.htm [Accessed 14-07-2017].

HRUBY, A. & HU, F. B. 2015. The Epidemiology of Obesity: A Big Picture. *Pharmacoeconomics,* 33**,** 673-89.

HSING, A. W., SAKODA, L. C. & CHUA, S., JR. 2007. Obesity, metabolic syndrome, and prostate cancer. *Am J Clin Nutr,* 86**,** s843-57.

HU, F. B., LI, T. Y., COLDITZ, G. A., WILLETT, W. C. & MANSON, J. E. 2003. Television watching and other sedentary behaviors in relation to risk of obesity and type 2 diabetes mellitus in women. *Jama,* 289**,** 1785-91.

HU, M. B., XU, H., HU, J. M., ZHU, W. H., YANG, T., JIANG, H. W. & DING, Q. 2016. Genetic polymorphisms in leptin, adiponectin and their receptors affect risk and aggressiveness of prostate cancer: evidence from a meta-analysis and pooled-review. *Oncotarget,* 7**,** 81049-81061.

HUANG, K. C., DOLPH, M., DONNELLY, B. & BISMAR, T. A. 2014. ERG expression is associated with increased risk of biochemical relapse following radical prostatectomy in early onset prostate cancer. *Clin Transl Oncol,* 16**,** 973-9.

INOUE, K., SLATON, J. W., EVE, B. Y., KIM, S. J., PERROTTE, P., BALBAY, M. D., YANO, S., BAR-ELI, M., RADINSKY, R., PETTAWAY, C. A. & DINNEY, C. P. 2000. Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. *Clin Cancer Res,* 6**,** 2104-19.

IRWIN, M. L., MCTIERNAN, A., BERNSTEIN, L., GILLILAND, F. D., BAUMGARTNER, R., BAUMGARTNER, K. & BALLARD-BARBASH, R. 2005. Relationship of obesity

and physical activity with c-peptide, leptin, and insulin-like growth factors in breast cancer survivors. *Cancer Epidemiol Biomarkers Prev,* 14**,** 2881-8.
ITO, Y., ISHIGURO, H., KOBAYASHI, N., HASUMI, H., WATANABE, M., YAO, M. & UEMURA, H. 2015. Adipocyte-derived monocyte chemotactic protein-1 (MCP-1) promotes prostate cancer progression through the induction of MMP-2 activity. *Prostate,* 75**,** 1009-19.
IUNA 2011. National Adult Nutrition Survey: Food and Nutrient Intakes, Physical Measurements, Physical Activity Patterns and Food Choice Motives.
JANAKIRAM, N. B. & RAO, C. V. 2014. The role of inflammation in colon cancer. *Adv Exp Med Biol,* 816**,** 25-52.
JIN, J. K., DAYYANI, F. & GALLICK, G. E. 2011. Steps in prostate cancer progression that lead to bone metastasis. *Int J Cancer,* 128**,** 2545-61.
JONES, A. G. & HATTERSLEY, A. T. 2013. The clinical utility of C-peptide measurement in the care of patients with diabetes. *Diabet Med,* 30**,** 803-17.
JONES, S. B. 2013. Effect of Exercise on Markers of Inflammation in Breast Cancer Survivors: The Yale Exercise and Survivorship Study. 6.
JOURDAN, M., VEYRUNE, J. L., VOS, J. D., REDAL, N., COUDERC, G. & KLEIN, B. 2003. A major role for Mcl-1 antiapoptotic protein in the IL-6-induced survival of human myeloma cells. *Oncogene,* 22**,** 2950-9.
KADOWAKI, T. 2006. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. 116**,** 1784-92.
KANG, M., BYUN, S. S., LEE, S. E. & HONG, S. K. 2018. Clinical Significance of Serum Adipokines according to Body Mass Index in Patients with Clinically Localized Prostate Cancer Undergoing Radical Prostatectomy. *World J Mens Health,* 36**,** 57-65.
KANG, Z., JANNE, O. A. & PALVIMO, J. J. 2004. Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor. *Mol Endocrinol,* 18**,** 2633-48.
KAPLAN, R. N., RIBA, R. D., ZACHAROULIS, S., BRAMLEY, A. H., VINCENT, L., COSTA, C., MACDONALD, D. D., JIN, D. K., SHIDO, K., KERNS, S. A., ZHU, Z., HICKLIN, D., WU, Y., PORT, J. L., ALTORKI, N., PORT, E. R., RUGGERO, D., SHMELKOV, S. V., JENSEN, K. K., RAFII, S. & LYDEN, D. 2005. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature,* 438**,** 820-7.
KARAN, D. & DUBEY, S. 2016. From Inflammation to Prostate Cancer: The Role of Inflammasomes. *Adv Urol,* 2016**,** 3140372.
KARIN, M. 2006. Nuclear factor-kappaB in cancer development and progression. *Nature,* 441**,** 431-6.
KARIN, M. 2009. NF-κB as a Critical Link Between Inflammation and Cancer. *Cold Spring Harb Perspect Biol,* 1.
KEAVER, L., WEBBER, L., DEE, A., SHIELY, F., MARSH, T., BALANDA, K. & PERRY, I. J. 2013. Application of the UK foresight obesity model in Ireland: the health and economic consequences of projected obesity trends in Ireland. *PLoS One,* 8**,** e79827.
KENFIELD, S. A., STAMPFER, M. J., GIOVANNUCCI, E. & CHAN, J. M. 2011. Physical activity and survival after prostate cancer diagnosis in the health professionals follow-up study. *J Clin Oncol,* 29**,** 726-32.
KIM, H. J., LEE, Y. S., WON, E. H., CHANG, I. H., KIM, T. H., PARK, E. S., KIM, M. K., KIM, W. & MYUNG, S. C. 2011. Expression of resistin in the prostate and its stimulatory effect on prostate cancer cell proliferation. *BJU Int,* 108**,** E77-83.
KITAHARA, C. M., FLINT, A. J., BERRINGTON DE GONZALEZ, A., BERNSTEIN, L., BROTZMAN, M., MACINNIS, R. J., MOORE, S. C., ROBIEN, K., ROSENBERG, P. S., SINGH, P. N., WEIDERPASS, E., ADAMI, H. O., ANTON-CULVER, H.,

BALLARD-BARBASH, R., BURING, J. E., FREEDMAN, D. M., FRASER, G. E., BEANE FREEMAN, L. E., GAPSTUR, S. M., GAZIANO, J. M., GILES, G. G., HAKANSSON, N., HOPPIN, J. A., HU, F. B., KOENIG, K., LINET, M. S., PARK, Y., PATEL, A. V., PURDUE, M. P., SCHAIRER, C., SESSO, H. D., VISVANATHAN, K., WHITE, E., WOLK, A., ZELENIUCH-JACQUOTTE, A. & HARTGE, P. 2014. Association between class III obesity (BMI of 40-59 kg/m2) and mortality: a pooled analysis of 20 prospective studies. *PLoS Med,* 11**,** e1001673.

KLOTZ, L. 2005. Active surveillance for prostate cancer: for whom? *J Clin Oncol,* 23**,** 8165-9.

KOELWYN, G. J., WENNERBERG, E., DEMARIA, S. & JONES, L. W. 2015. Exercise in Regulation of Inflammation-Immune Axis Function in Cancer Initiation and Progression. *Oncology (Williston Park),* 29.

KONIG, A., VILSMAIER, T., RACK, B., FRIESE, K., JANNI, W., JESCHKE, U., ANDERGASSEN, U., TRAPP, E., JUCKSTOCK, J., JAGER, B., ALUNNI-FABBRONI, M., FRIEDL, T. & WEISSENBACHER, T. 2016. Determination of Interleukin-4, -5, -6, -8 and -13 in Serum of Patients with Breast Cancer Before Treatment and its Correlation to Circulating Tumor Cells. *Anticancer Res,* 36**,** 3123-30.

KORNILUK, A., KOPER, O., KEMONA, H. & DYMICKA-PIEKARSKA, V. 2017. From inflammation to cancer. *Irish Journal of Medical Science (1971 -),* 186**,** 57-62.

KORPAL, M., KORN, J. M., GAO, X., RAKIEC, D. P., RUDDY, D. A., DOSHI, S., YUAN, J., KOVATS, S. G., KIM, S., COOKE, V. G., MONAHAN, J. E., STEGMEIER, F., ROBERTS, T. M., SELLERS, W. R., ZHOU, W. & ZHU, P. 2013. An F876L mutation in androgen receptor confers genetic and phenotypic resistance to MDV3100 (enzalutamide). *Cancer Discov,* 3**,** 1030-43.

KREGEL, S., CHEN, J. L., TOM, W., KRISHNAN, V., KACH, J., BRECHKA, H., FESSENDEN, T. B., ISIKBAY, M., PANER, G. P., SZMULEWITZ, R. Z. & VANDER GRIEND, D. J. 2016. Acquired resistance to the second-generation androgen receptor antagonist enzalutamide in castration-resistant prostate cancer. *Oncotarget,* 7**,** 26259-74.

KRIEGER, J. N., LEE, S. W. H., JEON, J., CHEAH, P. Y., LIONG, M. L. & RILEY, D. E. 2008. Epidemiology of prostatitis. *Int J Antimicrob Agents,* 31**,** S85-90.

KRUSPE, S., DICKEY, D. D., URAK, K. T., BLANCO, G. N., MILLER, M. J., CLARK, K. C., BURGHARDT, E., GUTIERREZ, W. R., PHADKE, S. D., KAMBOJ, S., GINADER, T., SMITH, B. J., GRIMM, S. K., SCHAPPET, J., OZER, H., THOMAS, A., MCNAMARA, J. O., 2ND, CHAN, C. H. & GIANGRANDE, P. H. 2017. Rapid and Sensitive Detection of Breast Cancer Cells in Patient Blood with Nuclease-Activated Probe Technology. *Mol Ther Nucleic Acids,* 8**,** 542-557.

KUMAR-SINHA, C., TOMLINS, S. A. & CHINNAIYAN, A. M. 2008. Recurrent gene fusions in prostate cancer. *Nat Rev Cancer,* 8**,** 497-511.

KUSKE, A., GORGES, T. M., TENNSTEDT, P., TIEBEL, A. K., POMPE, R., PREISSER, F., PRUES, S., MAZEL, M., MARKOU, A., LIANIDOU, E., PEINE, S., ALIX-PANABIERES, C., RIETHDORF, S., BEYER, B., SCHLOMM, T. & PANTEL, K. 2016. Improved detection of circulating tumor cells in non-metastatic high-risk prostate cancer patients. *Sci Rep,* 6**,** 39736.

LABELLE, M., BEGUM, S. & HYNES, R. O. 2011. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer Cell,* 20**,** 576-90.

LAI, G. Y., GIOVANNUCCI, E. L., POLLAK, M. N., PESKOE, S. B., STAMPFER, M. J., WILLETT, W. C. & PLATZ, E. A. 2014. Association of C-peptide and leptin with prostate cancer incidence in the Health Professionals Follow-up Study. *Cancer Causes Control,* 25**,** 625-32.

LANDSKRON, G., DE LA FUENTE, M., THUWAJIT, P., THUWAJIT, C. & HERMOSO, M. A. 2014. Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res,* 2014**,** 149185.

LARSEN, S. B., BRASSO, K., IVERSEN, P., CHRISTENSEN, J., CHRISTIANSEN, M., CARLSSON, S., LILJA, H., FRIIS, S., TJØNNELAND, A. & DALTON, S. O. 2013. Baseline PSA measurements and subsequent prostate cancer risk in the Danish Diet, Cancer and Health cohort. *Eur J Cancer,* 49**,** 3041-8.

LAZENNEC, G. & RICHMOND, A. 2010. Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol Med,* 16**,** 133-44.

LEE, S., LEE, H. C., KWON, Y. W., LEE, S. E., CHO, Y., KIM, J., LEE, S., KIM, J. Y., LEE, J., YANG, H. M., MOOK-JUNG, I., NAM, K. Y., CHUNG, J., LAZAR, M. A. & KIM, H. S. 2014. Adenylyl cyclase-associated protein 1 is a receptor for human resistin and mediates inflammatory actions of human monocytes. *Cell Metab,* 19**,** 484-97.

LEVINE, T. B., LEVINE, A.B. 2013. *Metabolic Syndrome and Cardiovascular Disease*, Wiley-Blackwell.

LI, H., STAMPFER, M. J., MUCCI, L., RIFAI, N., QIU, W., KURTH, T. & MA, J. 2010. A 25-year prospective study of plasma adiponectin and leptin concentrations and prostate cancer risk and survival. *Clin Chem,* 56**,** 34-43.

LI, Y., CHAN, S. C., BRAND, L. J., HWANG, T. H., SILVERSTEIN, K. A. & DEHM, S. M. 2013. Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines. *Cancer Res,* 73**,** 483-9.

LI, Y. C., ZOU, J. M., LUO, C., SHU, Y., LUO, J., QIN, J., WANG, Y., LI, D., WANG, S. S., CHI, G., GUO, F., ZHANG, G. M. & FENG, Z. H. 2017. Circulating tumor cells promote the metastatic colonization of disseminated carcinoma cells by inducing systemic inflammation. *Oncotarget,* 8**,** 28418-30.

LIGTHART, S. T., COUMANS, F. A., BIDARD, F. C., SIMKENS, L. H., PUNT, C. J., DE GROOT, M. R., ATTARD, G., DE BONO, J. S., PIERGA, J. Y. & TERSTAPPEN, L. W. 2013. Circulating Tumor Cells Count and Morphological Features in Breast, Colorectal and Prostate Cancer. *PLoS One,* 8**,** e67148.

LIHN, A. S., PEDERSEN, S. B. & RICHELSEN, B. 2005. Adiponectin: action, regulation and association to insulin sensitivity. *Obes Rev,* 6**,** 13-21.

LILJA, H., ULMERT, D. & VICKERS, A. J. 2008. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. *Nat Rev Cancer,* 8**,** 268-78.

LIN, G., YANG, R., BANIE, L., WANG, G., NING, H., LI, L. C., LUE, T. F. & LIN, C. S. 2010. Effects of transplantation of adipose tissue-derived stem cells on prostate tumor. *Prostate,* 70**,** 1066-73.

LIU, Q., RUSSELL, M. R., SHAHRIARI, K., JERNIGAN, D. L., LIONI, M. I., GARCIA, F. U. & FATATIS, A. 2013. Interleukin-1beta promotes skeletal colonization and progression of metastatic prostate cancer cells with neuroendocrine features. *Cancer Res,* 73**,** 3297-305.

LIU, T., ZHANG, L., JOO, D. & SUN, S. C. 2017. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther,* 2.

LIU, Y., ZHAO, X., SUN, X., LI, Y., WANG, Z., JIANG, J., HAN, H., SHEN, W., CORRIGAN, C. J. & SUN, Y. 2015. Expression of IL-17A, E, and F and their receptors in human prostatic cancer: Comparison with benign prostatic hyperplasia. *Prostate,* 75**,** 1844-56.

LOEB, S., BJURLIN, M., NICHOLSON, J., TAMMELA, T. L., PENSON, D., CARTER, H. B., CARROLL, P. & ETZIONI, R. 2014. Overdiagnosis and Overtreatment of Prostate Cancer. *Eur Urol,* 65**,** 1046-55.

LONERGAN, P. E. & TINDALL, D. J. 2011. Androgen receptor signaling in prostate cancer development and progression. *J Carcinog,* 10**,** 20.

LOPEZ FONTANA, C. M., MASELLI, M. E., PEREZ ELIZALDE, R. F., DI MILTA MONACO, N. A., UVILLA RECUPERO, A. L. & LOPEZ LAUR, J. D. 2011. Leptin increases prostate cancer aggressiveness. *J Physiol Biochem,* 67**,** 531-8.

LORENTE, D., OLMOS, D., MATEO, J., BIANCHINI, D., SEED, G., FLEISHER, M., DANILA, D. C., FLOHR, P., CRESPO, M., FIGUEIREDO, I., MIRANDA, S., BAETEN, K., MOLINA, A., KHEOH, T., MCCORMACK, R., TERSTAPPEN, L. W., SCHER, H. I. & DE BONO, J. S. 2016. Decline in Circulating Tumor Cell Count and Treatment Outcome in Advanced Prostate Cancer. *Eur Urol,* 70**,** 985-992.

LOU, X. L., SUN, J., GONG, S. Q., YU, X. F., GONG, R. & DENG, H. 2015. Interaction between circulating cancer cells and platelets: clinical implication. *Chin J Cancer Res,* 27**,** 450-60.

LU, S. H., TSAI, W. S., CHANG, Y. H., CHOU, T. Y., PANG, S. T., LIN, P. H., TSAI, C. M. & CHANG, Y. C. 2016. Identifying cancer origin using circulating tumor cells. *Cancer Biol Ther,* 17**,** 430-8.

LUBKOWSKA, A., DOLEGOWSKA, B. & BANFI, G. 2012. Growth factor content in PRP and their applicability in medicine. *J Biol Regul Homeost Agents,* 26**,** 3s-22s.

LUBKOWSKA, A., RADECKA, A., BRYCZKOWSKA, I., ROTTER, I., LASZCZYŃSKA, M. & DUDZIŃSKA, W. 2015. Serum Adiponectin and Leptin Concentrations in Relation to Body Fat Distribution, Hematological Indices and Lipid Profile in Humans. *Int J Environ Res Public Health,* 12**,** 11528-48.

MA, G., REN, Y., WANG, K. & HE, J. 2011. SRC-3 Has a Role in Cancer Other Than as a Nuclear Receptor Coactivator. *Int J Biol Sci,* 7**,** 664-72.

MAAS, M., HEGEMANN, M., RAUSCH, S., BEDKE, J., STENZL, A. & TODENHOFER, T. 2017. Circulating tumor cells and their role in prostate cancer. *Asian J Androl*.

MAEDA, N., SHIMOMURA, I., KISHIDA, K., NISHIZAWA, H., MATSUDA, M., NAGARETANI, H., FURUYAMA, N., KONDO, H., TAKAHASHI, M., ARITA, Y., KOMURO, R., OUCHI, N., KIHARA, S., TOCHINO, Y., OKUTOMI, K., HORIE, M., TAKEDA, S., AOYAMA, T., FUNAHASHI, T. & MATSUZAWA, Y. 2002. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med,* 8**,** 731-7.

MAERTENS, Y., HUMBERG, V., ERLMEIER, F., STEFFENS, S., STEINESTEL, J., BÖGEMANN, M., SCHRADER, A. J. & BERNEMANN, C. 2017. Comparison of isolation platforms for detection of circulating renal cell carcinoma cells. *Oncotarget,* 8**,** 87710-7.

MAHESWARAN, S. & HABER, D. A. 2010. Circulating tumor cells: a window into cancer biology and metastasis. *Curr Opin Genet Dev,* 20**,** 96-9.

MALO, E., UKKOLA, O., JOKELA, M., MOILANEN, L., KAHONEN, M., NIEMINEN, M. S., SALOMAA, V., JULA, A. & KESANIEMI, Y. A. 2011. Resistin is an indicator of the metabolic syndrome according to five different definitions in the Finnish Health 2000 survey. *Metab Syndr Relat Disord,* 9**,** 203-10.

MANI, R.-S. 2014. The emerging role of speckle-type POZ protein (SPOP) in cancer development. *Drug discovery today,* 19**,** 1498-1502.

MASSAGUE, J. & OBENAUF, A. C. 2016. Metastatic colonization by circulating tumour cells. *Nature,* 529**,** 298-306.

MATEO, J., BOYSEN, G., BARBIERI, C. E., BRYANT, H. E., CASTRO, E., NELSON, P. S., OLMOS, D., PRITCHARD, C. C., RUBIN, M. A. & DE BONO, J. S. 2017. DNA Repair in Prostate Cancer: Biology and Clinical Implications. *Eur Urol,* 71**,** 417-425.

MATTHEWS, C. E. 2008. Amount of Time Spent in Sedentary Behaviors in the United States, 2003–2004. 167**,** 875-81.

MCANENA, P., BROWN, J. A. & KERIN, M. J. 2017. Circulating Nucleosomes and Nucleosome Modifications as Biomarkers in Cancer. *Cancers (Basel),* 9.

MCCALL, P., WITTON, C. J., GRIMSLEY, S., NIELSEN, K. V. & EDWARDS, J. 2008. Is PTEN loss associated with clinical outcome measures in human prostate cancer? *Br J Cancer,* 99**,** 1296-301.

MCCRORY, R. L. A. C. 2011. Growing up in Ireland, National Longtitudinal Study of Children: Overweight and Obesity among 9-Year-Olds.

MEGO, M., GAO, H., COHEN, E. N., ANFOSSI, S., GIORDANO, A., TIN, S., FOUAD, T. M., DE GIORGI, U., GIULIANO, M., WOODWARD, W. A., ALVAREZ, R. H., VALERO, V., UENO, N. T., HORTOBAGYI, G. N., CRISTOFANILLI, M. & REUBEN, J. M. 2017. Circulating tumor cells (CTCs) are associated with abnormalities in peripheral blood dendritic cells in patients with inflammatory breast cancer. *Oncotarget,* 8**,** 35656-35668.

MEHNER, C., HOCKLA, A., MILLER, E., RAN, S., RADISKY, D. C. & RADISKY, E. S. 2014. Tumor cell-produced matrix metalloproteinase 9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer. *Oncotarget,* 5**,** 2736-49.

MEI, Z., LIU, Y., LIU, C., CUI, A., LIANG, Z., WANG, G., PENG, H., CUI, L. & LI, C. 2014. Tumour-infiltrating inflammation and prognosis in colorectal cancer: systematic review and meta-analysis. *Br J Cancer,* 110**,** 1595-605.

MENG, Y., LI, H., XU, P. & WANG, J. 2015. Do tumor volume, percent tumor volume predict biochemical recurrence after radical prostatectomy? A meta-analysis. *Int J Clin Exp Med,* 8**,** 22319-27.

MICHALAKI, V., SYRIGOS, K., CHARLES, P. & WAXMAN, J. 2004. Serum levels of IL-6 and TNF-alpha correlate with clinicopathological features and patient survival in patients with prostate cancer. *Br J Cancer,* 90**,** 2312-6.

MICHALAKIS, K., WILLIAMS, C. J., MITSIADES, N., BLAKEMAN, J., BALAFOUTA-TSELENIS, S., GIANNOPOULOS, A. & MANTZOROS, C. S. 2007. Serum Adiponectin Concentrations and Tissue Expression of Adiponectin Receptors Are Reduced in Patients with Prostate Cancer: A Case Control Study. *Cancer Epidemiology Biomarkers & Prevention,* 16**,** 308.

MIKOLAJCZYK, S. D., MILLAR, L. S., TSINBERG, P., COUTTS, S. M., ZOMORRODI, M., PHAM, T., BISCHOFF, F. Z. & PIRCHER, T. J. 2011. Detection of EpCAM-Negative and Cytokeratin-Negative Circulating Tumor Cells in Peripheral Blood. *J Oncol,* 2011**,** 252361.

MISTRY, T., DIGBY, J. E., DESAI, K. M. & RANDEVA, H. S. 2007. Obesity and prostate cancer: a role for adipokines. *Eur Urol,* 52**,** 46-53.

MOHAMED, A. A., TAN, S. H., SUN, C., SHAHEDUZZAMAN, S., HU, Y., PETROVICS, G., CHEN, Y., SESTERHENN, I. A., LI, H., SREENATH, T., MCLEOD, D. G., DOBI, A. & SRIVASTAVA, S. 2011. ERG oncogene modulates prostaglandin signaling in prostate cancer cells. *Cancer Biol Ther,* 11**,** 410-7.

MOJIMINIYI, O. A. & ABDELLA, N. A. 2007. Associations of resistin with inflammation and insulin resistance in patients with type 2 diabetes mellitus. *Scand J Clin Lab Invest,* 67**,** 215-25.

MOROTE, J., ROPERO, J., PLANAS, J., BASTAROS, J. M., DELGADO, G., PLACER, J., CELMA, A., DE TORRES, I. M., CARLES, J., REVENTOS, J. & DOLL, A. 2013. Metabolic syndrome increases the risk of aggressive prostate cancer detection. *BJU Int,* 111**,** 1031-6.

MORRELL, C. N., AGGREY, A. A., CHAPMAN, L. M. & MODJESKI, K. L. 2014. Emerging roles for platelets as immune and inflammatory cells. *Blood,* 123**,** 2759-67.

MOSCHINI, M., CARROLL, P. R., EGGENER, S. E., EPSTEIN, J. I., GRAEFEN, M., MONTIRONI, R. & PARKER, C. 2017. Low-risk Prostate Cancer: Identification, Management, and Outcomes. *Eur Urol*.

MU, Z., WANG, C., YE, Z., AUSTIN, L., CIVAN, J., HYSLOP, T., PALAZZO, J. P., JASLOW, R., LI, B., MYERS, R. E., JIANG, J., XING, J., YANG, H. & CRISTOFANILLI, M.

2015. Prospective assessment of the prognostic value of circulating tumor cells and their clusters in patients with advanced-stage breast cancer. *Breast Cancer Research and Treatment,* 154**,** 563-571.
MUNZ, M., BAEUERLE, P. A. & GIRES, O. 2009. The emerging role of EpCAM in cancer and stem cell signaling. *Cancer Res,* 69**,** 5627-9.
MURPHY, S. J., KARNES, R. J., KOSARI, F., CASTELLAR, B. E., KIPP, B. R., JOHNSON, S. H., TERRA, S., HARRIS, F. R., HALLING, G. C., KLEIN, J. L., NASIR, A., BERGSTRAHL, E., RANGEL, L. J., SUKOV, W. R., VASMATZIS, G. & CHEVILLE, J. C. 2016. Integrated analysis of the genomic instability of PTEN in clinically insignificant and significant prostate cancer. *Mod Pathol,* 29**,** 143-56.
NAKAMURA, K., FUSTER, J. J. & WALSH, K. 2014. Adipokines: a link between obesity and cardiovascular disease. *J Cardiol,* 63**,** 250-9.
NAKAZAWA, M. 2017. Mechanisms of Therapeutic Resistance in Prostate Cancer. 19**,** 13.
NAKAZAWA, M., PALLER, C. & KYPRIANOU, N. 2017. Mechanisms of Therapeutic Resistance in Prostate Cancer. *Curr Oncol Rep,* 19**,** 13.
NAM, R. K., SUGAR, L., YANG, W., SRIVASTAVA, S., KLOTZ, L. H., YANG, L., STANIMIROVIC, A., ENCIOIU, E., NEILL, M., LOBLAW, D. A., TRACHTENBERG, J., NAROD, S. A. & SETH, A. 2007. Expression of the TMPRSS2:ERG fusion gene predicts cancer recurrence after surgery for localised prostate cancer. *Br J Cancer,* 97**,** 1690-5.
NCI 2011. National Cancer Institute Treatment Choices for Men with Early-Stage Prostate Cancer.
NCRI 2017. National Cancer Registry Ireland Cancer Factsheet Prostate.
NELSON, W. G., DE MARZO, A. M. & ISAACS, W. B. 2003. Prostate cancer. *N Engl J Med,* 349**,** 366-81.
NEVEU, B., MOREEL, X., DESCHÊNES-ROMPRÉ, M. P., BERGERON, A., LARUE, H., AYARI, C., FRADET, Y. & FRADET, V. 2014. IL-8 secretion in primary cultures of prostate cells is associated with prostate cancer aggressiveness. *Res Rep Urol,* 6**,** 27-34.
NEWTON, R. U., KENFIELD, S. A., HART, N. H., CHAN, J. M., COURNEYA, K. S., CATTO, J., FINN, S. P., GREENWOOD, R., HUGHES, D. C., MUCCI, L., PLYMATE, S. R., PRAET, S. F. E., GUINAN, E. M., VAN BLARIGAN, E. L., CASEY, O., BUZZA, M., GLEDHILL, S., ZHANG, L., GALVAO, D. A., RYAN, C. J. & SAAD, F. 2018. Intense Exercise for Survival among Men with Metastatic Castrate-Resistant Prostate Cancer (INTERVAL-GAP4): a multicentre, randomised, controlled phase III study protocol. *BMJ Open,* 8**,** e022899.
NGUYEN, D. P., LI, J. & TEWARI, A. K. 2014. Inflammation and prostate cancer: the role of interleukin 6 (IL-6). *BJU Int,* 113**,** 986-92.
NICOLAZZO, C., RAIMONDI, C., FRANCESCANGELI, F., CECCARELLI, S., TRENTA, P., MAGRI, V., MARCHESE, C., ZEUNER, A., GRADILONE, A. & GAZZANIGA, P. 2017. EpCAM-expressing circulating tumor cells in colorectal cancer. *Int J Biol Markers,* 32**,** e415-e420.
NIEBAUER, J. & COOKE, J. P. 1996. Cardiovascular effects of exercise: role of endothelial shear stress. *J Am Coll Cardiol,* 28**,** 1652-60.
NIESWANDT, B., HAFNER, M., ECHTENACHER, B. & MANNEL, D. N. 1999. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. *Cancer Res,* 59**,** 1295-300.
NIH. 2018a. *Metabolic Syndrome* [Online]. Available: www.nhlbi.nih.gov/health-topics/metabolic-syndrome [Accessed 08-04-2018].
NIH 2018b. Physical Activity and Cancer.

NIJHUIS, J., RENSEN, S. S., SLAATS, Y., VAN DIELEN, F. M., BUURMAN, W. A. & GREVE, J. W. 2009. Neutrophil activation in morbid obesity, chronic activation of acute inflammation. *Obesity (Silver Spring),* 17**,** 2014-8.

NOBLE, M., RUSSELL, C., KRAEMER, L. & SHARRATT, M. 2012. UW WELL-FIT: the impact of supervised exercise programs on physical capacity and quality of life in individuals receiving treatment for cancer. *Support Care Cancer,* 20**,** 865-73.

NODA, T., KIKUGAWA, T., TANJI, N., MIURA, N., ASAI, S., HIGASHIYAMA, S. & YOKOYAMA, M. 2015. Longterm exposure to leptin enhances the growth of prostate cancer cells. *Int J Oncol,* 46**,** 1535-42.

NOH, B. J., SUNG, J. Y., KIM, Y. W., CHANG, S. G. & PARK, Y. K. 2016. Prognostic value of ERG, PTEN, CRISP3 and SPINK1 in predicting biochemical recurrence in prostate cancer. *Oncol Lett,* 11**,** 3621-3630.

NORTH, W. G. 2000. Gene regulation of vasopressin and vasopressin receptors in cancer. *Exp Physiol,* 85 Spec No**,** 27s-40s.

NPAGSG 2009. The National Guidelines on Physical Activity for Ireland.

O'SHANNESSY, D. J., DAVIS, D. W., ANDERES, K. & SOMERS, E. B. 2016. Isolation of Circulating Tumor Cells from Multiple Epithelial Cancers with ApoStream(®) for Detecting (or Monitoring) the Expression of Folate Receptor Alpha. *Biomark Insights,* 11**,** 7-18.

OH W.K., H. M., D'AMICO A. V., RICHIE J.P., AND KANTOFF P.W. 2003. Biology of Prostate Cancer. *In:* ROBERT A. WOLFF, J. A. A. D. B. E. (ed.) *Holland-Frei Cancer Medicince.* 6th ed. Hamilton: BC Decker Inc.

OLMOS, D., ARKENAU, H. T., ANG, J. E., LEDAKI, I., ATTARD, G., CARDEN, C. P., REID, A. H., A'HERN, R., FONG, P. C., OOMEN, N. B., MOLIFE, R., DEARNALEY, D., PARKER, C., TERSTAPPEN, L. W. & DE BONO, J. S. 2009. Circulating tumour cell (CTC) counts as intermediate end points in castration-resistant prostate cancer (CRPC): a single-centre experience. *Ann Oncol,* 20**,** 27-33.

ORGEL, E. & MITTELMAN, S. D. 2013. The Links Between Insulin Resistance, Diabetes, and Cancer. *Curr Diab Rep,* 13**,** 213-22.

OSORIO, C. F., SOUZA, D. B., GALLO, C. B., COSTA, W. S. & SAMPAIO, F. J. 2014. Leptin and leptin receptor expressions in prostate tumors may predict disease aggressiveness? *Acta Cir Bras,* 29 Suppl 3**,** 44-8.

OWEN, J. L., CRISCITIELLO, M. F., LIBREROS, S., GARCIA-AREAS, R., GUTHRIE, K., TORROELLA-KOURI, M. & IRAGAVARAPU-CHARYULU, V. 2011. Expression of the inflammatory chemokines CCL2, CCL5 and CXCL2 and the receptors CCR1–3 and CXCR2 in T lymphocytes from mammary tumor- bearing mice. *Cell Immunol,* 270**,** 172-82.

PAL, S. K., HE, M., CHEN, L., YANG, L., PILLAI, R., TWARDOWSKI, P., HSU, J., KORTYLEWSKI, M. & JONES, J. O. 2017. Synaptophysin expression on circulating tumor cells in patients with castration resistant prostate cancer undergoing treatment with abiraterone acetate or enzalutamide. *Urol Oncol*.

PALLER, C. J. & ANTONARAKIS, E. S. 2013. Management of Biochemically Recurrent Prostate Cancer After Local Therapy: Evolving Standards of Care and New Directions. *Clin Adv Hematol Oncol,* 11**,** 14-23.

PALUMBO, J. S., TALMAGE, K. E., MASSARI, J. V., LA JEUNESSE, C. M., FLICK, M. J., KOMBRINCK, K. W., JIROUSKOVA, M. & DEGEN, J. L. 2005. Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. *Blood,* 105**,** 178-85.

PARK, H. K. & AHIMA, R. S. 2013. Resistin in rodents and humans. *Diabetes Metab J,* 37**,** 404-14.

PARK, H. K. & AHIMA, R. S. 2014. Leptin signaling. *F1000Prime Rep,* 6.

PARK, S., ANG, R. R., DUFFY, S. P., BAZOV, J., CHI, K. N., BLACK, P. C. & MA, H. 2014. Morphological differences between circulating tumor cells from prostate cancer patients and cultured prostate cancer cells. *PLoS One,* 9**,** e85264.

PATEL, L., BUCKELS, A. C., KINGHORN, I. J., MURDOCK, P. R., HOLBROOK, J. D., PLUMPTON, C., MACPHEE, C. H. & SMITH, S. A. 2003. Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators. *Biochem Biophys Res Commun,* 300**,** 472-6.

PERNAR, C. H., FALL, K., RIDER, J. R., MARKT, S. C., ADAMI, H. O., ANDERSSON, S. O., VALDIMARSDOTTIR, U., ANDREN, O. & MUCCI, L. A. 2017. A Walking Intervention Among Men With Prostate Cancer: A Pilot Study. *Clin Genitourin Cancer*.

PFITZENMAIER, J., VESSELLA, R., HIGANO, C. S., NOTEBOOM, J. L., WALLACE, D., JR. & COREY, E. 2003. Elevation of cytokine levels in cachectic patients with prostate carcinoma. *Cancer,* 97**,** 1211-6.

PHIN, S., MOORE, M. W. & COTTER, P. D. 2013. Genomic Rearrangements of PTEN in Prostate Cancer. *Front Oncol,* 3**,** 240.

PODOLAK, J., EILERS, K., NEWBY, T., SLOTTKE, R., TUCKER, E., OLSON, S. B., LUE, H. W., YOUNGREN, J., AGGARWAL, R., SMALL, E. J., GRAFF, J. N., ALUMKAL, J. J., BEER, T. M. & THOMAS, G. V. 2017. Androgen receptor amplification is concordant between circulating tumor cells and biopsies from men undergoing treatment for metastatic castration resistant prostate cancer. *Oncotarget,* 8**,** 71447-71455.

POIRIER, P., GILES, T. D., BRAY, G. A., HONG, Y., STERN, J. S., PI-SUNYER, F. X. & ECKEL, R. H. 2006. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss: an update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. *Circulation,* 113**,** 898-918.

PROCTOR, M. J., MCMILLAN, D. C., HORGAN, P. G., FLETCHER, C. D., TALWAR, D. & MORRISON, D. S. 2015. Systemic inflammation predicts all-cause mortality: a glasgow inflammation outcome study. *PLoS One,* 10**,** e0116206.

RAMALLAL, R., TOLEDO, E., MARTINEZ, J. A., SHIVAPPA, N., HEBERT, J. R., MARTINEZ-GONZALEZ, M. A. & RUIZ-CANELA, M. 2017. Inflammatory potential of diet, weight gain, and incidence of overweight/obesity: The SUN cohort. *Obesity (Silver Spring),* 25**,** 997-1005.

REGMI, S., FU, A. & LUO, K. Q. 2017. High Shear Stresses under Exercise Condition Destroy Circulating Tumor Cells in a Microfluidic System. *Sci Rep,* 7.

RIDER, J. R., FIORENTINO, M., KELLY, R., GERKE, T., JORDAHL, K., SINNOTT, J. A., GIOVANNUCCI, E. L., LODA, M., MUCCI, L. A. & FINN, S. 2015. Tumor expression of adiponectin receptor 2 and lethal prostate cancer. *Carcinogenesis,* 36**,** 639-47.

ROBERTS, E., COSSIGNY, D. A. & QUAN, G. M. 2013. The role of vascular endothelial growth factor in metastatic prostate cancer to the skeleton. *Prostate Cancer,* 2013**,** 418340.

ROBINSON, D., VAN ALLEN, E. M., WU, Y. M., SCHULTZ, N., LONIGRO, R. J., MOSQUERA, J. M., MONTGOMERY, B., TAPLIN, M. E., PRITCHARD, C. C., ATTARD, G., BELTRAN, H., ABIDA, W., BRADLEY, R. K., VINSON, J., CAO, X., VATS, P., KUNJU, L. P., HUSSAIN, M., FENG, F. Y., TOMLINS, S. A., COONEY, K. A., SMITH, D. C., BRENNAN, C., SIDDIQUI, J., MEHRA, R., CHEN, Y., RATHKOPF, D. E., MORRIS, M. J., SOLOMON, S. B., DURACK, J. C., REUTER, V. E., GOPALAN, A., GAO, J., LODA, M., LIS, R. T., BOWDEN, M., BALK, S. P., GAVIOLA, G., SOUGNEZ, C., GUPTA, M., YU, E. Y., MOSTAGHEL, E. A., CHENG, H. H., MULCAHY, H., TRUE, L. D., PLYMATE, S. R., DVINGE, H., FERRALDESCHI,

R., FLOHR, P., MIRANDA, S., ZAFEIRIOU, Z., TUNARIU, N., MATEO, J., PEREZ-LOPEZ, R., DEMICHELIS, F., ROBINSON, B. D., SBONER, A., SCHIFFMAN, M., NANUS, D. M., TAGAWA, S. T., SIGARAS, A., ENG, K. W., ELEMENTO, O., SBONER, A., HEATH, E. I., SCHER, H. I., PIENTA, K. J., KANTOFF, P., DE BONO, J. S., RUBIN, M. A., NELSON, P. S., GARRAWAY, L. A., SAWYERS, C. L. & CHINNAIYAN, A. M. 2015. Integrative Clinical Genomics of Advanced Prostate Cancer. *Cell,* 162**,** 454.

ROCA, H., CRAIG, M. J., YING, C., VARSOS, Z. S., CZARNIESKI, P., ALVA, A. S., HERNANDEZ, J., FULLER, D., DAIGNAULT, S., HEALY, P. N. & PIENTA, K. J. 2012. IL-4 induces proliferation in prostate cancer PC3 cells under nutrient-depletion stress through the activation of the JNK-pathway and survivin up-regulation. *J Cell Biochem,* 113**,** 1569-80.

ROCA, H., JONES, J. D., PURICA, M. C., WEIDNER, S., KOH, A. J., KUO, R., WILKINSON, J. E., WANG, Y., DAIGNAULT-NEWTON, S., PIENTA, K. J., MORGAN, T. M., KELLER, E. T., NOR, J. E., SHEA, L. D. & MCCAULEY, L. K. 2018. Apoptosis-induced CXCL5 accelerates inflammation and growth of prostate tumor metastases in bone. *J Clin Invest,* 128**,** 248-266.

ROCA, H. & MCCAULEY, L. K. 2015. Inflammation and skeletal metastasis. *Bonekey Rep,* 4**,** 706.

RODRIGUEZ-HERNANDEZ, H., SIMENTAL-MENDIA, L. E., RODRIGUEZ-RAMIREZ, G. & REYES-ROMERO, M. A. 2013. Obesity and inflammation: epidemiology, risk factors, and markers of inflammation. *Int J Endocrinol,* 2013**,** 678159.

RODRIGUEZ-VIDA, A., GALAZI, M., RUDMAN, S., CHOWDHURY, S. & STERNBERG, C. N. 2015. Enzalutamide for the treatment of metastatic castration-resistant prostate cancer. *Drug Des Devel Ther,* 9**,** 3325-39.

RODRIGUEZ, C., FREEDLAND, S. J., DEKA, A., JACOBS, E. J., MCCULLOUGH, M. L., PATEL, A. V., THUN, M. J. & CALLE, E. E. 2007. Body mass index, weight change, and risk of prostate cancer in the Cancer Prevention Study II Nutrition Cohort. *Cancer Epidemiol Biomarkers Prev,* 16**,** 63-9.

ROEHRBORN, C. G. 2005. Benign prostatic hyperplasia: an overview. *Rev Urol,* 7 Suppl 9**,** S3-s14.

ROGERS, L. Q., FOGLEMAN, A., TRAMMELL, R., HOPKINS-PRICE, P., VICARI, S., RAO, K., EDSON, B., VERHULST, S., COURNEYA, K. S. & HOELZER, K. 2013. Effects of a physical activity behavior change intervention on inflammation and related health outcomes in breast cancer survivors: pilot randomized trial. *Integr Cancer Ther,* 12**,** 323-35.

ROJAS, A., LIU, G., COLEMAN, I., NELSON, P. S., ZHANG, M., DASH, R., FISHER, P. B., PLYMATE, S. R. & WU, J. D. 2011. IL-6 promotes prostate tumorigenesis and progression through autocrine cross-activation of IGF-IR. *Oncogene,* 30**,** 2345-55.

ROOMI, M. W., MONTERREY, J. C., KALINOVSKY, T., RATH, M. & NIEDZWIECKI, A. 2009. Patterns of MMP-2 and MMP-9 expression in human cancer cell lines. *Oncol Rep,* 21**,** 1323-33.

RUBIN, M. A. 2012. ETS rearrangements in prostate cancer. *Asian J Androl,* 14**,** 393-9.

SAHIBZADA, H. A., KHURSHID, Z., KHAN, R. S., NASEEM, M., SIDDIQUE, K. M., MALI, M. & ZAFAR, M. S. 2017. Salivary IL-8, IL-6 and TNF-alpha as Potential Diagnostic Biomarkers for Oral Cancer. *Diagnostics (Basel),* 7.

SAINI, S. 2016. PSA and beyond: alternative prostate cancer biomarkers. *Cell Oncol (Dordr),* 39**,** 97-106.

SARIOGLU, A. F., ACETO, N., KOJIC, N., DONALDSON, M. C., ZEINALI, M., HAMZA, B., ENGSTROM, A., ZHU, H., SUNDARESAN, T. K., MIYAMOTO, D. T., LUO, X., BARDIA, A., WITTNER, B. S., RAMASWAMY, S., SHIODA, T., TING, D. T., STOTT, S. L., KAPUR, R., MAHESWARAN, S., HABER, D. A. & TONER, M. 2015. A

microfluidic device for label-free, physical capture of circulating tumor cell clusters. *Nat Methods,* 12**,** 685-91.

SCHER, H. I., FIZAZI, K., SAAD, F., TAPLIN, M. E., STERNBERG, C. N., MILLER, K., DE WIT, R., MULDERS, P., CHI, K. N., SHORE, N. D., ARMSTRONG, A. J., FLAIG, T. W., FLECHON, A., MAINWARING, P., FLEMING, M., HAINSWORTH, J. D., HIRMAND, M., SELBY, B., SEELY, L. & DE BONO, J. S. 2012. Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med,* 367**,** 1187-97.

SFANOS, K. S. & DE MARZO, A. M. 2012. Prostate cancer and inflammation: the evidence. *Histopathology,* 60**,** 199-215.

SHAFIQUE, K., PROCTOR, M. J., MCMILLAN, D. C., QURESHI, K., LEUNG, H. & MORRISON, D. S. 2012. Systemic inflammation and survival of patients with prostate cancer: evidence from the Glasgow Inflammation Outcome Study. *Prostate Cancer Prostatic Dis,* 15**,** 195-201.

SHAH, S. P., MORIN, R. D., KHATTRA, J., PRENTICE, L., PUGH, T., BURLEIGH, A., DELANEY, A., GELMON, K., GULIANY, R., SENZ, J., STEIDL, C., HOLT, R. A., JONES, S., SUN, M., LEUNG, G., MOORE, R., SEVERSON, T., TAYLOR, G. A., TESCHENDORFF, A. E., TSE, K., TURASHVILI, G., VARHOL, R., WARREN, R. L., WATSON, P., ZHAO, Y., CALDAS, C., HUNTSMAN, D., HIRST, M., MARRA, M. A. & APARICIO, S. 2009. Mutational evolution in a lobular breast tumour profiled at single nucleotide resolution. *Nature,* 461**,** 809-13.

SHEN, M. M. & ABATE-SHEN, C. 2010. Molecular genetics of prostate cancer: new prospects for old challenges. *Genes Dev,* 24**,** 1967-2000.

SHEPHARD, R. J. 2017. Physical Activity and Prostate Cancer: An Updated Review. *Sports Med,* 47**,** 1055-1073.

SHIAO, S. L., CHU, G. C.-Y. & CHUNG, L. W. K. 2015. Regulation of prostate cancer progression by the tumor microenvironment. *Cancer Letters,* 380**,** 340-348.

SHINKO, D., DIAKOS, C. I., CLARKE, S. J. & CHARLES, K. A. 2017. Cancer-related systemic inflammation: The challenges and therapeutic opportunities for personalized medicine. *Clin Pharmacol Ther*.

SHOAG, J. & BARBIERI, C. E. 2016. Clinical variability and molecular heterogeneity in prostate cancer. *Asian J Androl,* 18**,** 543-8.

SILVA, F. M., DE ALMEIDA, J. C. & FEOLI, A. M. 2011. Effect of diet on adiponectin levels in blood. *Nutr Rev,* 69**,** 599-612.

SINHA, M. K., OPENTANOVA, I., OHANNESIAN, J. P., KOLACZYNSKI, J. W., HEIMAN, M. L., HALE, J., BECKER, G. W., BOWSHER, R. R., STEPHENS, T. W. & CARO, J. F. 1996. Evidence of free and bound leptin in human circulation. Studies in lean and obese subjects and during short-term fasting. *J Clin Invest,* 98**,** 1277-82.

SKERENOVA, M., MIKULOVA, V., CAPOUN, O., ZIMA, T. & TESAROVA, P. 2017. Circulating tumor cells and serum levels of MMP-2, MMP-9 and VEGF as markers of the metastatic process in patients with high risk of metastatic progression. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub,* 161**,** 272-280.

SMALL, E. J., SMITH, M. R., SEAMAN, J. J., PETRONE, S. & KOWALSKI, M. O. 2003. Combined analysis of two multicenter, randomized, placebo-controlled studies of pamidronate disodium for the palliation of bone pain in men with metastatic prostate cancer. *J Clin Oncol,* 21**,** 4277-84.

STARK, T., LIVAS, L. & KYPRIANOU, N. 2015. Inflammation in prostate cancer progression and therapeutic targeting. *Transl Androl Urol,* 4**,** 455-63.

STEARNS, M. E., RHIM, J. & WANG, M. 1999. Interleukin 10 (IL-10) inhibition of primary human prostate cell-induced angiogenesis: IL-10 stimulation of tissue inhibitor of metalloproteinase-1 and inhibition of matrix metalloproteinase (MMP)-2/MMP-9 secretion. *Clin Cancer Res,* 5**,** 189-96.

STEGNER, D., DUTTING, S. & NIESWANDT, B. 2014. Mechanistic explanation for platelet contribution to cancer metastasis. *Thromb Res,* 133 Suppl 2**,** S149-57.

STEPHENSON, A. J., KATTAN, M. W., EASTHAM, J. A., DOTAN, Z. A., BIANCO, F. J., JR., LILJA, H. & SCARDINO, P. T. 2006. Defining biochemical recurrence of prostate cancer after radical prostatectomy: a proposal for a standardized definition. *J Clin Oncol,* 24**,** 3973-8.

STEPPAN, C. M., BAILEY, S. T., BHAT, S., BROWN, E. J., BANERJEE, R. R., WRIGHT, C. M., PATEL, H. R., AHIMA, R. S. & LAZAR, M. A. 2001. The hormone resistin links obesity to diabetes. *Nature,* 409**,** 307-12.

STONE, J. P. & WAGNER, D. D. 1993. P-selectin mediates adhesion of platelets to neuroblastoma and small cell lung cancer. *J Clin Invest,* 92**,** 804-13.

STOREY, D. J., MCLAREN, D. B., ATKINSON, M. A., BUTCHER, I., FREW, L. C., SMYTH, J. F. & SHARPE, M. 2012. Clinically relevant fatigue in men with hormone-sensitive prostate cancer on long-term androgen deprivation therapy. *Ann Oncol,* 23**,** 1542-9.

STRAUB, R. H. & SCHRADIN, C. 2016. Chronic inflammatory systemic diseases: An evolutionary trade-off between acutely beneficial but chronically harmful programs. *Evol Med Public Health,* 2016**,** 37-51.

STRINE, T. W., CHAPMAN, D. P., BALLUZ, L. S., MORIARTY, D. G. & MOKDAD, A. H. 2008. The Associations Between Life Satisfaction and Health-related Quality of Life, Chronic Illness, and Health Behaviors among U.S. Community-dwelling Adults. *Journal of Community Health,* 33**,** 40-50.

TAAFFE, D. R., NEWTON, R. U., SPRY, N., JOSEPH, D., CHAMBERS, S. K., GARDINER, R. A., WALL, B. A., CORMIE, P., BOLAM, K. A. & GALVAO, D. A. 2017. Effects of Different Exercise Modalities on Fatigue in Prostate Cancer Patients Undergoing Androgen Deprivation Therapy: A Year-long Randomised Controlled Trial. *Eur Urol*.

TAICHMAN, R. S., LOBERG, R. D., MEHRA, R. & PIENTA, K. J. 2007. The evolving biology and treatment of prostate cancer. *J Clin Invest,* 117**,** 2351-61.

TERABE, M., PARK, J. M. & BERZOFSKY, J. A. 2004. Role of IL-13 in regulation of anti-tumor immunity and tumor growth. *Cancer Immunol Immunother,* 53**,** 79-85.

THALGOTT, M., RACK, B., MAURER, T., SOUVATZOGLOU, M., EIBER, M., KRESS, V., HECK, M. M., ANDERGASSEN, U., NAWROTH, R., GSCHWEND, J. E. & RETZ, M. 2013. Detection of circulating tumor cells in different stages of prostate cancer. *J Cancer Res Clin Oncol,* 139**,** 755-63.

THEURILLAT, J. P., UDESHI, N. D., ERRINGTON, W. J., SVINKINA, T., BACA, S. C., POP, M., WILD, P. J., BLATTNER, M., GRONER, A. C., RUBIN, M. A., MOCH, H., PRIVE, G. G., CARR, S. A. & GARRAWAY, L. A. 2014. Prostate cancer. Ubiquitylome analysis identifies dysregulation of effector substrates in SPOP-mutant prostate cancer. *Science,* 346**,** 85-89.

TIAN, L., LU, H. J., LIN, L. & HU, Y. 2016. Effects of aerobic exercise on cancer-related fatigue: a meta-analysis of randomized controlled trials. *Support Care Cancer,* 24**,** 969-983.

TIMMONS, B. W. & CIESLAK, T. 2008. Human natural killer cell subsets and acute exercise: a brief review. *Exerc Immunol Rev,* 14**,** 8-23.

TSIOTRA, P. C., BOUTATI, E., DIMITRIADIS, G. & RAPTIS, S. A. 2013. High Insulin and Leptin Increase Resistin and Inflammatory Cytokine Production from Human Mononuclear Cells. *Biomed Res Int,* 2013.

TURVEY, S. E. & BROIDE, D. H. 2010. Innate immunity. *J Allergy Clin Immunol,* 125.

VADAY, G. G., PEEHL, D. M., KADAM, P. A. & LAWRENCE, D. M. 2006. Expression of CCL5 (RANTES) and CCR5 in prostate cancer. *Prostate,* 66**,** 124-34.

VAINIO, P., LEHTINEN, L., MIRTTI, T., HILVO, M., SEPPANEN-LAAKSO, T., VIRTANEN, J., SANKILA, A., NORDLING, S., LUNDIN, J., RANNIKKO, A., ORESIC, M., KALLIONIEMI, O. & ILJIN, K. 2011. Phospholipase PLA2G7, associated with aggressive prostate cancer, promotes prostate cancer cell migration and invasion and is inhibited by statins. *Oncotarget,* 2**,** 1176-90.

VAN CAUTER, E., MESTREZ, F., STURIS, J. & POLONSKY, K. S. 1992. Estimation of insulin secretion rates from C-peptide levels. Comparison of individual and standard kinetic parameters for C-peptide clearance. *Diabetes,* 41**,** 368-77.

VAN DIELEN, F. M., VAN'T VEER, C., SCHOLS, A. M., SOETERS, P. B., BUURMAN, W. A. & GREVE, J. W. 2001. Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals. *Int J Obes Relat Metab Disord,* 25**,** 1759-66.

VAN SOEST, R. J., DE MORREE, E. S., KWELDAM, C. F., DE RIDDER, C. M., WIEMER, E. A., MATHIJSSEN, R. H., DE WIT, R. & VAN WEERDEN, W. M. 2015. Targeting the Androgen Receptor Confers In Vivo Cross-resistance Between Enzalutamide and Docetaxel, But Not Cabazitaxel, in Castration-resistant Prostate Cancer. *Eur Urol,* 67**,** 981-5.

VANSAUN, M. N. 2013. Molecular pathways: adiponectin and leptin signaling in cancer. *Clin Cancer Res,* 19**,** 1926-32.

VIDAL, A. C., HOWARD, L. E., MOREIRA, D. M., CASTRO-SANTAMARIA, R., ANDRIOLE, G. L., JR. & FREEDLAND, S. J. 2014. Obesity increases the risk for high-grade prostate cancer: results from the REDUCE study. *Cancer Epidemiol Biomarkers Prev,* 23**,** 2936-42.

VILLANUEVA, E. C. & MYERS, M. G., JR. 2008. Leptin receptor signaling and the regulation of mammalian physiology. *Int J Obes (Lond),* 32 Suppl 7**,** S8-12.

VISSER, M., BOUTER, L. M., MCQUILLAN, G. M., WENER, M. H. & HARRIS, T. B. 1999. Elevated C-reactive protein levels in overweight and obese adults. *Jama,* 282**,** 2131-5.

WABITSCH, M., BRENNER, R. E., MELZNER, I., BRAUN, M., MOLLER, P., HEINZE, E., DEBATIN, K. M. & HAUNER, H. 2001. Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. *Int J Obes Relat Metab Disord,* 25**,** 8-15.

WANG, J. S. & WENG, T. P. 2011. Hypoxic exercise training promotes antitumour cytotoxicity of natural killer cells in young men. *Clin Sci (Lond),* 121**,** 343-53.

WANG, Z., WANG, Y., ZHANG, J., HU, Q., ZHI, F., ZHANG, S., MAO, D., ZHANG, Y. & LIANG, H. 2017. Significance of the TMPRSS2:ERG gene fusion in prostate cancer. *Mol Med Rep,* 16**,** 5450-8.

WANNAMETHEE, S. G., LOWE, G. D., WHINCUP, P. H., RUMLEY, A., WALKER, M. & LENNON, L. 2002. Physical activity and hemostatic and inflammatory variables in elderly men. *Circulation,* 105**,** 1785-90.

WARK, L., KLONISCH, T., AWE, J., LECLERC, C., DYCK, B., QUON, H. & MAI, S. 2017. Dynamics of three-dimensional telomere profiles of circulating tumor cells in patients with high-risk prostate cancer who are undergoing androgen deprivation and radiation therapies. *Urol Oncol,* 35**,** 112.e1-112.e11.

WARRINGTON, R., WATSON, W., KIM, H. L. & ANTONETTI, F. R. 2011. An introduction to immunology and immunopathology. *Allergy, Asthma & Clinical Immunology,* 7**,** S1.

WATSON, P. A., ARORA, V. K. & SAWYERS, C. L. 2015. Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. *Nat Rev Cancer,* 15**,** 701-11.

WHO. 2011. *Global Strategy on Diet, Physical Activity and Health, Recommended levels of physical activity for adults aged 18 - 64 years* [Online]. Available:

http://www.who.int/dietphysicalactivity/factsheet_adults/en/ [Accessed 13/07/2017].
WHO. 2016. *Obesity and Overweight Fact Sheet* [Online]. Available: http://www.who.int/mediacentre/factsheets/fs311/en/ [Accessed 21/06/2017].
WISSMANN, M., YIN, N., MULLER, J. M., GRESCHIK, H., FODOR, B. D., JENUWEIN, T., VOGLER, C., SCHNEIDER, R., GUNTHER, T., BUETTNER, R., METZGER, E. & SCHULE, R. 2007. Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. *Nat Cell Biol,* 9**,** 347-53.
WOLVERS, M. D. J., BUSSMANN, J. B. J., BRUGGEMAN-EVERTS, F. Z., BOEREMA, S. T., VAN DE SCHOOT, R. & VOLLENBROEK-HUTTEN, M. M. R. 2017. Physical Behavior Profiles in Chronic Cancer-Related Fatigue. *Int J Behav Med*.
WU, Y. & ZHOU, B. P. 2009. Inflammation: a driving force speeds cancer metastasis. *Cell Cycle,* 8**,** 3267-73.
WYATT, A. W., ANNALA, M., AGGARWAL, R., BEJA, K., FENG, F., YOUNGREN, J., FOYE, A., LLOYD, P., NYKTER, M., BEER, T. M., ALUMKAL, J. J., THOMAS, G. V., REITER, R. E., RETTIG, M. B., EVANS, C. P., GAO, A. C., CHI, K. N., SMALL, E. J. & GLEAVE, M. E. 2017. Concordance of Circulating Tumor DNA and Matched Metastatic Tissue Biopsy in Prostate Cancer. *J Natl Cancer Inst,* 109.
XIE, H., XIE, B., LIU, C., WANG, J. & XU, Y. 2017. Association of PTEN expression with biochemical recurrence in prostate cancer: results based on previous reports. *Onco Targets Ther,* 10**,** 5089-5097.
XIE, T., DONG, B., YAN, Y., HU, G. & XU, Y. 2016. Association between MMP-2 expression and prostate cancer: A meta-analysis. *Biomed Rep,* 4**,** 241-5.
XING, Y., LIU, M., DU, Y., QU, F., LI, Y., ZHANG, Q., XIAO, Y., ZHAO, J., ZENG, F. & XIAO, C. 2008. Tumor cell-specific blockade of CXCR4/SDF-1 interactions in prostate cancer cells by hTERT promoter induced CXCR4 knockdown: A possible metastasis preventing and minimizing approach. *Cancer Biol Ther,* 7**,** 1839-48.
XU, L., ZHAO, Q., HUANG, S., LI, S., WANG, J. & LI, Q. 2015. Serum C-reactive protein acted as a prognostic biomarker for overall survival in metastatic prostate cancer patients. *Tumour Biol,* 36**,** 669-73.
YAMAUCHI, T., IWABU, M., OKADA-IWABU, M. & KADOWAKI, T. 2014. Adiponectin receptors: a review of their structure, function and how they work. *Best Pract Res Clin Endocrinol Metab,* 28**,** 15-23.
YANAGITA, M., LUKE, J. J., HODI, F. S., JANNE, P. A. & PAWELETZ, C. P. 2018. Isolation and characterization of circulating melanoma cells by size filtration and fluorescent in-situ hybridization. *Melanoma Res,* 28**,** 89-95.
YANASE, T. 2017. Impact of metabolic disorders on prostate cancer growth: Androgen and insulin resistance perspectives. 16**,** 252-7.
YANG, G., XIAO, X., ROSEN, D. G., CHENG, X., WU, X., CHANG, B., LIU, G., XUE, F., MERCADO-URIBE, I., CHIAO, P., DU, X. & LIU, J. 2011. The biphasic role of NF-kappaB in progression and chemoresistance of ovarian cancer. *Clin Cancer Res,* 17**,** 2181-94.
YANG, Y. C., BANUELOS, C. A., MAWJI, N. R., WANG, J., KATO, M., HAILE, S., MCEWAN, I. J., PLYMATE, S. & SADAR, M. D. 2016. Targeting Androgen Receptor Activation Function-1 with EPI to Overcome Resistance Mechanisms in Castration-Resistant Prostate Cancer. *Clin Cancer Res,* 22**,** 4466-77.
YOUNG, L., SUNG, J., STACEY, G. & MASTERS, J. R. 2010. Detection of Mycoplasma in cell cultures. *Nat Protoc,* 5**,** 929-34.
YU, H., PARDOLL, D. & JOVE, R. 2009. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer,* 9**,** 798-809.

YUSUF, S., HAWKEN, S., OUNPUU, S., DANS, T., AVEZUM, A., LANAS, F., MCQUEEN, M., BUDAJ, A., PAIS, P., VARIGOS, J. & LISHENG, L. 2004. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet,* 364**,** 937-52.

ZHANG, P., WANG, D., ZHAO, Y., REN, S., GAO, K., YE, Z., WANG, S., PAN, C. W., ZHU, Y., YAN, Y., YANG, Y., WU, D., HE, Y., ZHANG, J., LU, D., LIU, X., YU, L., ZHAO, S., LI, Y., LIN, D., WANG, Y., WANG, L., CHEN, Y., SUN, Y., WANG, C. & HUANG, H. 2017. Intrinsic BET inhibitor resistance in SPOP-mutated prostate cancer is mediated by BET protein stabilization and AKT-mTORC1 activation. *Nat Med,* 23**,** 1055-1062.

ZHANG, X., ZHOU, G., SUN, B., ZHAO, G., LIU, D., SUN, J., LIU, C. & GUO, H. 2015. Impact of obesity upon prostate cancer-associated mortality: A meta-analysis of 17 cohort studies. *Oncol Lett,* 9**,** 1307-12.

ZHANG, Y., PROENCA, R., MAFFEI, M., BARONE, M., LEOPOLD, L. & FRIEDMAN, J. M. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* 372**,** 425-32.

ZOPF, E. M., NEWTON, R. U., TAAFFE, D. R., SPRY, N., CORMIE, P., JOSEPH, D., CHAMBERS, S. K., BAUMANN, F. T., BLOCH, W. & GALVAO, D. A. 2016. Associations between aerobic exercise levels and physical and mental health outcomes in men with bone metastatic prostate cancer: a cross-sectional investigation. *Eur J Cancer Care (Engl)*.

Appendices

Appendix I: ExPeCT trial protocol

Background

Prostate cancer

Prostate cancer (PrCa) is the most common cancer found in men in the developed world [1]. Many men present with locally advanced or metastatic cancer for whom curative surgery is inappropriate [2]. For these men, increases in progression-free and overall survival and quality of life (QoL) are the primary management objectives, and new therapies and assisting lifestyle alterations are increasingly needed.

Metabolic syndrome and prostate cancer

Obesity, known to be associated with a pro-inflammatory, pro-thrombotic humoral milieu, confers a worse prognosis in PrCa. Between 1990 and 2002, Irish male obesity increased from 8% to 20%, with a further 47% of men overweight [3]. Metabolic syndrome (MS) is a constellation of risk factors for cardiovascular disease, with central adiposity and insulin resistance being the most important components. Male hypogonadism, due to androgen deprivation therapy (ADT)—the mainstay of treatment for locally advanced and metastatic PrCa—is an independent risk factor for the various components of MS [4–8]. MS is present in 50% of all men undergoing long-term ADT [9] and is associated with progression of PrCa [10]. This may explain the excess non-cancer mortality in this population [11].

MS is characterised by low-level chronic systemic inflammation. Increasing evidence suggests that substantial cross-talk occurs between molecular pathways involved in inflammation, coagulation, and obesity [12]. Elucidation of how these pathways interact with PrCa cells may shed light on why obesity disimproves PrCa prognosis.

Circulating tumour cells and prostate cancer

Circulating tumour cells (CTCs) are identified in the blood in advanced cancer. Epithelial cells circulating in the blood of patients with carcinoma can be identified using various techniques including the ScreenCell® system (ScreenCell, Paris, France). Increasing evidence suggests that numbers of CTCs may have a prognostic role in advanced PrCa. A prospective study of castration-resistant PrCa found that ≥ 5 CTCs per 7.5 mL of blood correlated with a poor prognosis [13]. When a variety of clinical, serological, and pathological parameters were considered, the model best predictive of survival was based on baseline lactate dehydrogenase (LDH), baseline CTC count, and fold-change in CTC count at monthly intervals [14].

Natural killer cells and obesity

Natural killer (NK) cell numbers in blood and in solid organs, as well as NK cell cytotoxicity and cytokine secretion, are known to be reduced in obesity [15]. In addition, obese people with hypertension, raised fasting glucose, and an unfavourable lipid profile have less NK cells than "metabolically healthy" obese patients. Obese subjects have lower numbers of hepatic NK cells and leptin receptor-positive cells compared with those of normal weight [16]. The NK cell fraction of white blood cells is sensitive to exercise [17], and five-fold increases in NK concentrations following acute exercise have been noted. Brief exercise upregulates molecular pathways in circulating NK cells associated with cancer and cell communication [18]. In healthy young men, hypoxic exercise training leads to enhanced in-vitro NK cell cytotoxicity [19].

Interactions between platelets and circulating tumour cells

Despite the long-recognised association between cancer and thromboembolism, it has been unclear whether the thrombocytosis often seen in patients with metastases is a consequence or cause of widespread dissemination of the tumour. Accumulating evidence now shows that platelets support tumour metastasis by various mechanisms [20]. Platelets are involved in the arrest of CTCs in the vasculature and, through endothelial interactions, enable their extravasation. Platelets also secrete various pro-oncogenic factors including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), and mediate pro-survival signals in ovarian cancer cells [21].

Tumour cell-induced platelet aggregation correlates with metastatic potential, and may be due to "cloaking" of tumour cells by adherent platelets. The interaction between platelet cloaking of CTCs and tumour cell killing by NK cells is not completely understood. "Cloaking" of CTCs by adherent platelets may impede NK cell clearance of CTCs from the circulation, enhancing metastatic spread. Thrombocytopaenic mice exhibited reduced tumour metastatic burden when the tumour cells were NK cell sensitive, and in-vitro studies demonstrated reduced NK tumourilytic activity when platelets aggregated around tumour cells [22]. Platelets may enable evasion of immune editing by NK cells by conferring a "pseudonormal" phenotype on CTCs by encouraging high-level surface expression of normal major histocompatibility complex (MHC) class 1 antigen by the tumour cells [23].

In these pre-clinical studies there is an association between increased platelet-tumour cell interactions and endpoints of metastasis and death in animal models, but no clinical data exist as yet relating these interactions to outcomes in human disease. The current proposed study takes the current weight of evidence that platelet interactions are important in metastasis, and attempts to make the leap to demonstrate this in a clinical population. Platelet "cloaking" may be enhanced in obese

patients due to the pro-inflammatory, pro-thrombotic state, and may be a mechanism for worse cancer-specific outcomes in this group.

Prostate cancer and exercise

Several studies have shown that exercise may be protective against aggressive PrCa although there is no evidence that exercise protects against PrCa overall [24–27]. In PrCa patients there is solid evidence that exercise (especially group exercise) improves muscular and aerobic endurance, reduces fatigue, and improves overall quality of life [28].

Physical activity reduces levels of systemic inflammatory mediators [29], such as tumour necrosis factor (TNF)α, and so exercise may represent an accessible and cost-effective means of ameliorating the pro-inflammatory effects of obesity. This effect of physical activity depends on type, volume, and intensity, and does not depend directly on weight loss [30].

Obesity and its biochemical effects may be influenced by lifestyle changes such as exercise. As physical activity reduces levels of systemic inflammatory mediators, aerobic exercise may represent an accessible and cost-effective means of ameliorating the pro-inflammatory effects of obesity.

Methods and design

ExPeCT study objectives

The overarching hypothesis is that enhanced platelet cloaking of CTCs in obese men with prostate cancer, due to increased systemic inflammation, is a mechanism underlying worse prognosis of cancer in these patients.

The aim is to test the following four hypotheses, dividing the experimental and analytical work into four separate projects:

1. Platelet cloaking of circulating PrCa tumour cells is more prominent in men with obesity than without.
2. Regular exercise can ameliorate platelet cloaking.
3. The degree of platelet cloaking varies with levels of systemic and primary tumour inflammation and coagulability.
4. Expression of an obesity-associated lethality gene signature leads to variation in platelet cloaking.

ExPeCT study design

This international multicentre prospective study will recruit men with metastatic PrCa from five Irish hospitals and one UK hospital. This study incorporates both an observational component, with exposed and non-exposed groups defined based on body mass index (BMI), and an exercise component, with randomization to exercise and control groups for a supervised exercise programme. Participants with metastatic prostate cancer will be recruited and divided into exposed (BMI ≥ 25 kg/m^2) and non-exposed groups (BMI < 25 kg/m^2). All exposed and non-exposed participants will be randomised to an exercise group or a control group, helping to minimise bias. The exercise group will participate in a 6-month exercise programme, comprising a weekly group exercise class and a home-based exercise programme. Participants will also be encouraged to complete activity diaries. From baseline (T0) to 3 months (T3), participants in the exercise arm will meet in small groups with a chartered physiotherapist for 1 h per week. At these sessions, participants will be educated about using the Polar heart rate monitors, prescribed their target exercise intensity, and complete a half-hour group aerobic exercise class. From T3 to 6 months (T6) continued aerobic exercise will be encouraged but classes will not be supervised by a chartered physiotherapist. All patients will be offered a personal exercise advice session at the study end to discuss long-term compliance to physical activity guidelines. Any patients demonstrating a need for further follow-up in relation to their physical activity levels will be advised to attend their general practitioner (GP) for a referral to the GP exercise scheme.

The study design consists of four main projects (Fig. 1):

Project 1: CTCs will be enumerated in the T0 samples. Adherent platelets will be quantified and compared between the exposed and non-exposed groups, and correlated with clinicopathological parameters.
Project 2: The exercise group will undertake a regular supervised aerobic exercise programme, whereas the control group will not. T3 and T6 blood samples will be assessed for CTC numbers and platelet cloaking. Changes will be compared with the T0 sample, and between exposed and non-exposed, and exercise and control groups. Participants will complete a detailed questionnaire to assess QoL and other parameters at each visit.
Project 3: Blood samples will be assessed for NK cell number and activation, markers of systemic inflammation, adipokines, and serum factors related to platelet activation. The prostate needle core biopsies (NCBs) will be examined microscopically for atrophy and inflammation by morphology and immunohistochemistry, with particular reference to NK cells. All variables will be correlated with platelet cloaking.
Project 4: NCBs will be assessed for expression of an obesity-associated lethality gene signature (whose genes are known to play a role in obesity or platelet aggregation and coagulation), and correlated with platelet cloaking of CTCs.

ExPeCT participant selection criteria

Inclusion criteria

1. Written informed consent obtained before any study-related procedures.

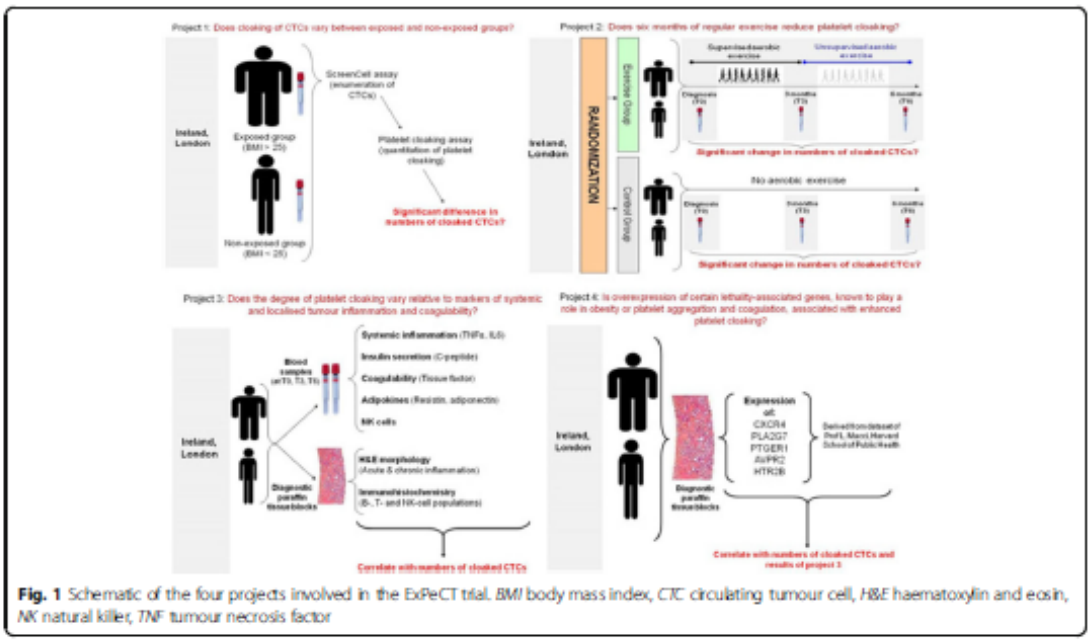


Fig. 1 Schematic of the four projects involved in the ExPeCT trial. *BMI* body mass index, *CTC* circulating tumour cell, *H&E* haematoxylin and eosin, *NK* natural killer, *TNF* tumour necrosis factor

2. Aged ≥ 18 years and male.
3. Histologically confirmed diagnosis of prostate adenocarcinoma.
4. M1 metastatic disease as confirmed by computed tomography (CT)/magnetic resonance imaging (MRI) or by bone scan, excluding patients who only have nodal metastatic disease.
5. Stable medical condition, including the absence of acute exacerbations of chronic illnesses, serious infections, or major surgery within 28 days prior to randomisation.
6. Capable of participating safely in the proposed exercise as assessed and signed off by a treating physician involved in ExPeCT recruitment.

Exclusion criteria

1. Patients with a history of radical prostatectomy.
2. Patients with other known malignancy (except non-melanoma skin cancers or fully excised carcinoma in situ at any site).

Participant enrolment procedure

Potential patients will be enrolled to the study on the basis of the inclusion/exclusion criteria. Enrolment of patients will be undertaken by staff at the medical oncology clinics at each recruiting site as well as members of the ExPeCT research team who have been delegated this task by the principle investigator (PI) (Fig. 2). Any queries about eligibility will be addressed directly to the Chief Investigator. Informed consent will be obtained by clinic staff or a member of the ExPeCT research team according to the requirements of International Conference on Harmonisation-Good Clinical Practice (ICH-GCP).

Upon registration of new participants, a signature confirming eligibility for the trial must be obtained from a treating physician involved in ExPeCT recruitment. Each registered patient will receive a unique participant identifier number (PIN). In order to ensure random allocation of participants to each study group, the computer programme Graphpad will be used to randomly assign a treatment group to each PIN. When issuing each PIN, two gatekeepers (1 in Ireland and 1 in the UK) will inform the research team of the treatment allocation of the participant. If a participant chooses to withdraw from the study, all data obtained up to the point of withdrawal will be carried forward unless requested otherwise.

Study methodology

Demographic and clinical characteristics

A datasheet will be completed for each participant after recruitment at T0 and at the T3 and T6 follow-up visits. Data gathered will include date of birth, anthropometric parameters (body weight, standing height, waist circumference), blood pressure, routine laboratory data (serum

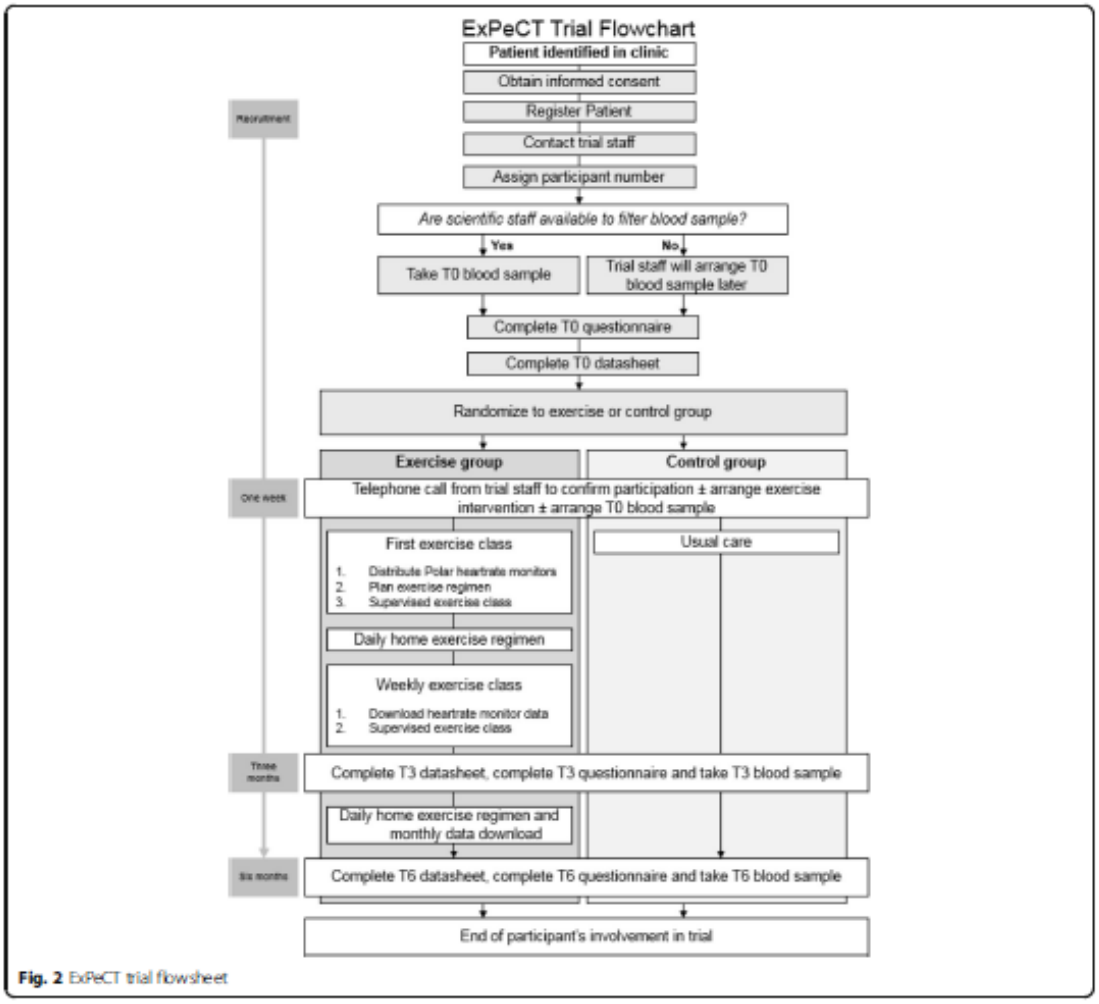


Fig. 2 ExPeCT trial flowsheet

prostate-specific antigen (PSA), haemoglobin, white cell and platelet counts), site of metastasis, and cancer-related data (stage and Gleason grade of cancer, details of current and previous systemic and radiation therapy). Data will also be recorded from three measures of physical function including balance, lower limb strength, and gait speed. These three measures will be completed with the patient by the chartered physiotherapist. Participants may also be asked to complete a structured interview session with the chartered physiotherapist exploring attitudes towards exercise. An overview of all data collected is included in Fig. 3.

Primary study endpoint

Platelet cloaking of circulating tumour cells

For each clinical review episode (at baseline and after 3 and 6 months), 12–16 mL of blood drawn from each patient into K_2-EDTA tubes will be filtered by a ScreenCell® Cyto kit within 4 h. CTC enrichment depends on vacuum-assisted filtration through a microporous membrane filter to separate CTCs from other blood cells on the basis of size. Three to five filters will be generated for each participant, two of which will be stained with May-Grunwald Giemsa, followed by a broad-spectrum epithelial marker, and one to three reserved for platelet cloaking assays and other relevant markers. CTCs will be enumerated cytologically. The degree of platelet adhesion to CTCs will be assessed by immunohistochemistry. The number of CTCs with adherent platelets will be counted, and the approximate number of platelets adherent to each cell will be estimated.

			Study Period			
			Enrolment	T0	T3	T6
		Eligibility Screen	X			
		Informed Consent	X			
		Allocation	X			
Type of Data	Details Collected	Instrument Used				
Demographic and Clinical Measures	Background Details (age at diagnosis, domiciliary situation, comorbidities, recent medications)	Physicians' Health Study Assessment		X	X	X
	Blood Pressure, BMI, Waist Circ.	Clinical Registration Form		X	X	X
Subjective Lifestyle Measures	Stress	Perceived Stress Scale – 4		X	X	X
	Memory and Cognition	Physicians' Health Study Assessment		X	X	X
	Pain	Brief Pain Inventory Scale		X	X	X
	Smoking and Alcohol	Physicians' Health Study Assessment		X	X	X
	Physical Activity	Physicians' Health Study Assessment		X	X	X
	Depression	PHQ-9		X	X	X
	Diet (dairy products, meat, vitamin D)	Physicians' Health Study Assessment		X	X	X
	Quality of Life	FACT-P		X	X	X
	Sleep	Pittsburgh Sleep Quality Index		X	X	X
Physical Function Measures	Physical Activity Measurements	Actigraph Accelerometers		X		
	Physical Performance Measurements	Short Physical Performance Battery		X	X	X
	Adherence to Exercise Programme	FT7 Polar Monitors, Patient Exercise Diaries		X	X	X
Biological Measures	Circulating Tumour Cells	ScreenCell® system		X	X	X
	Serum and Plasma	Vacuette® system		X	X	X
	Natural Killer Cells[a]	Vacuette® system		X	X	X
	Diagnostic Paraffin Tissue Blocks	Original diagnostic NCB paraffin tissue blocks	X			

Fig. 3 ExPeCT SPIRIT figure. [a]Natural killer cell testing will only be performed at Irish sites. *BMI* body mass index, *Circ.* Circumference, *FACT-P* Functional Assessment of Cancer Therapy scales for Men with Prostate Cancer, *NCB* needle core biopsy, *PHQ* Patient Health Questionnaire

Secondary study endpoints

Systemic and localized tumour inflammation and coagulability

This part of the project consists of measurement of systemic and prostate inflammation, markers of coagulation, cytokines, and NK cells. The substrates for this work will be blood samples taken from each participant at T0, T3, and T6, and the original diagnostic NCB paraffin tissue blocks. Examples of the serological and haematological tests include adiponectin, leptin, and resistin.

Expression of lethality-associated genes

This project will evaluate expression of selected genes known to be associated with PrCa progression, coagulation, and stem cell-like phenotype in diagnostic NCBs. Sections of formalin-fixed, paraffin-embedded tissue blocks will be cut from each patient's diagnostic prostate NCB specimen. These sections will be dissected by either laser capture microdissection or gross dissection. Ribonucleic acid (RNA) will be extracted from the microdissected tissue. Gene expression profiling will be undertaken on diagnostic biopsy material using custom-designed assays designed to detect only mRNA and to traverse the exonic junction. Assays for the genes CXCR4, PLA2G7, PTGER1, AVPR2, and HTR2B will be employed. Quantitation of results of polymerase chain reaction (PCR) will be undertaken using the ΔΔCt method, comparing the Ct values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. Diagnostic material may be used for further gene expression analysis associated with obesity as part of the trial.

Quality of life assessment

All participants will complete a detailed questionnaire after recruitment at T0, and again at T3 and T6. The sections of the questionnaire are as follows:

1. Background details (age at diagnosis, domiciliary situation, comorbidities, recent medications).

2. Smoking and alcohol.
3. Sleep (Pittsburgh Sleep Quality Index [31]).
4. Stress (Perceived Stress Scale – 4).
5. Depression (Patient Health Questionnaire (PHQ)-9) [32].
6. Quality of life (FACT-P) [33].
7. Memory and cognition.
8. Physical activity.
9. Diet (dairy products, meat, vitamin D).
10. Pain (Brief Pain Inventory Scale) [34].

Some sections of the questionnaire are stand-alone validated instruments (such as the Functional Assessment of Cancer Therapy scales for Men with Prostate Cancer (FACT-P), which is designed to assess health-related quality of life in this setting [33]). Others, such as the sections on physical activity and diet, are derived from a prostate cancer-specific questionnaire used in the large Physicians' Health Study based at Harvard University [35].

Exercise programme

The exercise group will participate in a 6-month moderate-to-vigorous intensity aerobic exercise programme comprising a weekly class and a home-based aerobic exercise programme. Participants will also be encouraged to complete weekly activity diaries. From T0 to T3, participants in the exercise arm will meet in small groups with a chartered physiotherapist for 1 h per week. During the first class the participants will receive an introduction to the format of the exercise and will be educated on safe exercise practices and strategies to monitor exercise exertion.

Each exercise participant will receive, and be educated about using, a Polar heart rate monitor for the duration of the study. Participants will exercise to a prescribed heart rate range during class and home sessions. Exercise prescription will progress in intensity and duration during months 1 and 2 of the programme to reach the target 3 h per week (180 min/week) of moderate-to-vigorous intensity activity from month 3 onwards (Table 1). This level of activity has been previously shown to be associated with a 33% reduction in all-cause mortality following prostate cancer [36]. Participants will be encouraged to achieve this target exercise in six 30-min sessions throughout the week. However, flexibility will be allowed to facilitate longer or shorter session to a total of 180 min/week. Each exercise session must be of at least 10 min duration. The research team has previously shown that similar aerobic activity intensities can be achieved in cancer survivors through a home-based walking programme and that a Polar heart rate monitor was an acceptable means of monitoring activity intensity [37].

During months 1–3, data from the Polar heart rate monitor will be downloaded weekly to monitor adherence. Participants will be scheduled to attend the research centre once monthly from T3 to T6 to download data and encourage ongoing adherence to the programme. In addition, participants will receive weekly telephone contact from the ExPeCT research team from T3 to T6 to encourage adherence.

The control group will not be given specific advice regarding exercise beyond that considered usual medical care, and will not be invited to participate in the aerobic exercise group. Participants will be reviewed at T3 and T6 following the baseline visit and anthropometric measurements and further blood samples taken. Participants assigned to the control group will be offered a personal exercise advice session following completion of the T6 assessment.

Table 1 Exercise intensity during supervised classes

Supervised exercise classes		Exercise intensity (% heart rate reserve) by baseline fitness group			Duration
		Poor	Fair	Average	(min)
Month 1	Week 1	40–50%	50–60%	55–65%	20
	Week 2	40–50%	50–60%	55–65%	20
	Week 3	45–55%	55–65%	60–70%	20
	Week 4	45–55%	55–65%	60–70%	30
Month 2	Week 5	50–60%	60–70%	65–75%	30
	Week 6	50–60%	60–70%	65–75%	30
	Week 7	55–65%	65–75%	65–75%	30
	Week 8	55–65%	65–75%	65–75%	30
Month 3	Week 9	60–70%	65–75%	65–75%	30
	Week 10	60–70%	65–75%	65–75%	30
	Week 11	60–75%	65–75%	65–75%	30
	Week 12	60–75%	65–75%	65–75%	30

Exercise prescription

Participants will be asked to self-rate their baseline activity levels as one of three categories as per American College of Sports Medicine (ACSM) guidelines:

1) Sedentary or minimally active, not completing any moderate to vigorous activity (equivalent to poor fitness levels).
2) Sporadic physical activity, suboptimal exercise (equivalent to fair fitness levels).
3) Habitual physical activity, regular moderate to vigorous exercise (equivalent to average fitness levels).

Exercise intensity will be prescribed using individualised heart rate reserve (HRR) ranges in accordance with the ACSM guidelines. The following formula will be used to calculate HRR and heart rate (HR) range prescriptions: (target % × [maximum HR − resting HR] + resting HR). For each participant, age-predicted maximal HR will be calculated using the following equation: (206.9 − [0.67 × age]) [38]. Participants with self-rated 'poor' fitness levels (category 1) will commence the programme at an aerobic intensity of 40–50% HRR. Those with self-rated 'fair' fitness levels (category 2) will commence the programme at an aerobic intensity of 50–60% HRR, and those with self-rated 'average' fitness levels (category 3) will commence the programme at 55–65% HRR. The duration and frequency of the home exercise programme sessions is outlined in Table 2.

Patients will also be encouraged to use the Borg Breathlessness Scale. Using this scale, participants will give a subjective rating of perceived exertion. It is a widely used and reliable indicator to monitor and guide exercise intensity [39]. The scale allows individuals to subjectively rate their level of exertion during exercise and can be used to correlate exertion levels with exercise heart rates [40]. The Borg scale will be particularly valuable with participants on beta blockers as measures of exercise intensity are inaccurate or dampened on these medications and polar monitors may not reflect an accurate heart rate during exercise.

Forms of aerobic exercise undertaken at the supervised exercise classes will specifically avoid activities which may be associated with higher risk (e.g. the use of rowing machines in participants with lumbar spinal metastases). Walking on treadmills is a low-risk exercise activity.

Exercise follow-up

Participants will be invited to attend outpatient departments 6 months after T0 and the trial datasheet, questionnaire, and physical function measures will again be completed. Blood samples will be obtained in the same fashion as for the T0 visit. All patients will be offered a personal exercise advice session at study end to discuss long-term compliance to physical activity guidelines. Any patients demonstrating a need for further follow-up in relation to their physical activity levels will be advised to attend their GP for a referral to the GP exercise scheme. After this visit, participants will be thanked for their involvement and discharged from the study.

Table 2 Home-based exercise intensity

Home-based walking programme		Exercise intensity (% heart rate reserve) by baseline fitness group			Time	
		Poor	Fair	Average	Days/week	Duration (min)
Month 1	Week 1	40–50%	50–60%	55–65%	2	20
	Week 2	40–50%	50–60%	55–65%	3	20
	Week 3	45–55%	55–65%	60–70%	3	20
	Week 4	45–55%	55–65%	60–70%	3	30
Month 2	Week 5	50–60%	60–70%	65–75%	3	30
	Week 6	50–60%	60–70%	65–75%	4	30
	Week 7	55–65%	65–75%	65–75%	4	30
	Week 8	55–65%	65–75%	65–75%	5	30
Month 3	Week 9	60–70%	65–75%	65–75%	5	30
	Week 10	60–70%	65–75%	65–75%	5	30
	Week 11	60–75%	65–75%	65–75%	5	30
	Week 12	60–75%	65–75%	65–75%	5	30
Month 4	Weeks 13–16	60–75%	65–75%	65–75%	6	30
Month 5	Weeks 17–20	60–75%	65–75%	65–75%	6	30
Month 6	Weeks 12–24	60–75%	65–75%	65–75%	6	30

Study duration

The study is scheduled to last for 4 years; initial funding was drawn down in April 2014. Enrolment commenced in November 2014 and closed in May 2017 in order to allow enrolled participants to complete their 6 months of follow-up and exercise programme and for all laboratory work and analysis to be finished before the study completion date.

Patient withdrawal and off-study procedure

Patients are free to withdraw from participation in the study at any time upon request. An off-study form must be completed and sent to the ExPeCT research team if a patient withdraws from the study or leaves due to another reason (e.g. study completion, extraordinary medical circumstances, lost to follow-up).

Incident reporting

The occurrence and severity of any incidents from the time of consent to completion of the programme at 6 months will be recorded by the chartered physiotherapist on a standardised reporting form (e.g. adverse events occurring as a result of exercise or adverse reactions to study blood draws). All incidents will be reported to the site PI. Incidents will be followed until resolution or until a patient withdraws from the study or leaves due to another reason (e.g. study completion, extraordinary medical circumstances, lost to follow-up). Recurrent incidents in the same patient will be counted as separate incidents.

Data management

The ExPeCT research team will be the only people with access to the data collected in the course of this project. Data analysis will be performed at St. James's Hospital by the in-house bioinformatics team and other members of the ExPeCT research team. At the end of the study period, when all analysis is complete, data will be retained by the ExPeCT research team. Data will be securely stored for up to 10 years with the option of requesting ethical permission for a prolonged storage time.

Sample size

We will recruit 200 participants over the lifetime of the study, evenly divided between the exercise group and the control group. To calculate the power of the study, we used data from a previous study of ovarian cancer cell lines which showed approximately 2% platelet adhesion [21]. A standard deviation (SD) varying from 2% to 10% would enable us to detect a difference of platelet cloaking of between 0.79% and 3.9%. Research into this area is at an early stage and the clinical importance of specific incremental changes in the degree of platelet cloaking is as yet uncertain, but its elucidation is beyond the scope of this study.

With regard to the detection of changes in platelet cloaking with time, and taking the same assumptions regarding SD of platelet adhesion in PrCa CTCs as in project 1, we will be able to detect a change of 1.8% platelet cloaking between any two time points in the 100 participants in each of the exercise and the control groups, determined by paired t testing. A SD varying from 2% to 10% would enable us to detect a difference of platelet cloaking of between 0.56% and 2.8%. Generalised linear mixed models will be employed in order to account for the correlation between multiple measurements in the same experimental subject.

Statistical analysis

Project 1 will compare the number of cloaked platelets, comparing healthy weight and overweight men using either the t test or the non-parametric Mann-Whitney test, depending on the normality of the data. Linear regression models will be used to test the association between obesity and extent of platelet cloaking, adjusting for potential confounders such as age, use of medications, and smoking. If the data are not normally distributed then a log transformation will be employed. In addition to comparing overweight and healthy weight men as a binary exposure, BMI will be modelled as an ordinal variable (<18.5, 18.5–24.9, 25.0–27.4, 27.5–29.9, 30+) and as a continuous variable and to test for linear trends with the log likelihood ratio test of nested models.

Project 2 will compare measurements of platelet cloaking at baseline and months 3 and 6 follow-up time points among men randomised to the exercise and control arms, in both the exposed (BMI ≥ 25) and non-exposed (BMI < 25) groups. Intention-to-treat analyses will use linear mixed-effect models to incorporate each biomarker for a given participant over time. BMI will also be stratified to look at potential effect modification. To estimate longitudinal changes in quality of life scores from baseline, the primary analysis will be carried out using a mixed-effects model for repeated measures.

Project 3 will examine the extent of the inflammatory infiltrate in diagnostic NCBs. All variables will be correlated with CTC numbers and platelet cloaking using basic descriptive statistics such as Pearson correlation coefficients for continuous variables and simple t tests for categorical variables. In the event of skewed distributions or sparse data, we will use non-parametric tests such as the Spearman correlation and Mann-Whitney. Moreover, a principal component analysis will be undertaken to estimate the proportion of variability in platelet cloaking and CTC number which is explained as a function of the obesity inflammatory biomarkers. The biomarkers will be modelled as principal components in the

linear regression and adjusted for potential confounders such as age, smoking, and other factors.

Project 4: Generalized linear regression models will be used to examine whether obesity is associated with expression of each of the five markers in the tumour tissue, adjusting for potential confounders such as age and smoking status, as well as clinical features. Obesity will be dichotomised as BMI greater or less than 25, and we will also model BMI as a continuous variable and examine tests for trend. The expression of each marker will be assessed with respect to the extent of platelet cloaking (high, intermediate, and low). The categorisation of platelet cloaking as high, intermediate, and low is dependent on the proportion of CTCs with adherent platelets (high > 75%, intermediate 25–75%, low < 25%). A gene score will be created by ranking individuals across expression of each gene in tertiles, assigning points for each marker as lowest tertile = 0, middle tertile = 1, upper tertile = 2, and calculating a summary score.

Ethics and research governance

The study protocol and other documentation have been approved by NRES Committee London—Camden & Islington (REC reference 14/LO/1859), The Mater Misericordia Hospital Research Ethics Committee, Dublin (REC reference: 1/378/1760), Beaumont Hospital Ethics (Medical Research) Committee, Dublin (REC Reference 15/73), SJH/AMNCH Research Ethics Committee, Dublin (REC Reference: 2014-11 List 41 (6)) and St Luke's Radiation Oncology Network, Dublin (REC Number not assigned. Trial referred to as ICORG 15-21 (sponsorship identifier)).

Cancer Trials Ireland is the sponsor for the Irish sites on this study (Protocol Number CTRIAL-IE (ICORG) 15-21).

Discussion

Many patients diagnosed with PrCa are not suitable for radical therapy because of the extent or grade of disease. In those patients who have potentially curable disease, obesity and its complications may make radical surgery impractical. ADT is itself a cause of obesity and metabolic syndrome. For all of these reasons, men with PrCa who are obese are less likely to be treated with curative intent. Medical therapy is improving for the cardiovascular complications of obesity which are the major competing cause of death in these men. As control of obesity-related cardiovascular risk factors improves, aggressiveness of PrCa becomes more important in determining the cause of mortality. It is known that obese men have a worse outlook regarding cancer-related mortality than non-obese men. The combination of an ageing population with an increased PrCa incidence, increasing obesity prevalence, and improved management of cardiovascular risk factors means that in the future, simply put, more men are going to die as a result of the deleterious effect of being overweight in advanced PrCa. Demonstration that platelet cloaking is a mechanism by which obesity disimproves PrCa survival would suggest that therapies targeted at points along the pathway of platelet activation could be efficacious. For example, adiponectin supplementation or blockade of interleukin (IL)-6 or TNFα could be useful. Comparison of the expression of lethality-associated genes between the primary site and CTCs could highlight genes which are upregulated as part of the metastatic pathway, with potential for targeted therapy.

ExPeCT aims to elucidate a potential mechanism by which obesity confers a worse prognosis in PrCa, two increasingly prevalent diseases in the Western world. ExPeCT hopes to show that a low-cost, accessible exercise programme can improve QoL and potentially ameliorate the effects of obesity through alterations in the systemic adipokine and inflammatory mediator profile.

Trial status

ExPeCT trial protocol Version 1.5, 28 July 2016. Recruitment was initiated in October 2014 and continued until May 2017. Data collection is ongoing for enrolled participants and is expected to conclude in November 2017.

Abbreviations

ACSM: American College of Sports Medicine; ADT: Androgen deprivation therapy; BMI: Body mass index; CT: Computed tomography; CTC: Circulating tumour cell; FACT-P: Functional Assessment of Cancer Therapy scales for Men with Prostate Cancer; GP: General practitioner; HR: Heart rate; HRR: Heart rate reserve; ICH-GCP: International Conference on Harmonisation-Good Clinical Practice; IL: Interleukin; K_2-EDTA: Ethylenediaminetetraacetic acid; LDH: Lactate dehydrogenase; MRI: Magnetic resonance imaging; MS: Metabolic syndrome; NCB: Needle core biopsy; NK: Natural killer; PCR: Polymerase chain reaction; PDGF: Platelet-derived growth factor; PHQ: Patient Health Questionnaire; PI: Principal Investigator; PIN: Participant identifier number; PrCa: Prostate cancer; PSA: Prostate-specific antigen; QoL: Quality of life; RNA: Ribonucleic acid; SD: Standard deviation; T0: Baseline; T3: Three months; T6: Six months; TNF: Tumour necrosis factor; VEGF: Vascular endothelial growth factor

Acknowledgements

The authors would like to acknowledge their partnership with the Transdisciplinary Prostate Cancer Partnership (ToPCaP; www.topcapteam.org).

Funding

The ExPeCT trial is funded by the World Cancer Research Fund, grant reference number: 2013/1003.

Availability of data and materials

Not applicable to this study.

Authors' contributions

All authors have read and approved the final manuscript. SF, DMOD, RMD, JH, BH, and JOL are co-applicants on the initial grant proposal. BH and EG contributed to the original grant proposal and protocol. GS and LB contributed to the production of this manuscript and to protocol development. OC contributed to study coordination.

Ethics approval and consent to participate
The study protocol and other documentation have been approved by NRES Committee London—Camden & Islington (REC reference 14/LO/1859), The Mater Misericordia Hospital Research Ethics Committee, Dublin (REC reference: 1/378/1760), Beaumont Hospital Ethics (Medical Research) Committee, Dublin (REC Reference 15/73), SJH/AMNCH Research Ethics Committee, Dublin (REC Reference: 2014-11 List 41 (6)) and St Luke's Radiation Oncology Network, Dublin (REC Number not assigned. Trial referred to as ICORG 15-21 (sponsorship identifier)). Written informed consent will be obtained from each participant before any study-related procedures.

Consent for publication
Not applicable to this study.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
[1]Discipline of Physiotherapy, School of Medicine, Trinity College Dublin, Dublin, Ireland. [2]Department of Histopathology and Morbid Anatomy, Trinity Translational Medicine Institute, Dublin, Ireland. [3]School of Medicine, Trinity College Dublin, Dublin, Ireland. [4]Department of Histopathology, Cork University Hospital, Wilton, Cork, Ireland. [5]Cancer Trials Ireland, Dublin, Ireland. [6]Institute of Pathology, University Hospital Basel, Basel, Switzerland. [7]King's College London, School of Cancer and Pharmaceutical Sciences, Translational Oncology & Urology Research (TOUR), London, UK. [8]Guy's and St Thomas' NHS Foundation Trust, London, UK. [9]Department of Radiation Oncology, St Luke's Hospital, Dublin, Ireland. [10]Department of Oncology, Beaumont Hospital, Dublin, Ireland. [11]Department of Urology, St James's Hospital, Dublin, Ireland. [12]Department of Oncology, Mater Misericordiae, Dublin, Ireland. [13]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, USA. [14]Department of Histopathology, St James's Hospital, Dublin, Ireland. [15]HOPE Directorate, St James's Hospital, Dublin, Ireland. [16]Department of Oncology, Adelaide and Meath Hospital incorporating the National Children's Hospital, Dublin, Ireland.

Received: 29 March 2017 Accepted: 6 September 2017
Published online: 04 October 2017

References
1. Ferlay J, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127(12):2893–917.
2. Jani AB. Management strategies for locally advanced prostate cancer. Drugs Aging. 2006;23(2):119–29.
3. McCarthy SN, Gibney MJ, Flynn A. Overweight, obesity and physical activity levels in Irish adults: evidence from the North/South Ireland food consumption survey. Proc Nutr Soc. 2002;61(1):3–7.
4. Smith JC, et al. The effects of induced hypogonadism on arterial stiffness, body composition, and metabolic parameters in males with prostate cancer. J Clin Endocrinol Metab. 2001;86(9):4261–7.
5. Dockery F, et al. Testosterone suppression in men with prostate cancer leads to an increase in arterial stiffness and hyperinsulinaemia. Clin Sci (Lond). 2003;104(2):195–201.
6. Smith MR, Lee H, Nathan DM. Insulin sensitivity during combined androgen blockade for prostate cancer. J Clin Endocrinol Metab. 2006;91(4):1305–8.
7. Keating NL, O'Malley AJ, Smith MR. Diabetes and cardiovascular disease during androgen deprivation therapy for prostate cancer. J Clin Oncol. 2006;24(27):4448–56.
8. Keating NL, et al. Diabetes and cardiovascular disease during androgen deprivation therapy: observational study of veterans with prostate cancer. J Natl Cancer Inst. 2010;102(1):39–46.
9. Braga-Basaria M, et al. Metabolic syndrome in men with prostate cancer undergoing long-term androgen-deprivation therapy. J Clin Oncol. 2006;24(24):3979–83.
10. Smith MR, et al. Diabetes and mortality in men with locally advanced prostate cancer: RTOG 92-02. J Clin Oncol. 2008;26(26):4333–9.
11. Van Hemelrijck M, et al. Absolute and relative risk of cardiovascular disease in men with prostate cancer: results from the population-based PCBaSe Sweden. J Clin Oncol. 2010;28(21):3448–56.
12. Zhang N, Lawrence DA. Tissue factor and obesity, a two-way street. Nat Med. 2011;17(11):1343–4.
13. de Bono JS, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res. 2008;14(19):6302–9.
14. Scher HI, et al. Circulating tumour cells as prognostic markers in progressive, castration-resistant prostate cancer: a reanalysis of IMMC38 trial data. Lancet Oncol. 2009;10(3):233–9.
15. Lynch LA, et al. Are natural killer cells protecting the metabolically healthy obese patient? Obesity (Silver Spring). 2009;17(3):601–5.
16. Lautenbach A, et al. Human obesity reduces the number of hepatic leptin receptor (ob-R) expressing NK cells. Endocr Res. 2011;36(4):158–66.
17. Timmons BW, Cieslak T. Human natural killer cell subsets and acute exercise: a brief review. Exerc Immunol Rev. 2008;14:8–23.
18. Radom-Aizik S, et al. Impact of brief exercise on peripheral blood NK cell gene and microRNA expression in young adults. J Appl Physiol (1985). 2013;114(5):628–36.
19. Wang JS, Weng TP. Hypoxic exercise training promotes antitumour cytotoxicity of natural killer cells in young men. Clin Sci (Lond). 2011;121(8):343–53.
20. Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. Nat Rev Cancer. 2011;11(2):123–34.
21. Egan K, et al. Platelet adhesion and degranulation induce pro-survival and pro-angiogenic signalling in ovarian cancer cells. PLoS One. 2011;6(10):e26125.
22. Nieswandt B, et al. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. Cancer Res. 1999;59(6):1295–300.
23. Placke T, et al. Platelet-derived MHC class I confers a pseudonormal phenotype to cancer cells that subverts the antitumor reactivity of natural killer immune cells. Cancer Res. 2012;72(2):440–8.
24. Giovannucci EL, et al. A prospective study of physical activity and incident and fatal prostate cancer. Arch Intern Med. 2005;165(9):1005–10.
25. Patel AV, et al. Recreational physical activity and risk of prostate cancer in a large cohort of US men. Cancer Epidemiol Biomarkers Prev. 2005;14(1):275–9.
26. Nilsen TI, Romundstad PR, Vatten LJ. Recreational physical activity and risk of prostate cancer: a prospective population-based study in Norway (the HUNT study). Int J Cancer. 2006;119(12):2943–7.
27. Johnsen NF, et al. Physical activity and risk of prostate cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. Int J Cancer. 2009;125(4):902–8.
28. Keogh JW, MacLeod RD. Body composition, physical fitness, functional performance, quality of life, and fatigue benefits of exercise for prostate cancer patients: a systematic review. J Pain Symptom Manage. 2012;43(1):96–110.
29. Ho SS, et al. Effects of chronic exercise training on inflammatory markers in Australian overweight and obese individuals in a randomized controlled trial. Inflammation. 2013;36(3):625–32.
30. Balducci S, et al. Anti-inflammatory effect of exercise training in subjects with type 2 diabetes and the metabolic syndrome is dependent on exercise modalities and independent of weight loss. Nutr Metab Cardiovasc Dis. 2010;20(8):608–17.
31. Buysse DJ, et al. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. Psychiatry Res. 1989;28(2):193–213.
32. Kroenke K, Spitzer RL, Williams JB. The PHQ-9: validity of a brief depression severity measure. J Gen Intern Med. 2001;16(9):606–13.
33. Esper P, et al. Measuring quality of life in men with prostate cancer using the functional assessment of cancer therapy-prostate instrument. Urology. 1997;50(6):920–8.
34. Cleeland CS, Ryan KM. Pain assessment: global use of the Brief Pain Inventory. Ann Acad Med Singapore. 1994;23(2):129–38.
35. Deo P, Nayak R, Rajpura J. Women's attitudes and health beliefs toward osteoporosis screening in a community pharmacy. J Osteoporos. 2013;2013:650136.
36. Kenfield SA, et al. Physical activity and survival after prostate cancer diagnosis in the health professionals follow-up study. J Clin Oncol. 2011;29(6):726–32.
37. Broderick JM, et al. Feasibility and efficacy of a supervised exercise intervention in de-conditioned cancer survivors during the early survivorship phase: the PEACH trial. J Cancer Surviv. 2013;7(4):551–62.

38. Kohl HW, et al. An empirical evaluation of the ACSM guidelines for exercise testing. Med Sci Sports Exerc. 1990;22(4):533–9.
39. Wilson RC, Jones PW. Long-term reproducibility of Borg scale estimates of breathlessness during exercise. Clin Sci (Lond). 1991;80(4):309–12.
40. Borg GA. Psychophysical bases of perceived exertion. Med Sci Sports Exerc. 1982;14(5):377–81.

Appendix II: Representative ExPeCT patient information leaflet

ExPeCT Trial (Exercise, Prostate cancer and Circulating Tumour cells)

Patient Information Leaflet

Site Doctor/Principal Investigator:	Prof John McCaffrey
Study Doctor Address:	Mater Misericordiae University Hospital, Dublin 7
ICORG Study Number:	ICORG 15-21
Name of Institution leading the research:	Trinity College Dublin
Chief Investigator's Name:	Prof Stephen Finn

Sponsor/Supporter Name and Address: ICORG – the All Ireland Cooperative Oncology Research Group (ICORG), 60 Fitzwilliam Square North, Dublin 2, Ireland.

Introduction:

You are being invited to take part in a research study taking place in St. James's Hospital, Dublin 8. Before you decide whether or not you wish to take part, you should carefully read the information provided below, and if you wish, discuss this with your family, friends or GP. Please take time to ask questions. Do not feel rushed or under any obligation to decide quickly. You should clearly understand the risks and benefits of participating in this study so that you can make a decision that is right for you. This process is known as Informed Consent.

Why is this study being done?

The human body, including the prostate gland, is made up of billions of tiny cells. Sometimes a small number of these cells start to grow too rapidly and become cancer cells. In men with metastatic prostate cancer, these cancer cells

can spread beyond the prostate gland and can be found floating in the blood, where they are called “circulating tumour cells” (CTCs). Very small blood particles (called platelets) become stuck to these CTCs, an occurrence which is called “platelet cloaking”. Platelet cloaking may prevent the body’s defence systems from recognising and killing the cancer cells and allowing them to spread around the body.

Men who are overweight are more likely to develop blood clots, because their platelets are stickier than in men of normal weight. Exercise can improve quality of life for men with cancer and can reduce the stickiness of platelets; therefore, we anticipate that exercise might result in reduced platelet cloaking and, therefore help to reduce or even prevent the spread of cancer cells in overweight men.

This study aims to investigate this by measuring platelet cloaking of CTCs in overweight and normal-weight men with prostate cancer, and the effect of exercise on platelet cloaking and other markers, such as protein or DNA, in the blood which are associated with platelet cloaking.

Who is organising and funding this study?

This study has been organised by Prof Stephen Finn and researchers in Trinity College Dublin in collaboration with the Irish Clinical Oncology Research Group (ICORG). The project is funded by the World Cancer Research Fund, a global network of charities which fund research into the links between cancer, diet, exercise and other lifestyle factors.

What will happen to me if I agree to take part?

If you decide to join the study you will be asked**:**

1. To travel to St. James’s Hospital to provide a blood sample on three occasions, each three months apart. Less than 2 tablespoons of blood will be drawn on each occasion.

These blood samples will be taken by a member of the research team in the Clinical Research Facility in St James’s Hospital in Dublin. The research laboratory will then analyse the blood sample for key elements such as proteins, DNA, CTCs and platelet cloaking. Your doctor will not be informed about any results and your treatment will not be affected by this study.

2. To complete a questionnaire at each occasion of blood draw while in St James’s Hospital.

This will gather information regarding your quality of life, your diet, how well you are sleeping, any medications you take and your psychological well-being. Cancer is a disease which touches every aspect of a man’s life, which should be captured through the questionnaire. The

questionnaire will take approximately 60 minutes to complete. The total time for each appointment will be 90 minutes.

3. This study is a randomized study. This means that, if you agree to join the study, a random decision will be made as to whether or not you will be also asked to participate in an exercise programme (see paragraph 4 below).

Before you agree to join the study, neither the doctors, who are treating you, nor the researchers conducting the study will know whether you will be randomized to participate in this exercise programme or be part of the control group. It is, therefore, important for you to decide whether or not you would be happy to participate in the exercise programme before you agree to join the study.

4. If you are randomised to take part in the exercise programme you will be asked to participate in a regular exercise programme. This will involve a weekly one-hour class with a physiotherapist and several other men with prostate cancer in the Clinical Research Facility in St James's Hospital. This part of the exercise programme will last for three months.

You do not need to be someone who already takes regular exercise in order to be able to participate – the programme is suitable for all fitness levels and will be tailored to your abilities. The exercise programme will focus on aerobic exercise, eg. Walking , running, cycling. If you are asked to participate in the exercise programme you will also be given a small machine to measure your heartrate, and encouraged to do some exercise every day. After completing a three months program you will no longer have a weekly exercise class, and will be encouraged to continue exercising every day.

5. If you do agree to join the study, some of your medical details will also be collected by the researchers, and your height, weight and waist circumference will be recorded at each threemonthly check-up.

6. In addition, the tissue from your prostate biopsy which provided your original diagnosis of prostate cancer will be retrieved from the laboratory in the Mater Hospital and analysed by the research team in St James's Hospital as part of the research.

Who and how many people will take part in the study?

Men with prostate cancer who are known to have metastatic disease will be invited to take part. We will invite about 200 patients to take part in this study, 133 from Ireland and 67 from London.

How long will I be on the study?

Your total involvement in the study would be 6 months.

Do I have to take part?

You do not have to take part in this study and if you decide not to take part, it will have no effect on your care now or in the future.

Can I stop being on this study?

If you do decide to take part, you can change your mind at any time without having to give a reason and without any effect on the care you will receive from the medical staff.

Can anyone else stop me from being in this study?

The study doctor or physiotherapist may stop you from taking part in our research at any time if:

- It is in your best interest.
- You do not follow your study responsibilities.
- You do not meet the study criteria.
- The study is stopped by the sponsor.

What are the possible benefits of taking part?

If you take part in the study and agree to give samples, you may help scientists and doctors understand the significance of circulating tumour cells in the blood. This may improve treatment for cancer patients in the future.
It is important for you to realise that the research study is designed to increase knowledge and understanding of cancer, and so you yourself will not benefit from taking part in it. However, if you are asked to participate in the exercise programme, you may benefit from the experience of taking regular exercise. For some men, this may help to improve symptoms, sleep and general quality of life, as well as improving your general health.
Please note that your doctors will not be informed of the results. Your doctors will make decisions about your treatment independent from this study.

What are the possible risks of taking part?

The study involves having extra blood tests taken in the Clinical Research Facility in St James's

Hospital in addition to routine blood tests being taken at your Mater Hospital clinic appointment.
When you give blood, you may feel faint, or experience mild pain, bruising, irritation or redness at the site. In very rare cases, you may get an infection.
The exercise programme in which you may be asked to participate is carefully supervised by physiotherapists from Trinity College Dublin, and is very safe. You will only be invited to join the study if your doctors feel that you would be well enough to participate in the exercise programme if randomised into this arm.

What happens if I am injured because I took part in this study?

Your safety while taking part in a study is most important. If you feel that you have been injured because of taking part please tell your study doctor or physiotherapist. Our research is covered by an insurance policy in ICORG. The doctors, nurses and other clinical staff involved are covered under the Clinical Indemnity Scheme.

Will my taking part in this study be kept confidential?

All of the blood samples, questionnaires and other data will be coded with an identification number and will not be labelled with your name or any other information that directly identifies you. The connection between the code and you will be kept by the research team in St James's Hospital. Your blood samples and any paper-based data will be kept in secure storage. Electronic (computerised) data will be stored on password-protected machines and servers.

Who will have access to my sample?

The chief investigator, Prof Stephen Finn, his research team and their collaborators will have access to your samples. Anyone who works with your samples will hold your information and results in confidence.

Where will my sample be stored?

If you consent to the study your blood samples, questionnaires and other data will be securely stored in Prof Stephen Finn's laboratory in St James Hospital.

What about the future use of my sample for research?

Samples and data will be stored securely for ten years with the option to seek ethical permission for a longer storage time.

Future research may involve tests that your samples would be suitable for. We will not be able to contact you to ask permission for each individual future study but ask you now for your overall permission to use your donated samples for research purposes. Ethical approval from the St James's and Tallaght joint research ethics committee will be sought before any future research is carried out.
If information from this study is published or presented at scientific meetings, your name and other personal information will not be used.

What are the costs of taking part in this study?

You will not be charged for the cost of tests done for the purpose of this study. You will not be reimbursed for your travel costs or parking for your three visits. If you are asked to partake in the exercise aspect of the study you will be reimbursed for your parking fee when attending the classes.

Who has reviewed and approved this study?

This study has been approved by the Mater Hospital Research Ethics Committee.

Contact for further information

If you have any further questions about the study or if you wish to withdraw from the study please contact your doctor or the physiotherapist responsible for your exercise routine. If you wish to withdraw, you may do so without giving a reason and your future treatment will not be affected. Your samples will continue to be stored as part of the study, however you may request to have your samples destroyed and removed from the trial. In this case any remaining samples, which have not yet been analysed, will be destroyed.

For additional information now or any future time please contact:

Chief Investigator:	Prof. Stephen Finn	Telephone:	087 6577927
Physiotherapist:	Grainne Sheill	Telephone:	087 6577927

Thank you for your time.

Appendix III: Representative ExPeCT patient consent form

ExPeCT Trial (Exercise, Prostate cancer and Circulating Tumour cells)

Informed Consent Form

Site Doctor/Principal Investigator: Prof Liam Grogan

Study Doctor Address: Beaumont Hospital, Beaumont, Dublin 9.

ICORG Study Number: ICORG 15-21

Name of Institution leading the Research: Trinity College Dublin

Chief Investigator: Prof Stephen Finn

Sponsor/Supporter Name and Address: ICORG – the All Ireland Cooperative Oncology Research Group (ICORG), 60 Fitzwilliam Square North, Dublin 2, Ireland.

Please write your initials in each box

1. I confirm that I have been given a copy of the Patient Information Leaflet ICORG Version 1.0 10-Aug-2015, BH Version 2.0 07-Sept-2015. I have read the patient information leaflet or it has been read to me. This information was explained to me and my questions were answered. ☐

2. I understand and agree to provide a blood sample for research purposes. ☐

3. I understand that this is a randomised trial and that I may or may not be asked to participate in the exercise routine. I agree to take part in the exercise if I am requested to do so. ☐

4. I understand that my treating doctor will not be informed of the results unless it is relevant to my treatment. ☐

5. I understand that I must attend the Clinical Research Facility in St James's Hospital to donate blood for the study and if I am randomised to participate in the exercise aspect of the study. ☐

6. I understand that data related to me collected during this study will be processed and analysed as is required by this clinical research study in the department of Histopathology in Trinity College Dublin and St James's Hospital and according to the Data Protection Act. ☐

7. I understand that my samples may be used for research as described in the Patient Information Leaflet. ☐

8. I understand and agree to allow my data and samples to be used for future research. Before any future research is carried out the ethics committee must agree with the research. If you do not consent to the future use of your samples for research you may still participate in this study.

YES ☐

9. I give permission to access my archival tissue sample and to use this for research as described in the Patient Information Leaflet and that this may involve the consumption of any residual material. Any residual material will be returned to Beaumont Hospital. ☐

10. I understand that I am free to withdraw from the study at any time and that this will not affect my standard treatment. ☐

11. I agree to take part in the above study. ☐

_____ _____ _____

Name of Patient (Print) Signature of Patient Date

_____ _____

Name of Witness (Print) Signature of Witness Date

(if required)

Name of Study Doctor (Print)	Signature of Study Doctor
Or Study Co-ordinator (Print)	**Or** Study Co-ordinator
Or Research Nurse (Print)	**Or** Research Nurse
Or Person delegated by CI/PI (Print)	**Or** Person delegated by CI/PI

Appendix IV: Biological sample log

Biological Sample Log for ExPeCT Trial

Patient Data

Site of Recruitment

- Dublin (Tallaght) ☐
- Dublin (St James's) ☐
- Dublin (Beaumont) ☐
- Dublin (Mater) ☐
- Dublin (St Luke's) ☐
- London, UK ☐

Participant Identification Number _____

Date of Sample Collection _____

Study Time Point

- Recruitment (T0) ☐
- Three month review (T3) ☐
- Six month review (T6) ☐

Study Arm

- Exercise intervention group ☐
- Non-exercise comparison group ☐

Blood Sample Collection

Serum Collection

- Number of tubes ☐
- Time of Blood Draw ☐
- Time stored at -80 degrees Celsius ☐

Plasma Collection (Citrate Tube)

- Number of tubes ☐
- Time of Blood Draw ☐
- Time stored at -80 degrees Celsius ☐

Whole Blood for Circulating Tumour Cell Collection (K2EDTA Tube)

Number of Tubes ☐

Time of Blood Draw ☐

Time of Screencell Filtration ☐

Number of Filters ☐

Filter Identification Numbers _____

_____

_____

_____

Date of Storage at 4^{o}C _____

Additional Notes (e.g. clogging of filters, blood passage past yellow line on module A)

_____

_____

_____

Appendix V: ExPeCT case report form

Baseline (T0)
Patient Data Sheet

ExPeCT - ID
EXP ☐☐☐

Date of Birth (dd/mmm/yyyy) ☐☐ ☐☐☐ ☐☐☐☐

Date of Assessment (dd/mmm/yyyy) ☐☐ ☐☐☐ ☐☐☐☐

Demographics

Study Arm
- Exercise intervention group ☐
- Non-exercise comparison group ☐

Primary Hospital

- Dublin (St James's) ☐
- Dublin (Mater) ☐
- Dublin (Tallaght) ☐
- Dublin (Beaumont) ☐
- Dublin (St Luke's) ☐
- Guy's Hospital, UK ☐

Clinical Measurements

Height ☐☐☐ cm

Weight ☐☐☐.☐ Kg

Waist circumference ☐☐☐ cm

Blood pressure ☐☐☐ / ☐☐☐ mmHg
systolic *diastolic*

Details of Prostate Cancer

Date of histological diagnosis (dd/mmm/yyyy) ☐☐ ☐☐☐ ☐☐☐☐

Tumour histology, histologic type (Please tick relevant box)

- Adenocarcinoma ☐
- Small cell neuroendocrine carcinoma ☐
- Other (please specify) ☐

_____

Baseline (T0) Patient Data Sheet **ExPeCT- ID** **EXP** ☐☐☐ **Date of Birth** (dd/mmm/yyyy) ☐☐ ☐☐☐ ☐☐☐☐

Initial Gleason grade *3-5* ☐ + *3-5* ☐ = *6-10* ☐

Initial TNM status:

Primary tumour (T)

TX ☐
T0 ☐
T1 ☐
T2 ☐
T3 ☐
T4 ☐

Regional lymph nodes (N):

NX ☐
N0 ☐
N1 ☐
N2 ☐

Distant metastasis (M):

MX ☐
M0 ☐
M1 ☐

Initial treatment (Tick all that apply)

Surgery ☐ Radiotherapy ☐ Hormones ☐

Date of first relapse (dd/mmm/yyyy) ☐☐ ☐☐☐ ☐☐☐☐

Baseline (T0) Patient Data Sheet **ExPeCT - ID EXP** ☐☐☐ **Date of Birth** (dd/mmm/yyyy) ☐☐ ☐☐☐ ☐☐☐☐

Bone Scans ☐ YES ☐ NO (Tick box, if yes record date of scan, location of metastases and number of lesions)

Date (dd/mmm/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

Location of metastases (Tick all relevant boxes)

Pelvis ☐ Visceral ☐ Spinal ☐ Femur ☐ Other (specify) ☐ _____

Total number of lesions if available (NA if not available) ☐☐

Overall summary

Stable metastatic disease ☐ Increased disease burden (since previous scan) ☐

Other (specify) ☐ _____

Date (dd/mmm/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

Location of metastases (Tick all relevant boxes)

Pelvis ☐ Visceral ☐ Spinal ☐ Femur ☐ Other (specify) ☐ _____

Total number of lesions if available (NA if not available) ☐☐

Overall summary

Stable metastatic disease ☐ Increased disease burden (since previous scan) ☐

Other (specify) ☐ _____

Baseline (T0) Patient Data Sheet **ExPeCT- ID EXP** ☐☐☐ **Date of Birth** (dd/mmm/yyyy) ☐☐ ☐☐☐ ☐☐☐☐

Haematology

Date of sample (dd/mmm/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

Test (units)	If test is performed in different units, please specify units	Results	If outside lab normal range, please state significance
Total PSA (ng/mL)			NCS ☐ CS ☐
Haemoglobin (g/dL)			NCS ☐ CS ☐
White cell count (10^9/L)			NCS ☐ CS ☐
Platelet count (10^9/L)			NCS ☐ CS ☐

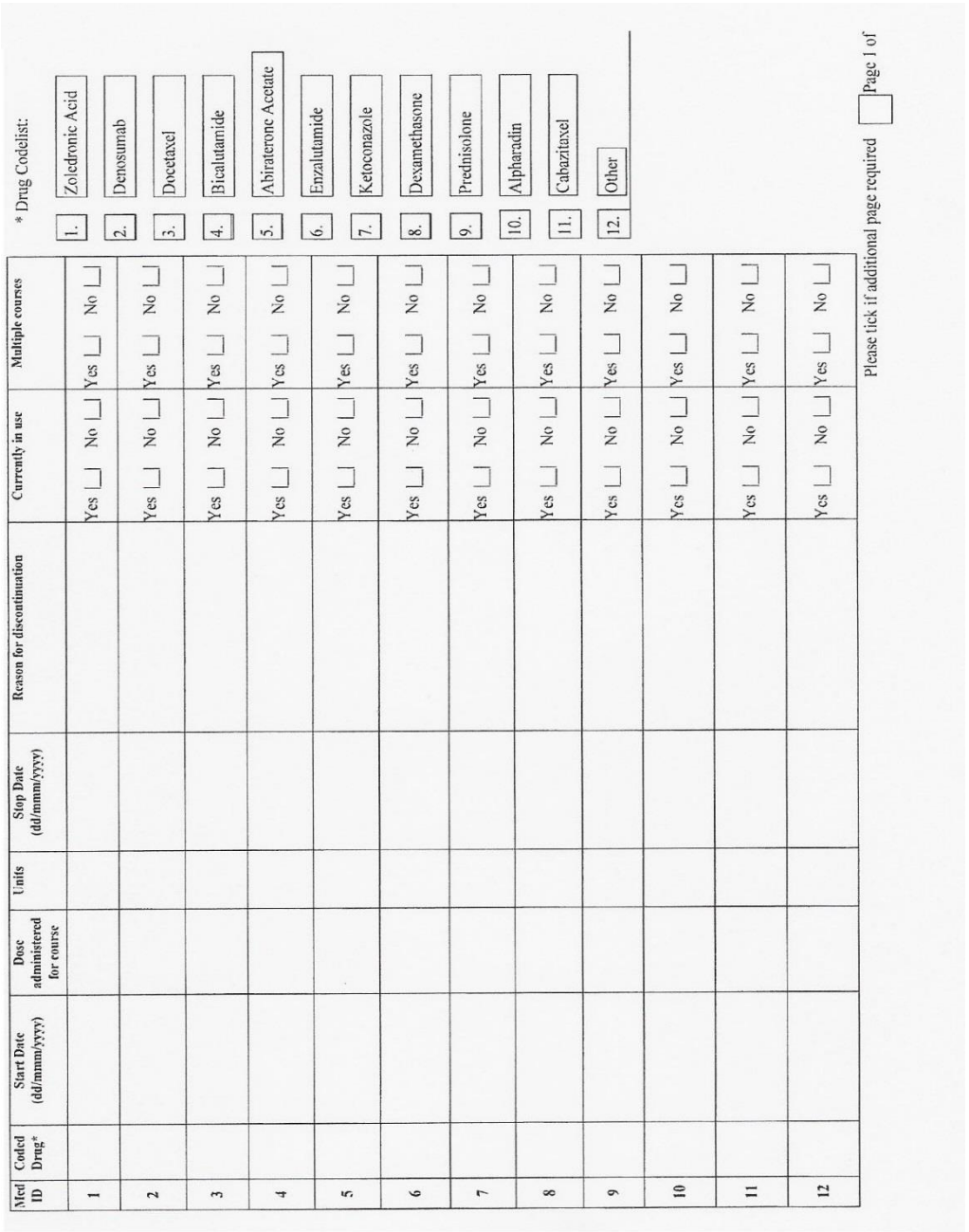
NCS Not Clinically Significant
CS Clinically Significant

Baseline (T0)
Patient Data Sheet

ExPeCT - ID
EXP ☐☐☐

Date of Birth (dd/mmm/yyyy)
☐☐ ☐☐☐ ☐☐☐☐

Details of Previous and Current Systemic Therapy



Med ID	Coded Drug*	Start Date (dd/mmm/yyyy)	Dose administered for course	Units	Stop Date (dd/mmm/yyyy)	Reason for discontinuation	Currently in use	Multiple courses
1							Yes ☐ No ☐	Yes ☐ No ☐
2							Yes ☐ No ☐	Yes ☐ No ☐
3							Yes ☐ No ☐	Yes ☐ No ☐
4							Yes ☐ No ☐	Yes ☐ No ☐
5							Yes ☐ No ☐	Yes ☐ No ☐
6							Yes ☐ No ☐	Yes ☐ No ☐
7							Yes ☐ No ☐	Yes ☐ No ☐
8							Yes ☐ No ☐	Yes ☐ No ☐
9							Yes ☐ No ☐	Yes ☐ No ☐
10							Yes ☐ No ☐	Yes ☐ No ☐
11							Yes ☐ No ☐	Yes ☐ No ☐
12							Yes ☐ No ☐	Yes ☐ No ☐

* Drug Codelist:
1. Zoledronic Acid
2. Denosumab
3. Docetaxel
4. Bicalutamide
5. Abiraterone Acetate
6. Enzalutamide
7. Ketoconazole
8. Dexamethasone
9. Prednisolone
10. Alpharadin
11. Cabazitaxel
12. Other

Please tick if additional page required ☐ Page 1 of

Appendix VI: ExPeCT Off Study Form

Off Study Form **ExPeCT - ID** **Date of Birth** (dd/mmm/yyyy)

EXP ☐☐☐ ☐☐ ☐☐☐ ☐☐☐☐

Primary Hospital

Dublin (St James's)	☐	Dublin (Beaumont)	☐
Dublin (Mater)	☐	Dublin (St Luke's)	☐
Dublin (Tallaght)	☐	Guy's Hospital, UK	☐

This off study form must be completed for all patients, whether they have completed the study or have withdrawn from the study or had to stop their participation for any reason.

Date Off Study (dd/mmm/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

PLEASE SPECIFY THE REASON WHY THE PATIENT IS NOW OFF THE STUDY (please tick one)

☐ Study completion

☐ Extraordinary medical circumstances Please specify _____

☐ Sponsor decision

☐ Lost to follow up Date of last contact (dd/mmm/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

☐ Withdrawal of patient consent Date of withdrawal (dd/mmm/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

Reason for withdrawal _____

☐ Death Date of death (dd/mmm/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

Cause of death _____

☐ Other

Please specify _____

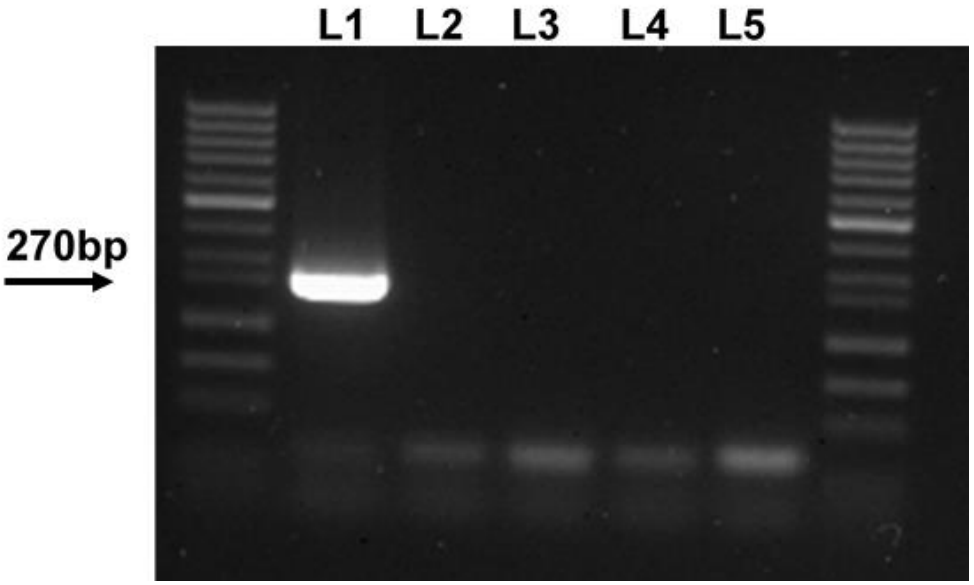
Previous assessment forms completed: ☐ YES ☐ NO ☐ N/A

Investigator's name _____

Investigator's signature: _____

Date (dd/mmm/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

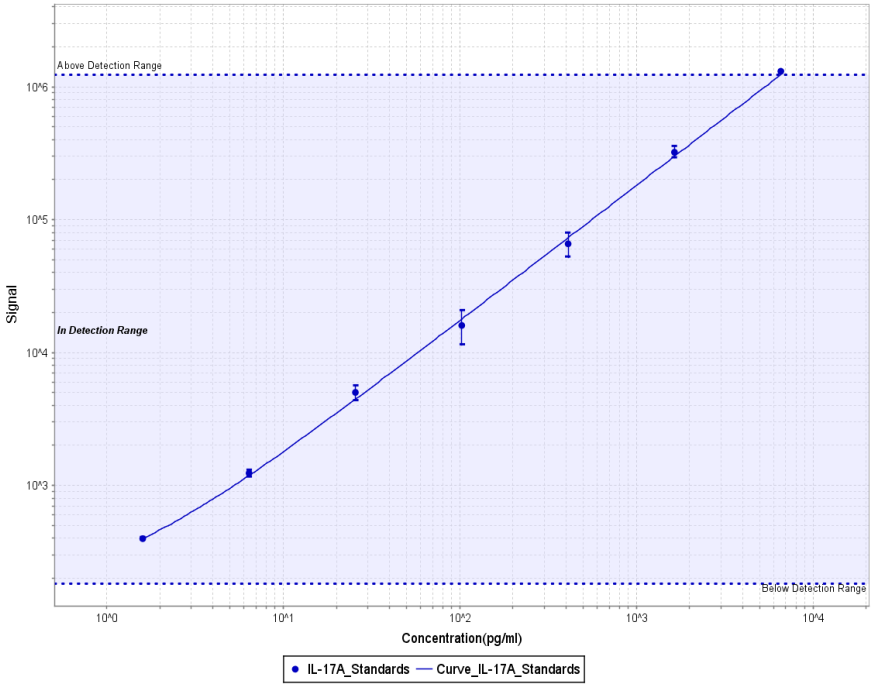
Appendix VII: Representative mycoplasma image



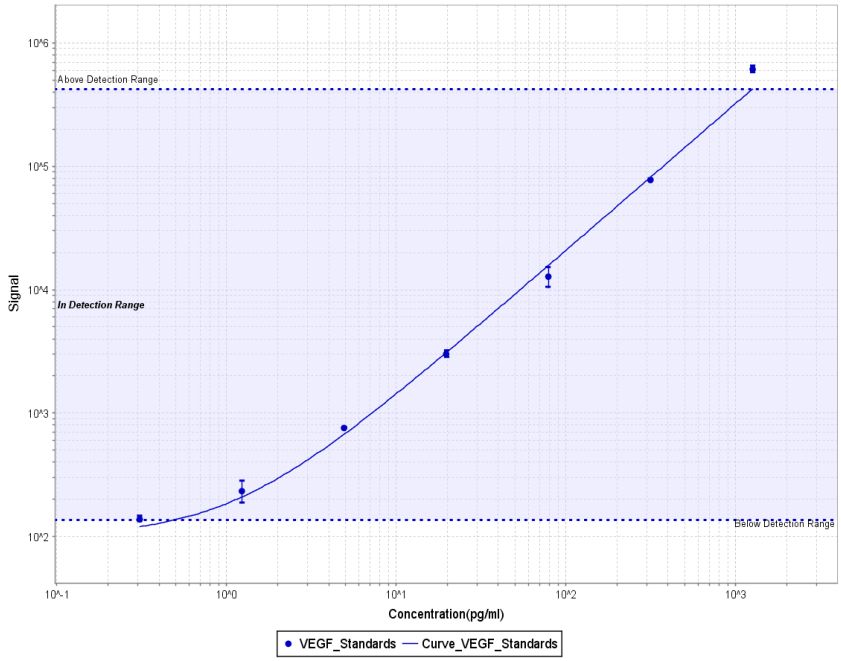
Representative image of mycoplasma testing. Arrow depicts positive mycoplasma control (270bp).

L- Lane

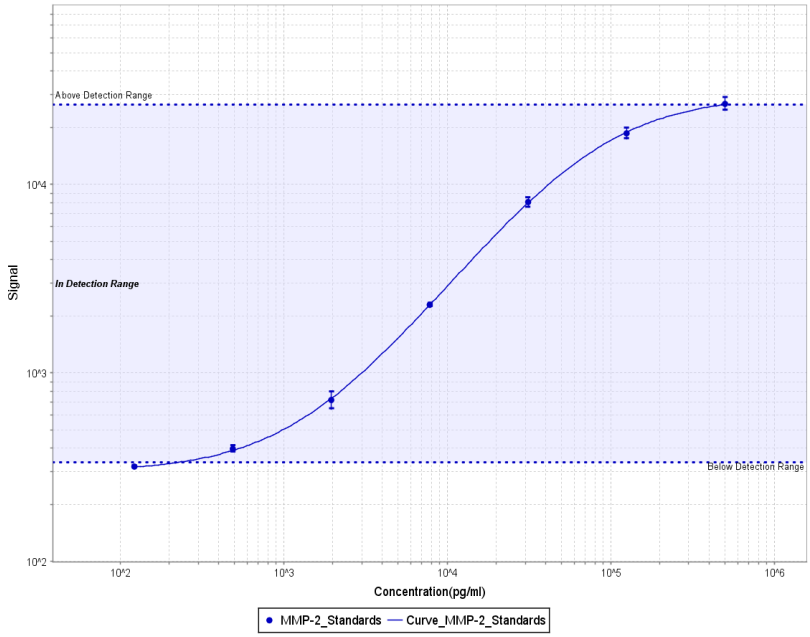
Appendix VIII: Standard curves from Meso-Scale Discovery assays



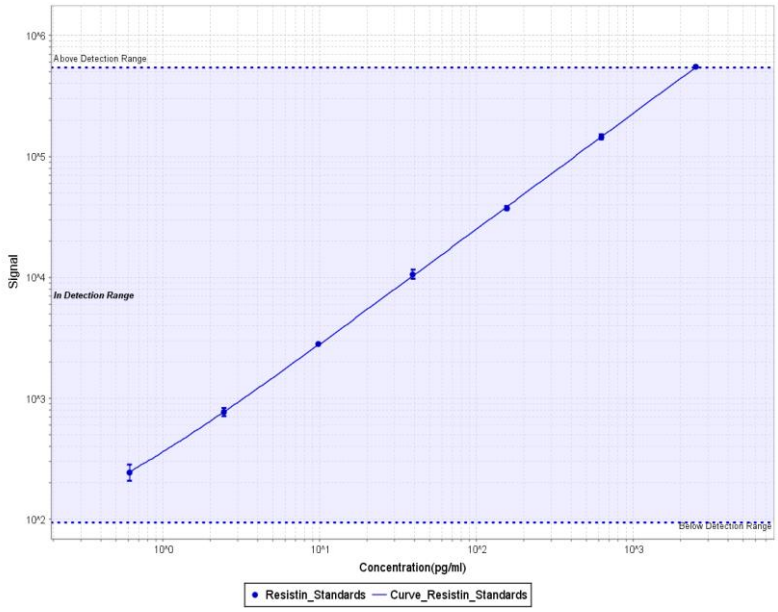
Representative standard curve from IL-17a Meso-Scale Discovery multiplex assay. The assay was used to quantify IL-17a concentration in ExPeCT serum samples and cell supernatant.



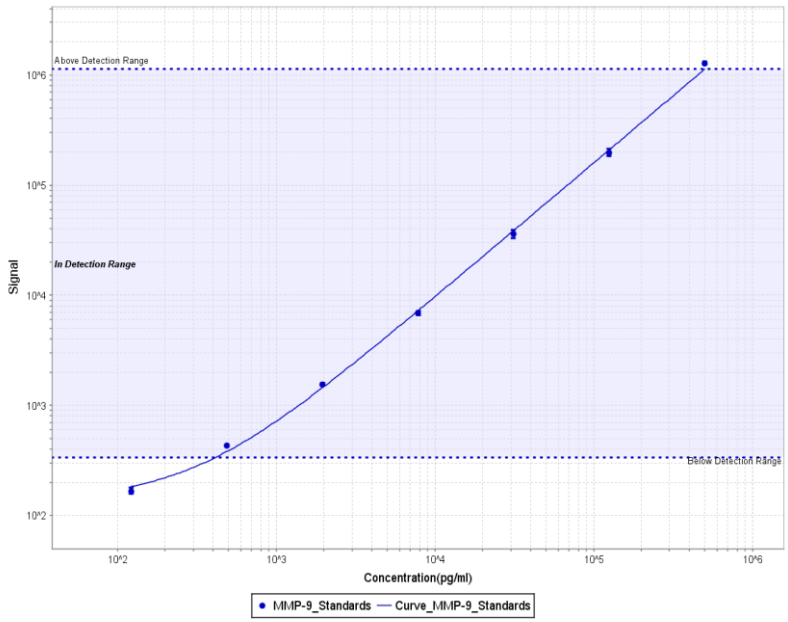
Representative standard curve from VEGF Meso-Scale Discovery multiplex assay. The assay was used to quantify VEGF concentration in ExPeCT serum samples and cell supernatant.



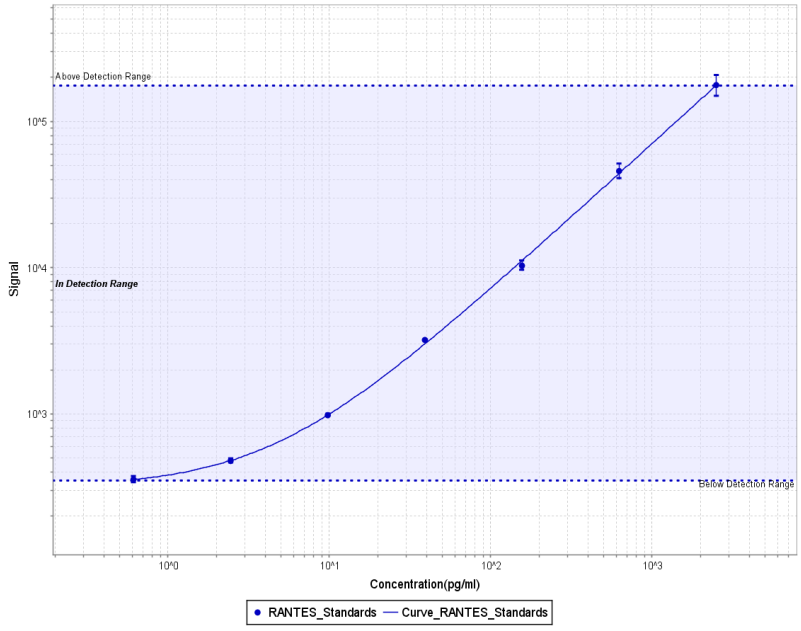
Representative standard curve from MMP2 Meso-Scale Discovery single-plex assay. The assay was used to quantify MMP2 concentration in ExPeCT serum samples and cell supernatant.



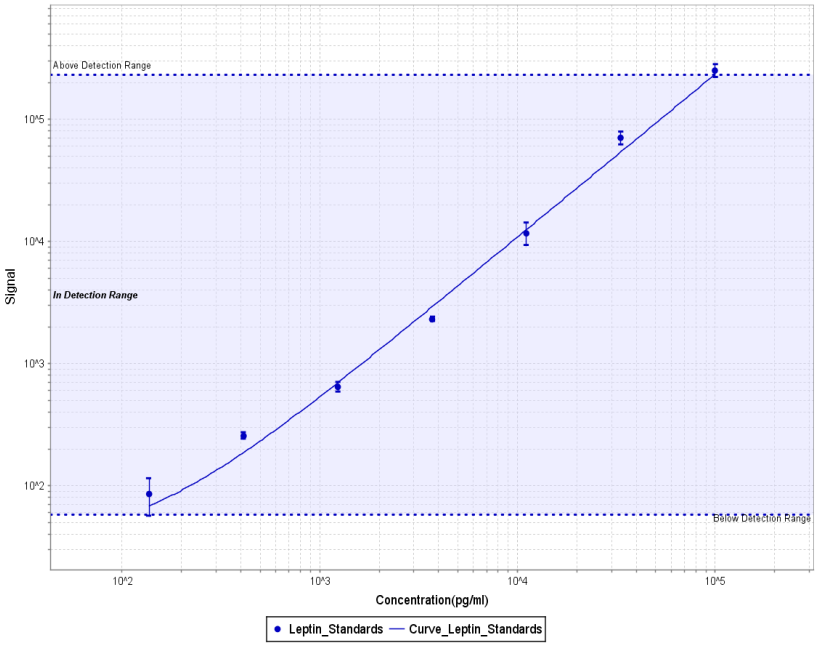
Representative standard curve from resistin Meso-Scale Discovery single-plex assay. The assay was used to quantify resistin concentration in ExPeCT serum samples and cell supernatant.



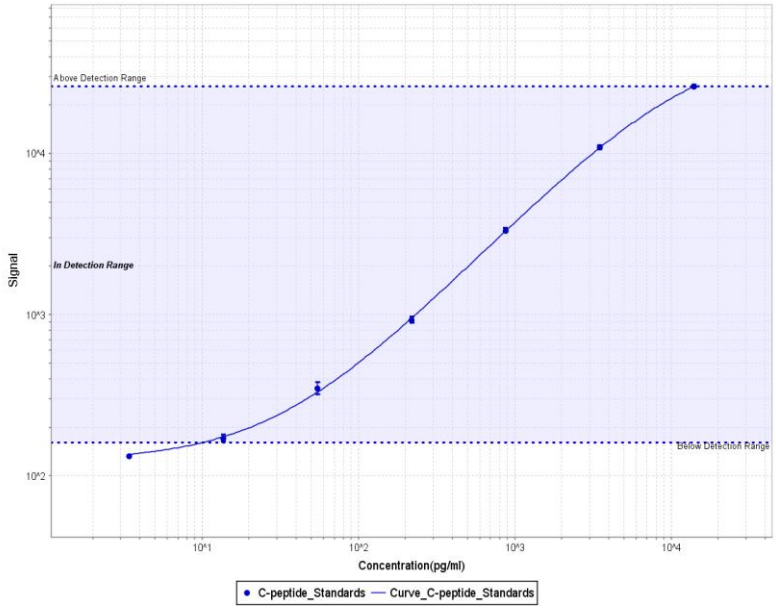
Representative standard curve from MMP9 Meso-Scale Discovery single-plex assay. The assay was used to quantify MMP9 concentration in ExPeCT serum samples and cell supernatant.



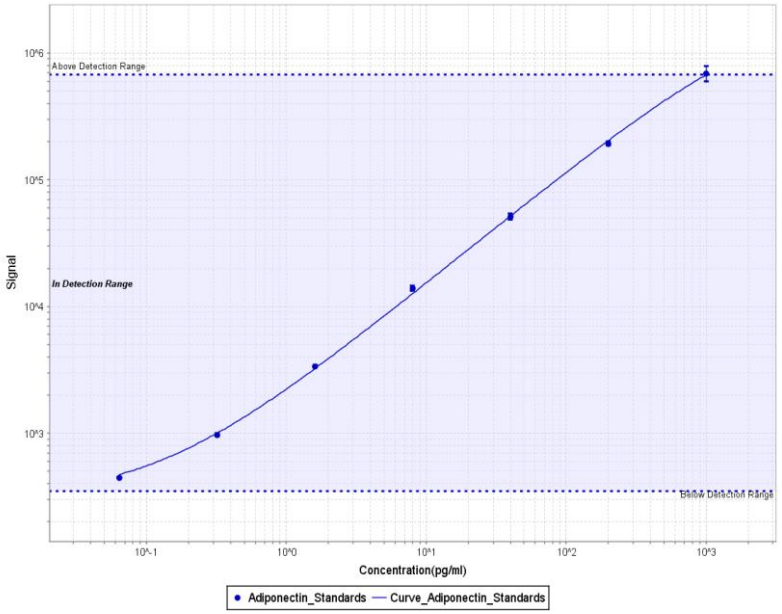
Representative standard curve from RANTES Meso-Scale Discovery single-plex assay. The assay was used to quantify RANTES concentration in ExPeCT serum samples and cell supernatant.



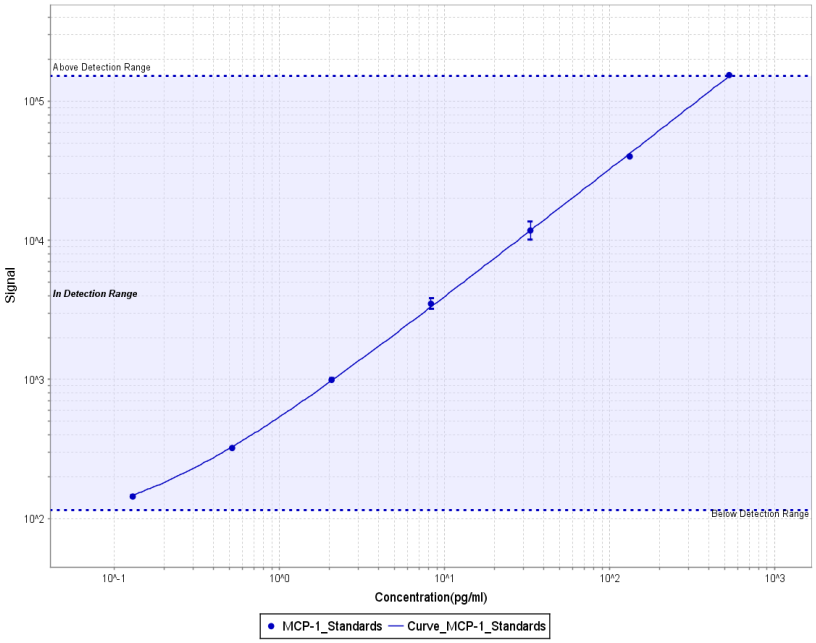
Representative standard curve from Leptin Meso-Scale Discovery single-plex assay. The assay was used to quantify Leptin concentration in ExPeCT serum samples and cell supernatant.



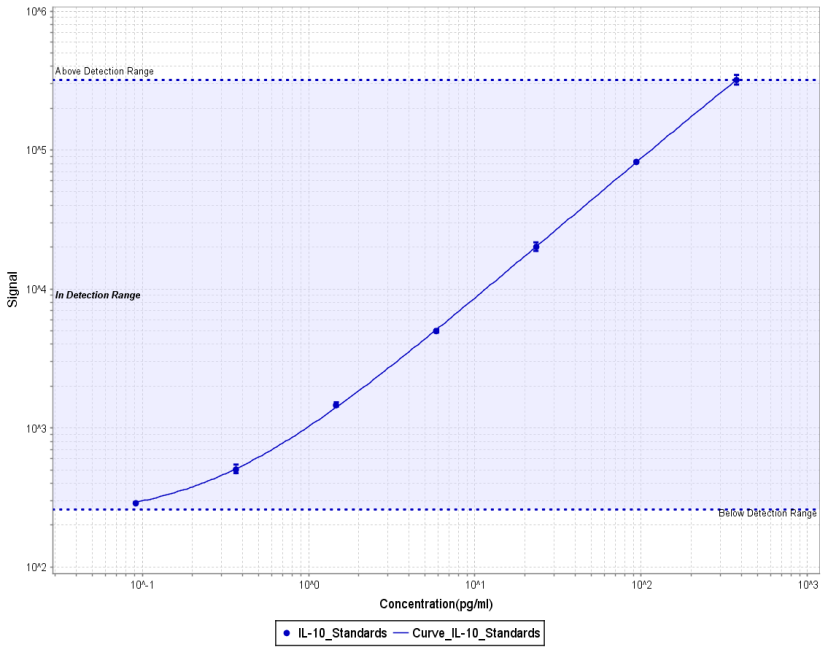
Representative standard curve from C-peptide Meso-Scale Discovery single-plex assay. The assay was used to quantify C-peptide concentration in ExPeCT plasma samples and cell supernatant.



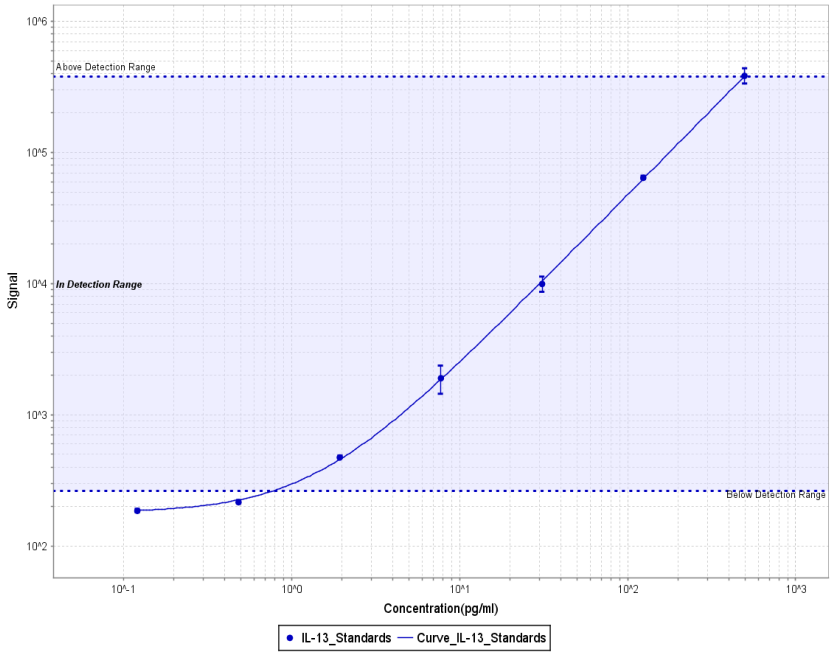
Representative standard curve from adiponectin Meso-Scale Discovery single-plex assay. The assay was used to quantify adiponectin concentration in ExPeCT serum samples and cell supernatant.



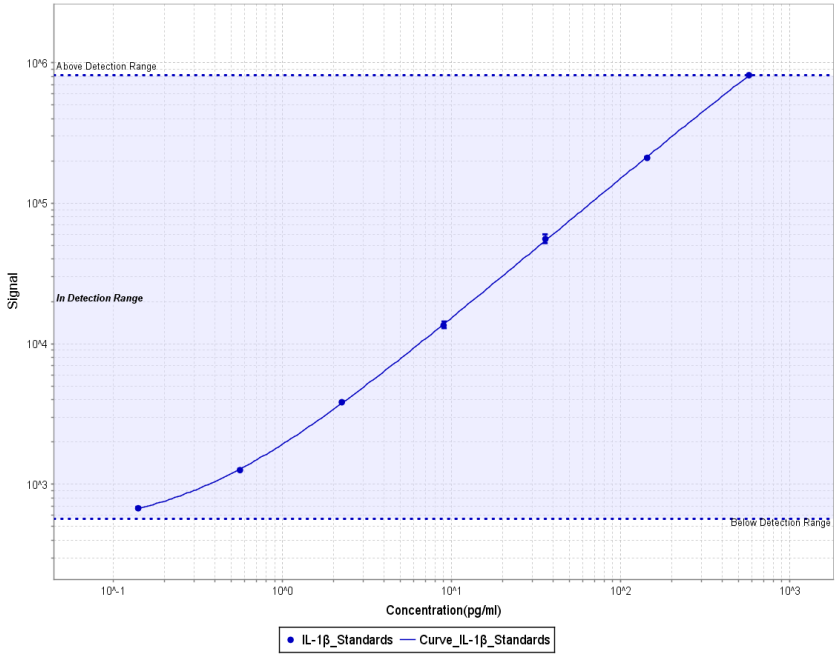
Representative standard curve from MCP-1 Meso-Scale Discovery single-plex assay. The assay was used to quantify MCP-1 concentration in ExPeCT serum samples and cell supernatant.



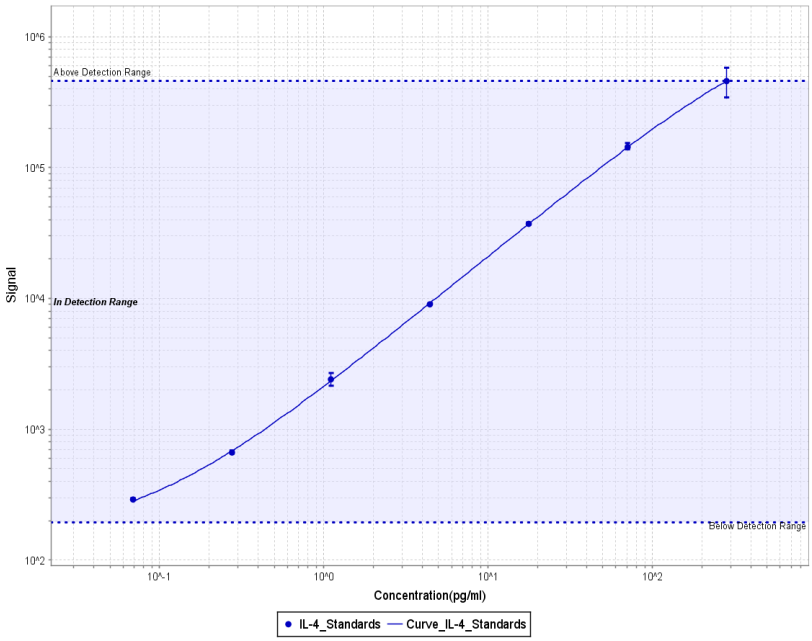
Representative standard curve from IL-10 Meso-Scale Discovery multiplex assay. The assay was used to quantify IL-10 concentration in ExPeCT serum samples and cell supernatant.



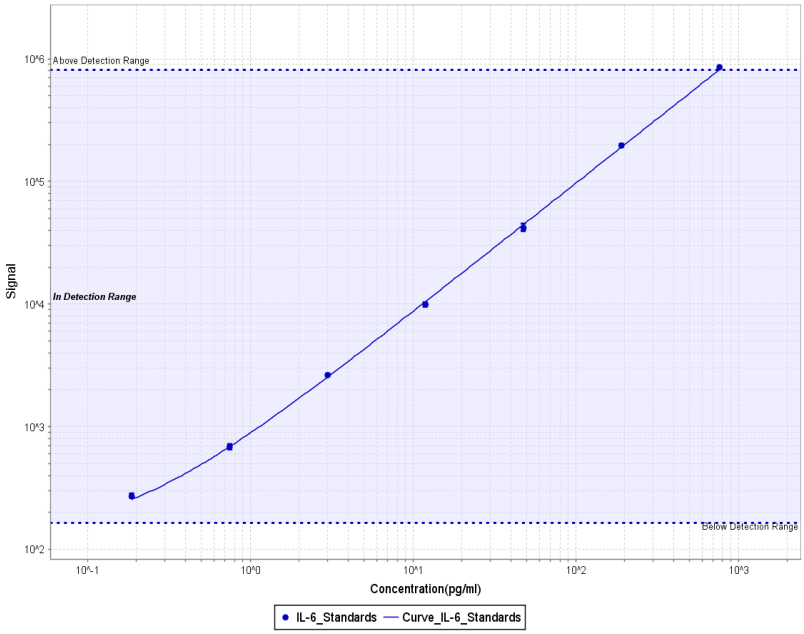
Representative standard curve from IL-13 Meso-Scale Discovery multiplex assay. The assay was used to quantify IL-13 concentration in ExPeCT serum samples and cell supernatant.



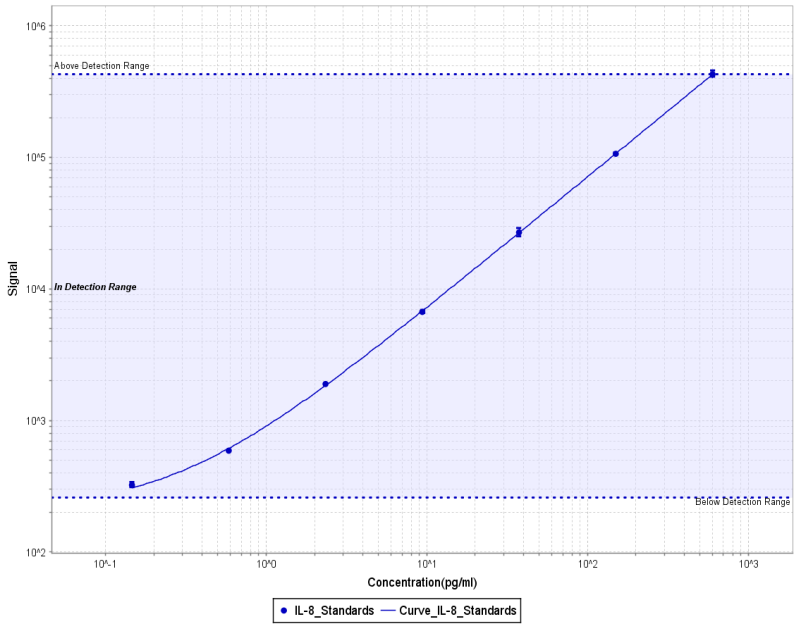
Representative standard curve from IL-1β Meso-Scale Discovery multi-plex assay. The assay was used to quantify IL-13 concentration in ExPeCT serum samples and cell supernatant.



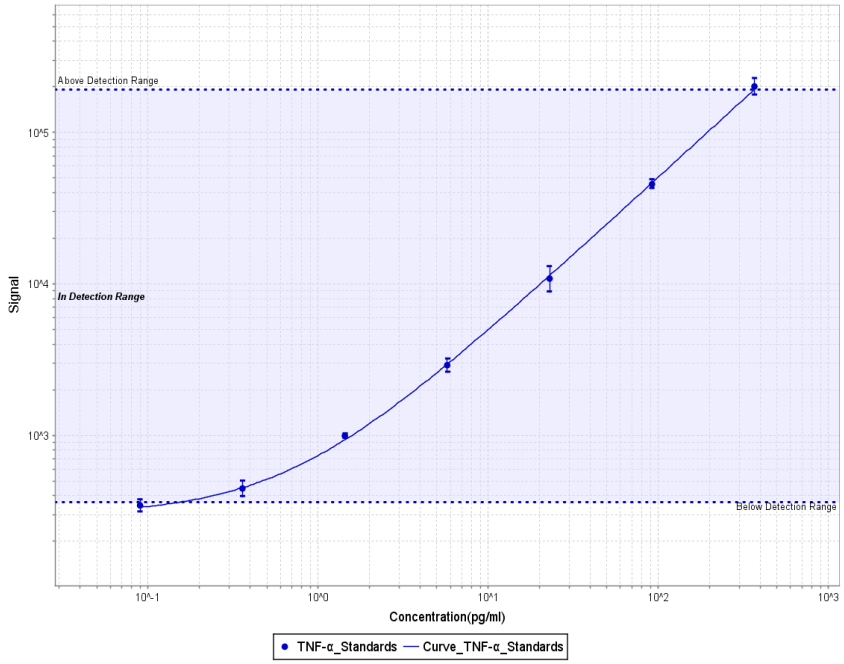
Representative standard curve from IL-4 Meso-Scale Discovery multi-plex assay. The assay was used to quantify IL-4 concentration in ExPeCT serum samples and cell supernatant.



Representative standard curve from IL-6 Meso-Scale Discovery multiplex assay. The assay was used to quantify IL-6 concentration in ExPeCT serum samples and cell supernatant.



Representative standard curve from IL-8 Meso-Scale Discovery multiplex assay. The assay was used to quantify IL-8 concentration in ExPeCT serum samples and cell supernatant.



Representative standard curve from TNFα Meso-Scale Discovery multiplex assay. The assay was used to quantify TNFα concentration in ExPeCT serum samples and cell supernatant.

Appendix IX: Lethality gene expression tables

CXCR4

	Estimate	SD	z_value	p_value
Exercise - Control	0.4304	0.9473	0.4543	0.6496
BMI	0.2341	0.4804	0.4873	0.6299
Weight	-0.0245	0.4965	-0.0493	0.9610
Hb	0.1597	0.4967	0.3216	0.7503
WCC	0.3285	0.4936	0.6656	0.5113
PSA	-1.0003	0.4439	-2.2536	0.0322
WCir	0.5283	0.4883	1.0818	0.2889
AvgCTCs	0.0183	0.0277	0.6597	0.5150
Adiponectin	-0.4190	0.5011	-0.8363	0.4113
C_peptide	0.2732	0.5058	0.5403	0.5940
CCL2	0.6229	0.4736	1.3151	0.2004
IL13	0.2330	0.5556	0.4194	0.6817
IL17A	-0.1514	0.4888	-0.3096	0.7594
IL1β	-0.3792	0.6037	-0.6281	0.5408
IL4	-0.5323	0.5713	-0.9317	0.3825
IL6	0.3568	0.4845	0.7365	0.4683
IL8	-0.1309	0.4890	-0.2678	0.7911
Leptin	-0.0231	0.5751	-0.0402	0.9683
MMP9	0.0798	0.4895	0.1630	0.8718
RANTES	-0.2110	0.4879	-0.4324	0.6692
Resistin	0.2341	0.4875	0.4802	0.6352
TNFa	0.0508	0.4896	0.1037	0.9183
VEGF	0.2831	0.5043	0.5614	0.5797
Control-Height	-1.2168	0.6341	-1.9191	0.0550
Exercise-Height	0.6210	0.7515	0.8263	0.4086
Control-Cloaking	1.7110	1.9278	0.8876	0.3748
Exercise-Cloaking	-3.1118	1.9080	-1.6309	0.1029
Control-IL10	0.2974	0.5590	0.5320	0.5947
Exercise-IL10	-1.8969	0.7663	-2.4754	0.0133
Control-MMP2	1.5957	0.8152	1.9575	0.0503
Exercise-MMP2	-0.4509	0.5775	-0.7809	0.4349

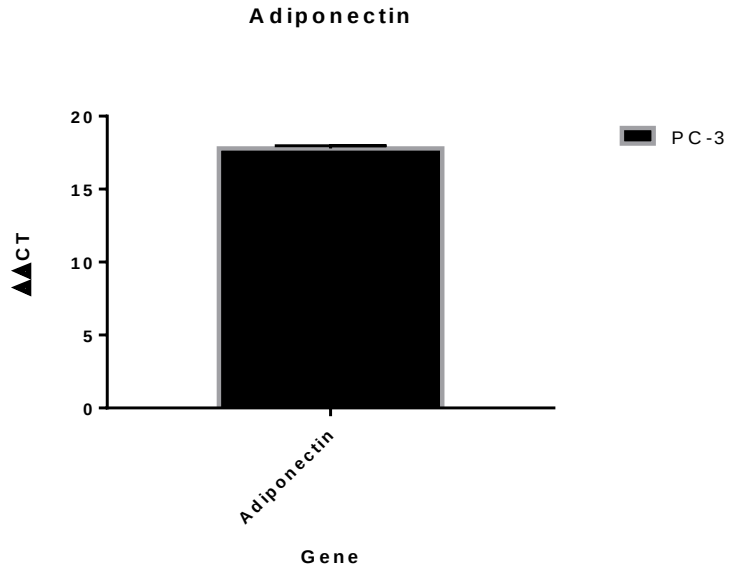
HTR2B

	Estimate	SD	z_value	p_value
Exercise - Control	-1.0342	0.9429	-1.0967	0.2728
BMI	0.5666	0.4734	1.1970	0.2414
Weight	0.4323	0.4784	0.9035	0.3740
Hb	-0.5139	0.4755	-1.0807	0.2890
PSA	-0.6244	0.4708	-1.3263	0.1955
WCir	0.4515	0.4951	0.9119	0.3699
AvgCTCs	-0.0067	0.0304	-0.2218	0.8261
CloakingTRUE	0.2714	1.4028	0.1934	0.8480
Adiponectin	-0.6544	0.4758	-1.3755	0.1822
C_peptide	0.5025	0.4489	1.1195	0.2745
CCL2	0.5425	0.4645	1.1679	0.2543
IL13	-1.0586	0.3997	-2.6486	0.0226
IL17A	0.0542	0.4950	0.1096	0.9137
IL1β	-0.2690	0.6376	-0.4219	0.6800
IL4	-0.1572	0.5389	-0.2917	0.7790
IL6	0.4278	0.4695	0.9114	0.3712
IL8	0.3104	0.4733	0.6557	0.5182
Leptin	-0.4370	0.5017	-0.8712	0.3940
MMP2	-0.6899	0.4563	-1.5120	0.1436
MMP9	-0.2688	0.4743	-0.5666	0.5762
RANTES	-0.1266	0.4768	-0.2656	0.7928
Resistin	-0.2934	0.4737	-0.6194	0.5415
TNFa	0.2035	0.4757	0.4277	0.6727
VEGF	-0.1070	0.4925	-0.2172	0.8300
Control-Height	-3.5218	2.3333	-1.5094	0.1312
Exercise-Height	0.8893	0.4542	1.9582	0.0502
Control-WCC	-0.4096	0.4646	-0.8817	0.3779
Exercise-WCC	3.5928	1.4484	2.4804	0.0131
Control-IL10	0.3854	0.5209	0.7400	0.4593
Exercise-IL10	-1.5629	0.7180	-2.1766	0.0295

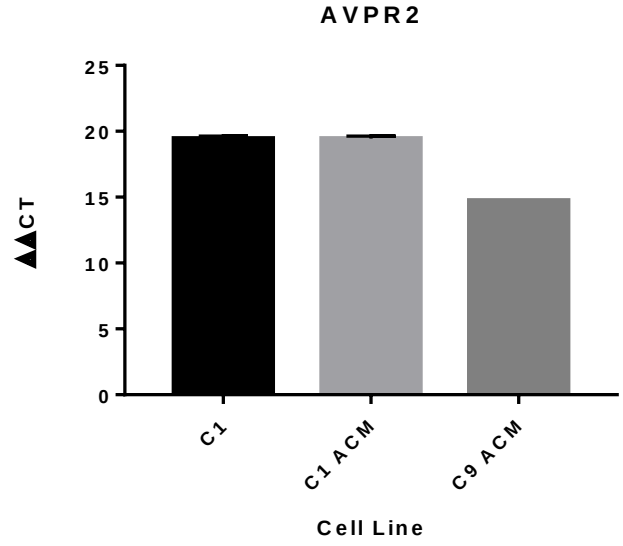
PLA2G7

	Estimate	SD	z_value	p_value
Exercise - Control	-1.4533	1.0273	-1.4146	0.1572
BMI	0.8169	0.5136	1.5905	0.1222
Weight	0.4474	0.5291	0.8456	0.4047
Height	0.6883	0.5198	1.3241	0.1955
WCC	-0.2671	0.5326	-0.5015	0.6197
PSA	-0.8499	0.5118	-1.6606	0.1072
AvgCTCs	-0.0017	0.0328	-0.0505	0.9601
Cloaking	-0.2637	1.5908	-0.1657	0.8695
Adiponectin	-0.7887	0.5595	-1.4096	0.1710
C_peptide	0.0927	0.5936	0.1562	0.8771
CCL2	0.0561	0.5754	0.0975	0.9231
IL10	-0.9794	0.5426	-1.8052	0.0826
IL13	-0.1614	0.8012	-0.2014	0.8433
IL17A	-0.4491	0.5688	-0.7896	0.4369
IL1β	-0.4985	0.8279	-0.6022	0.5574
IL6	0.2446	0.5735	0.4265	0.6732
Leptin	-0.2980	0.6703	-0.4445	0.6610
MMP2	0.1205	0.5751	0.2095	0.8357
MMP9	-0.4446	0.5689	-0.7815	0.4416
RANTES	-0.1223	0.5751	-0.2126	0.8333
Resistin	-0.6229	0.5624	-1.1074	0.2782
TNFa	-0.0444	0.5755	-0.0772	0.9390
VEGF	-0.4152	0.5884	-0.7057	0.4869
Control-Hb	0.4002	0.7517	0.5324	0.5944
Exercise-Hb	-1.3212	0.6925	-1.9079	0.0564
Control-IL4	1.8000	1.1052	1.6287	0.1034
Exercise-IL4	-1.1099	0.5450	-2.0367	0.0417
Control-IL8	1.7882	0.8491	2.1060	0.0352
Exercise-IL8	-0.7823	0.6443	-1.2142	0.2247

Appendix X: Adiponectin expression in PC-3 cells



Adiponectin expression in PC-3 cells. Data graphed as mean ± SEM ΔΔCT.



AVPR2 expression in C1, C1 ACM and C9 ACM cells. Data graphed as mean ± SEM ΔΔCT.