Characterisation of circulating tumour cells in men with advanced prostate cancer and correlation with numbers of circulating and tissue-based inflammatory cells

A thesis submitted to the University of Dublin

for the degree of Doctor in Medicine (M.D.)

2019

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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.

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Brian Hayes
SUMMARY

Despite the widespread availability of PSA screening for prostate cancer many men present with advanced stage disease, and in these men treatment is directed towards improved survival and quality of life rather than cure. Exercise therapies are recognised to have benefits in quality-of-life, all-cause and cancer-specific mortality in cancer patients, and prostate cancer is no exception. Obesity and metabolic syndrome are encountered with increasing frequency in prostate cancer patients, either as pre-existing comorbidities or as a side-effect of androgen deprivation therapy. The systemic inflammatory milieu which underpins the obese state has profoundly negative effects on cancer survivorship, but data show that control of obesity, including through managed exercise interventions, can improve outcomes.

Blood-borne circulating tumour cells are considered an intermediate step in the metastatic cascade and therefore a potentially useful target for therapy. Interventions (including exercise) targeted at improving the deleterious pro-oncogenic systemic consequences of obesity may in part exert their effects by interfering with interactions between platelets, circulating tumour cells and cells of the immune system. Adhesion of platelets to circulating tumour cells may impair the ability of NK-cells to destroy them, and it has been hypothesised that enhanced platelet cloaking of circulating tumour cells in obese men with prostate cancer, due to increased systemic inflammation, is a mechanism underlying the worse prognosis of cancer in these patients.

In order to test this hypothesis the interational multidisciplinary ExPeCT clinical trial (“Examining Exercise, Prostate Cancer and Circulating Tumour Cells”) was established. This recruited men with advanced prostate cancer from centres in Dublin and London and randomised them either to participation in a formal six-month supervised walking intervention (exercise group), or to usual care (control group). Blood samples were taken, quality-of-life questionnaires were completed and clinical data (including blood pressure, body mass index, waist circumference etc) were gathered at the time of recruitment (T0) and after three (T3) and six (T6) months. Participants were divided into “exposed” and “non-exposed” groups based on baseline body mass index ≥ 25 kg/m² or < 25kg/m² respectively. Blood samples were passed through microporous ScreenCell® Cyto filters, allowing isolation of circulating tumour cells on the basis of size, and examined by light microscopy using standard cytological techniques. Circulating tumour cells were enumerated on each filter and the presence or absence of platelet cloaking was recorded. The participants’ original diagnostic biopsy tissue blocks were acquired, stained with H&E and immunohistochemical antibodies for T-lymphocytes, NK-cells and macrophages. In addition flow
Summary

cytometry for numbers and subsets of circulating lymphocytes was undertaken in a subset of Dublin-based participants.

Participant accrual to the ExPeCT trial was slower than expected, with 67 men recruited over the course of the trial, and 61 having at least a T0 blood sample drawn. 30 participants were randomized to the exercise group, 31 to the control group. There were 11 participants in the non-exposed group and 50 in the exposed group. Circulating tumour cells were identified on the vast majority of ScreenCell® filters, and there was a significant reduction in their numbers between T0 and T3, with the change driven by differences in the control group and the exposed group. Platelet cloaking was significantly more frequently seen in blood draws from the control group than the exercise group participants, which might suggest that the exercise intervention played a role in altering platelet adhesion to those men who participated in the exercise programme. However morphological assessment of platelet cloaking was found to be difficult, as the majority of circulating tumour cells present on the filters lacked cytoplasm, perhaps consequent upon the shear forces exerted on the cells during the filtration process.

A higher circulating fraction of CD3-positive T-lymphocytes and a lower circulating fraction of B-lymphocytes and NK-cells were found in the exercise group when compared with the control group. Linear correlations were identified between circulating tumour cell numbers on the one hand and platelet count, total lymphocyte count, CD4-positive T-lymphocyte count and NK-cell count on the other hand, relationships which were found to be independent of one another by multiple regression analysis. The demonstration of a relationship with platelet count is the first such report in men with prostate cancer, providing clinical evidence to support the abundant published scientific data that circulating tumour cells interact with platelets in various ways to enhance their metastatic potential.

Atrophic prostatic lesions were identified in 43% of biopsy specimens, and 65% had an infiltrate of chronic inflammatory cells in the benign background prostatic tissue. 91% of biopsies had at least mild chronic inflammation within the tumour, with T-lymphocytes being the predominant cell type. No correlation was seen between circulating tumour cell numbers and the density of inflammatory cell subsets in core biopsy tissue.

Overall this study provides useful support for the hypothesis that metastasis, platelet function, systemic inflammation and hypercoagulability are closely linked in advanced cancer, and elucidates several potentially valuable directions for future research.
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This work is dedicated to my wife Aoife and to my daughters Anna and Eleanor

For thy sweet love remembered such wealth brings
That then I scorn to change my state with kings.
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<th>Definition</th>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ADT</td>
<td>Androgen deprivation therapy</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>CEC</td>
<td>Circulating epithelial cell</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
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<tr>
<td>CTC</td>
<td>Circulating tumour cell</td>
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<tr>
<td>DRE</td>
<td>Digital rectal examination</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ExPeCT</td>
<td>Exercise, Prostate Cancer and Circulating Tumour Cells</td>
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<tr>
<td>FNA</td>
<td>Fine needle aspiration</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<tr>
<td>HGPIN</td>
<td>High grade prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
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<td>HPF</td>
<td>High power (40x) field</td>
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<tr>
<td>ICH-GCP</td>
<td>International Conference on Harmonisation – Good Clinical Practice</td>
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<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>lcSFA</td>
<td>Long chain saturated fatty acid</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MGG</td>
<td>May Grunwald Giemsa</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MS</td>
<td>Metabolic syndrome</td>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>NCB</td>
<td>Needle core biopsy</td>
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<tr>
<td>NFκb</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>PA</td>
<td>Partial atrophy</td>
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<td>Postatrophic hyperplasia</td>
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<td>Pore diameter</td>
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<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
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<td>Proliferative inflammatory atrophy</td>
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<td>PLR</td>
<td>Platelet lymphocyte ratio</td>
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<td>Prostate cancer</td>
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<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>QoL</td>
<td>Quality of Life</td>
</tr>
<tr>
<td>SA</td>
<td>Simple atrophy</td>
</tr>
<tr>
<td>SACF</td>
<td>Simple atrophy with cyst formation</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combine immune deficiency</td>
</tr>
<tr>
<td>SII</td>
<td>Systemic immune-inflammation index</td>
</tr>
<tr>
<td>T0</td>
<td>Timepoint 0 months</td>
</tr>
<tr>
<td>T3</td>
<td>Timepoint 3 months</td>
</tr>
<tr>
<td>T6</td>
<td>Timepoint 6 months</td>
</tr>
<tr>
<td>TCIPA</td>
<td>Tumour cell induced platelet aggregation</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TURP</td>
<td>Transurethral resection of the prostate</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand Factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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PUBLICATIONS

Exercise and prostate cancer: evidence and proposed mechanisms for disease modification.

Hayes BD, Brady L, Pollak MN, Finn S.

The ExPeCT (Examining Exercise, Prostate Cancer and Circulating Tumour Cells) trial: study protocol for a randomized controlled trial.

Trials 2017;18:456

Examining the link between obesity, inflammation, and exercise in patients with metastatic prostate cancer—An interim analysis from the ExPeCT trial.


Lifestyle and health-related quality of life in men with metastatic prostate cancer.

ACKNOWLEDGEMENTS

The work presented in this thesis is but one angle of the large, international, multidisciplinary ExPeCT trial, and many people were involved in its delivery. I would like to thank in particular Stephen Finn who conceived and led this project. His encouragement to me at the early stages was really very valuable, in particular when we failed in our initial attempt to acquire funding and he refused to be disheartened. His mentoring and guidance at a formative stage of my career was tremendously important. In addition Orla Sheils and John O’Leary were very important in the initial planning and funding acquisition of the ExPeCT trial. Orla was a constant friendly and supportive presence during my years as a lecturer in Trinity. John took me under his wing as a first-year histopathology SpR and encouraged me to see myself as a researcher, and opened my eyes to all of the possibilities that an enquiring mind can see. The six months I spent with him in the Coombe were some of the most enjoyable of my training.

Thanks also to Lauren Brady, who did most of the filtering and staining of the Dublin ScreenCell® filters on-site in St James’s, and was endlessly patient when her own work was delayed by my tardiness in producing results! She and Tatiana Vlajnic did much early work in cell-line spiking experiments to validate the use of the ScreenCell® filters. Anne-Marie Baird was also closely involved in specimen filtration and staining. Lauren and Anne-Marie also acquired and organised staining of the paraffin tissue blocks from the participants’ needle core biopsies. Emer Guinan and Grainne Sheill ran the exercise component of the ExPeCT trial in Dublin and acquired blood samples. Jean Dunne and Dean Holden performed the flow cytometry analysis at the Department of Immunology at St James’s Hospital, and the staff of the histopathology labs at St James’s Hospital Dublin undertook H&E and immunohistochemistry staining work. I am very grateful for all of their help. In addition I would like to thank all other members of the ExPeCT team in Ireland (particularly Juliette Hussey, Moya Cunningham, Liam Grogan, Thomas Lynch, Rustom Manecksha, John McCaffrey, Dearbhaile O’Donnell, Ray McDermott), the UK (particularly Fidelma Cahill, Mieke Van Hemelrijck, Sarah Rudman, Nicola Peat) and the USA (particularly Lorelei Mucci and Bryan Stanfill, who provided invaluable statistical support). Thanks also to Ronan Leen for help with proofreading.

Finally my thanks to all of the men who participated in the ExPeCT trial, giving selflessly of themselves in order that we might make one small step further along the road to beating cancer.

This research was funded by the World Cancer Research Fund.
1. INTRODUCTION

1.1 GENERAL INTRODUCTION

Prostate cancer (PrCa), the most frequently diagnosed male cancer in the developed world (Jemal et al 2011), is a leading cause of male cancer death. Although prostate specific antigen (PSA) screening identifies many early cancers, numerous men still present with locally advanced or metastatic disease for whom radical surgery with curative intent is inappropriate. In this setting increased disease-free and overall survival and improved quality-of-life (QoL) are the primary management objectives, and new therapies and lifestyle alterations which can assist are increasingly needed.

The incorporation of exercise programmes into cancer care is increasingly recognised to have beneficial effects. A systematic review of exercise interventions in cancer survivors found improvements in strength, fatigue, fitness, functional QoL, self esteem and anxiety (Speck et al 2010). Much of this research relates to colon and breast cancer but increasing evidence demonstrates improvements in symptom control, all-cause mortality and cancer-specific mortality in PrCa also. Improved cancer-specific mortality suggests the potential for exciting developments in our understanding of the biology and aggressiveness of PrCa, together with novel therapeutic opportunities.

In those PrCa patients with potentially curable disease, obesity and its complications may make surgery impractical. Androgen deprivation therapy (ADT), the mainstay of systemic treatment for hormone responsive advanced PrCa, itself causes obesity and metabolic syndrome (MS). As medical therapy for obesity-related cardiovascular risk factors improves, aggressiveness of PrCa becomes more important than cardiovascular complications in determining the cause of mortality in these men. We know that obese men have a worse outlook regarding cancer-related mortality than non-obese men. The combination of an aging population with an increased PrCa incidence, increasing obesity prevalence and improved management of cardiovascular risk factors means that in the future more men are going to die as a result of the deleterious effect of overweight in advanced PrCa.

This thesis describes a series of experiments on material derived from men with advanced PrCa, intended to assess the link between obesity, exercise, systemic inflammation and coagulability
and circulating tumour cells. The material was derived from men who participated in the ExPeCT trial (Exercise, Prostate Cancer and Circulating Tumour Cells), a randomized controlled trial comparing various clinical and circulating biomarkers between men with advanced PrCa who participated in an organised exercise intervention, and those who did not.
1.2 THE PROSTATE GLAND

1.2.1 ANATOMY AND HISTOLOGY

The prostate gland is the largest accessory gland in the male reproductive system, and normally weighs approximately 20g. It is situated in the pelvis and shaped like an inverted pyramid, with its base applied to the trigone of the bladder and its apex pointing inferiorly. The prostatic urethra runs through the prostate gland. The glandular tissue can be divided into four regions on the basis of anatomy and function: the central zone, transitional zone, peripheral zone and an anterior region of fibromuscular stroma.

The microanatomy of the prostate comprises glands and supporting stroma. Microscopically the glands appear convoluted with papillary infoldings, and are lined by tall columnar cells with secretory features, having round basal nuclei and pale or clear cytoplasm (Young et al 2006). A layer of basal cells is also present at the basal aspect of the secretory cells – these can be

Figure 1.1: The prostate gland. Henry Gray. Anatomy of the Human Body. 1918 (source: Wikimedia commons)
highlighted with immunohistochemical stains (e.g. p63), and are a useful diagnostic adjunct in the assessment of individual glands as malignant or benign, as invasive glands lack a basal cell layer.

1.2.2 PROSTATIC HYPERPLASIA

Nodular hyperplasia is a very common disorder, generally affecting men over the age of 50, with the potential for compressive obstruction of the urethra and associated lower urinary tract symptoms. Microscopic evidence of hyperplasia rises in incidence from 20% at age 40 to 90% at age 70, but direct correlation with symptomatology is not a feature (Kumar et al 2005). Nodular hyperplasia (benign prostatic hyperplasia – BPH) tends to arise in the transitional zone of the gland. Surgical treatment of BPH by transurethral resection (TURP) can give rise to the incidental diagnosis of prostate cancer in the resected tissue.

1.2.3 PROSTATITIS AND PROSTATIC ATROPHY

Figure 1.2: Acute prostatitis (source: Wikimedia commons)

Acute bacterial prostatitis, which often follows catheterisation or surgical procedures on the lower urinary tract, is generally caused by gram negative organisms such as E. coli (Khan et al 2017). It presents with fever and dysuria. Chronic prostatitis on the other hand presents with ill-defined symptoms of back pain and discomfort, and may be difficult to recognise and treat effectively. It can be caused by chronic bacterial infection within the gland, which is relatively
resistant to systemic antimicrobial treatment, or can frequently be abacterial. Granulomatous prostatitis can be caused by tuberculosis, Bacille Calmette-Guerin treatment of bladder cancer, or can be nonspecific and idiopathic.

![Figure 1.3: Simple atrophy (centre) and simple atrophy with cyst formation (top) (source: Wikimedia commons)](image)

Prostatic atrophy is a decrease in the amount of cytoplasm in glandular epithelial cells, often accompanied by other cytological changes, and it can mimic carcinoma histologically. A consensus has emerged that four variants exist – simple atrophy (SA), simple atrophy with cyst formation (SACF), post-atrophic hyperplasia (PAH) and partial atrophy (PA) – and there is good interobserver reproducibility among pathologists for their diagnosis (De Marzo et al 2006). SA, the most common, is characterised by crowded angulated glands, sometimes with an infiltratrative distribution, and scant cytoplasm. Immunohistochemistry to highlight basal cells may be necessary to exclude carcinoma. SACF is more easily distinguished from carcinoma – the cysts are lined by flattened or low cuboidal epithelium, and are generally recognised in a lobular fashion. The cysts may be macroscopically identifiable. PAH comprises crowded acini surrounding a dilated duct, and like SA the paucity of cytoplasm imparts a basophilic impression on low power microscopy. PA most closely mimics carcinoma as it comprises closely packed glands with preserved cytoplasm, and therefore a pale appearance at low power.
Prostatic atrophy is often linked with chronic inflammation, to such an extent that SA and PAH are termed “proliferative inflammatory atrophy” (PIA). They are thought by some to be associated with high grade prostatic intraepithelial neoplasia (HGPIN), a probable precursor lesion for carcinoma (De Marzo et al 1999, Putzi and De Marzo 2000). The occurrence of most PIA lesions in the peripheral zone and the morphological identification of “merging” of PIA lesions with HGPIN are presented as supportive evidence (Wang et al 2009). In addition various somatic and epigenetic alterations seen in HGPIN are also seen in PIA lesions (Nakayama et al 2003). Many studies however show no association between atrophic lesions and carcinoma (Servian et al 2015), PrCa lethality (Davidsson et al 2011) or even show that baseline atrophy is protective against the subsequent development of malignancy (Moreira et al 2015).

1.2.4 PROSTATE CARCINOMA

1.2.4.1 Epidemiology

Prostatic acinar adenocarcinoma, by far the most common malignant neoplasm of the prostate gland, is a very common disease, but often clinically undetected. Autopsy studies have shown foci of carcinoma in completely-embedded prostate glands of up to 40% of men in western countries (Breslow et al 1977). An American man aged more than 50 years has a 9.5% lifetime risk of clinical detection of PrCa, and although many cancers never present clinically, PrCa is the second most common male malignancy worldwide, and the fifth most common cause of male cancer death (Humphrey et al 2016, Bray et al 2018). In Ireland, most Western European countries and most countries in North and South America, PrCa is the most frequently diagnosed cancer in men in 2018 (Bray et al 2018). There is a dramatic variation worldwide however, and while the age-standardised incidence rate is 85.7 per 100,000 in Western Europe, it is only 5 per 100,000 in south central Asia (Bray et al 2018). Currently identified risk factors for the development of PrCa are predominantly dietary and include ingestion of red meat and animal fats, and milk and dairy products. Tomatoes, fish, cauliflower and broccoli may be protective (Humphrey et al 2016).

1.2.4.2 PSA

In the developed world most PrCa is not symptomatic at diagnosis, and is rather detected on screening for the prostate-derived serine protease PSA. PSA can be elevated in various benign prostate diseases, including BPH and prostatitis, but low PSA levels (less than 1 ng/mL) indicate a very low risk of PrCa (Aus et al 2005). When integrated with clinical examination findings (i.e. digital rectal examination, DRE) PSA can be used clinically to determine the need for biopsy.
### 1.2.4.3 Pathological features

Tissue can be acquired for pathological diagnosis of PrCa through fine needle aspiration (FNA - no longer recommended for routine use), needle core biopsy (NCB - 10-12 systematic 18-gauge ultrasound-guided cores, with additional targeting of any clinically or ultrasonographically suspicious areas), transurethral resection (typically performed for management of BPH, with incidental detection of carcinoma in a minority of cases) or simple prostatectomy (for treatment of BPH). Radical prostatectomy as definitive treatment for PrCa is not undertaken without pre-operative histological diagnosis. Sometimes the primary diagnosis is made at the time of sampling of metastatic sites, such as targeted biopsy of bony lesions, or detection of carcinoma in bone reamings following pathological fracture.

![Figure 1.4: Morphological features of prostatic adenocarcinoma. Small malignant glands, closely packed, nucleolated, with blue luminal mucin (source: Wikimedia commons)](image)

Microscopic features of carcinoma include: crowded small glands, prominent nucleoli, nuclear enlargement and hyperchromatism, amphophilic cytoplasm, luminal crystalloids or blue-tinged mucin. In general the diagnosis rests on the assessment of multiple microscopic features but perineural invasion, glomerulations and mucinous fibroplasias (Baisden et al 1999) are considered highly specific for carcinoma on biopsy. Many morphological variants of acinar
adenocarcinoma exist, some of which have a worse prognosis (signet-ring carcinoma, pleomorphic giant cell carcinoma, sarcomatoid carcinoma – Humphrey et al 2012) and some of which can histologically mimic benign prostate epithelium (atrophic and foamy gland variants). In small foci, the absence of p63-positive, 34βE12-positive basal cells, combined with cytoplasmic positivity for P504S (AMACR / racemase), can support morphological assessment in making the diagnosis. Sometimes broader immunohistochemical panels are required to distinguish poorly-differentiated prostatic carcinoma from urothelial or colorectal carcinoma (Epstein et al 2014).

1.2.4.4 Prognosis

Various prognostic features, clinical, histological and molecular, have been identified. Gleason grading, first developed in the late 1960s (Gleason and Mellinger, 1974) remains the most useful histological factor. Gleason grading is based entirely on architectural rather than cytological features, and the Gleason score is assigned based on the two worst grades present. On NCB, the number of cores involved by tumour and the percentage of tissue involved also have prognostic significance (Srigley et al 2009). Pathological stage, primarily determined in radical prostatectomy specimens, is the second most useful pathological feature. The AJCC TNM 8th edition staging system is based on the presence and volume of tumour on one or both sides, extraprostatic extension (including seminal vesicle involvement) and invasion of adjacent structures (Amin et al 2017).

1.2.4.5 Management strategies

Management of PrCa is stratified depending on risk categories, usually defined as follows (D’Amico et al 1998, Heidenreich et al 2014, Heidenreich et al 2014):

- Low risk: cT1-T2a and Gleason score ≤ 6 and PSA < 10μg/L
- Intermediate risk: cT2b-T2c or Gleason score = 7 or PSA 10-20μg/L
- High risk: cT3a, Gleason score 8-10 or PSA > 20μg/L
- Very high risk: cT3b-T4 or cN1

Clinical guidelines in use in Ireland (Department of Health, 2015) indicate that active surveillance may be suitable for men with lowest risk PrCa, but this may be converted to active treatment in the event of changes in PSA or DRE findings, upgrade in disease on NCB or imaging findings consistent with disease progression. Radical prostatectomy is the treatment of choice for men with low and intermediate risk PrCa and a life expectancy exceeding ten years, and can also be considered in high risk disease often in combination with other therapies. Radical prostatectomy
may be accompanied by lymph node dissection if the estimated risk for node involvement exceeds 5%. Patients with positive margins or seminal vesicle involvement benefit from adjuvant radiotherapy, and salvage radiotherapy is used in patients who develop a detectable PSA without evidence of metastatic disease. Standard treatment at the time of biochemical relapse or metastatic recurrence is ADT. Other hormonal therapies (enzalutamide, abiraterone) can be considered in castration-resistant PrCa, and docetaxel-based chemotherapy is usually used in advanced or symptomatic disease. Radium-223 may benefit patients with predominantly osseous metastatic disease.
1.3 CIRCULATING TUMOUR CELLS

1.3.1 DEFINITION
Circulating tumour cells (CTCs) are an intermediate stage in the process of metastasis, whereby a cancer can spread from a primary site to set up secondary malignant growths at anatomically distant sites. In order to establish a metastatic deposit cancer cells need to invade into the circulation at the primary site, embolise within the blood and survive the various immunological threats posed to them in doing so, lodge within the circulation at a capillary bed within a distant site, invade into the surrounding tissues and grow within these tissues, whose microenvironment may be less conducive to neoplastic growth than the primary site. As metastases become more established or following resection of a primary tumour CTCs may increasingly originate within metastatic deposits rather than within the primary tumour itself (Micalizzi et al 2017). The CTC stage is a potentially useful target for therapy as intravascular cells are vulnerable to the cellular and humoral processes of the innate and adaptive immune systems. Haematogenous dissemination of CTCs should be considered separately from the process of metastasis to local / regional lymph nodes, by which malignant cells spread through lymphatic vessels.

1.3.2 STEMNESS AND CTCs
The cancer stem cell (CSC) model posits that carcinomas develop from mutated adult stem cells with high tumorigenicity, capacity for self-renewal and ability to recapitulate the heterogeneity of the original tumour when transplanted. CSCs represent a minority of cells within a tumour but are responsible for its aggressive behaviour (Lang et al 2009). ALDH1A1, part of the differentiation-related retinoic acid pathway, is found in CSCs in haematopoietic and lung cancers, and it has also been isolated from PrCa cell lines (Li et al 2010). ALDH1A1+ cells show many convincing features of PCa CSCs, including the capacity to recapitulate a heterogeneous tumour population when serially passaged in vivo. High ALDH1A1 expression was associated with poor cancer-specific survival.

1.3.3 MEANS OF IDENTIFICATION AND ENUMERATION
CTCs are rare in the circulation and the starting point for research into their biology and significance is the development of filtration or enrichment technologies capable of extracting the small numbers of cells of interest from several millilitres of blood. At the simplest level red blood cells can be lysed, which leaves white blood cells and CTCs for assessment (Hensler et al 2016), but the “signal” of small numbers of CTCs within the abundant background “noise” of leucocytes is so weak that this is unsuitable for most purposes.
The most popular and FDA-approved system for CTC enrichment involves CTC separation from leucocytes by virtue of their affinity for certain specific antibodies, for example EpCAM. Epithelial cells (with an EpCAM+, CD45- immunophenotype) rarely exceed one cell per 7.5ml of blood in patients without malignancy (Allard et al 2004). The CellSearch® Circulating Tumor Cell Kit contains a ferrofluid-based capture reagent, with a magnetic core and anti-EpCAM antibodies. Following immunomagnetic capture fluorescent reagents are added for identification of CTCs, including anti-CK-Phycoerythrin (specific for the intracellular protein cytokeratin, characteristic of epithelial cells), DAPI (which stains the cell nucleus), and anti-CD45-Allophycocyanin (specific for leukocytes). The reagent/sample mixture is dispensed by a proprietary CellTracks® AutoPrep® System into a cartridge that is inserted into a MagNest® cell presentation device, within which a strong magnetic field attracts the magnetically labeled epithelial cells to the cartridge surface. An analyser then scans the entire surface of the cartridge, acquires images and displays any event to the user with co-location of CK-PE and DAPI fluorescence. The CellSearch® system classifies an event as a CTC when its morphology is consistent with that of a tumor cell and it exhibits an EpCAM+, CK+, DAPI+ and CD45- phenotype.

Epithelial-mesenchymal transition (EMT) involves cellular loss of epithelial differentiation and switching towards a mesenchymal phenotype. It occurs in embryogenesis but also in the development of invasion in early stage carcinomas. The loss of epithelial surface markers by EMT-switched tumour cells invading the circulation may impede CTC identification by the CellSearch® system. Cancer cells which have acquired EMT share CSC characteristics and express CSC markers (Kong et al 2010). A drawback of CellSearch® is that cells which have successfully invaded the circulation will have undergone EMT, often losing epithelial surface antigens such as EpCAM in the process. It is thought that unfortunately many of the circulating CSC-like cells, which would be the fraction most likely to be biologically significant, undergo EMT as part of the metastatic cascade and thus slip beneath the radar (Chen et al 2012, Yu et al 2013). Other markers such as cell-surface vimentin have been reported to be expressed in CTCs which have undergone EMT, increasing the sensitivity of immunoaffinity-based assays (Satelli et al 2015).

Apart from differences in cell surface markers, CTCs also differ from leucocytes in their size, shape, deformability and other biophysical properties, differences which can be exploited for CTC identification and enrichment. For example, centrifugal forces can be used to sort circulating cells by size along a spiral microchannel (Hou et al 2013). The ScreenCell® system (ScreenCell® Paris, France) does not rely on EpCAM expression and so may be more useful in isolation of CTCs with
EMT gene expression. ScreenCell® filters use a microporous membrane filter and a vacuum tube to allow leucocytes to pass through the 7.5μm filter pores, while trapping the larger and less deformable CTCs on the filter (Desitter et al 2011). Several devices are available from ScreenCell® for isolating CTCs for different purposes – the “Cyto” filter isolates fixed cells for morphological assessment, including immunocytochemistry or FISH studies, the “CC” filter allows isolation of live cells for culture and the MB filter allows filtration of cells suitable for extraction of nucleic acids and further molecular genetic analysis. An additional advantage of size-based enrichment techniques such as this is the ability to detect CTC clusters, which may have greater capacity to establish metastases (Aceto et al 2014).

Flow cytometry, with its ability to characterise in detail the immunophenotype of small numbers of cells in fluid phase specimens, is an attractive method for detection of CTCs, and significant progress has been made in this area (Ulrich and Tarnok 2014). Both in cell line spiking experiments (Takao and Takeda 2011) and in clinical specimens from patients with head and neck squamous cell carcinoma (Hristozova et al 2012) flow cytometry techniques with antibodies against EpCAM and CK7 were found to be sensitive. A novel platform allows flow cytometric isolation of both single and clustered CTCs, followed by their molecular characterisation (Bhagwat et al 2018), in a mouse model of pancreatic cancer. Flow cytometry is likely in the future to be a powerful tool in translational and clinical research into CTCs in cancer patients.

1.3.4 PROGNOSTIC SIGNIFICANCE
CTC enumeration, using technologies such as the FDA-approved CellSearch® system or the filtration-based ScreenCell® system may have a prognostic role in advanced PrCa. Higher numbers of CTCs are found in patients with prostate and breast carcinoma than gastrointestinal carcinomas, suggesting that the portal circulation may act as an effective filter. Increasing evidence suggests that the concentration of CTCs may have a prognostic role in advanced PrCa. A prospective study of 231 men with castration-resistant disease found that more than or equal to five CTCs per 7.5ml of blood correlated with a poor prognosis, when assessed either before or after intitiation of a new line of chemotherapy [de Bono et al 2008]. When a variety of clinical, serological and pathological parameters were considered, the model best predictive of survival following chemotherapy was based on baseline LDH, baseline CTC count and fold change in CTC count at 4, 8 and 12 week intervals [Scher et al 2009]. There is accumulating evidence of the prognostic power of CTC counts in lung cancer (reviewed Syrigos et al 2018). In small cell lung carcinoma changes in CTC counts provide indication of survival following treatment (Cheng et al 2016). More recent studies have further emphasised the negative prognostic impact of numbers
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of EpCAM-expressing circulating tumour cells in metastatic prostate cancer, as detected by CellSearch® (de Wit et al 2018). Additional interrogation of CTCs in prostate cancer patients, including the detection of EGFR expression within prostate cancer CTCs, may further refine their prognostic significance above and beyond mere enumeration (Josefsson et al 2017).
1.4 COAGULATION

1.4.1 OVERVIEW OF COAGULATION

The normal functioning of the circulatory system requires that blood should be a free-flowing liquid within the blood vessels, but as these blood vessels are vulnerable to trauma a mechanism for plugging leaks in the system is required. Following vascular injury there is a localised but transient arteriolar vasoconstrictive response (Mitchell 2005). Exposure to the circulation of subendothelial extracellular matrix allows platelet adherence and activation, and further aggregation, to rapidly form a haemostatic plug. At the same time tissue factor (TF) release from damaged tissue and from platelets initiates a series of enzymatic conversions of proenzymes into active enzymes (the coagulation cascade), which ultimately leads to the activation of thrombin, which in turn converts the soluble plasma protein fibrinogen into insoluble fibrin. A permanent plug is formed by polymerised fibrin and platelets, and at this point regulatory systems are activated to ensure that coagulation is limited to the site of injury and does not become widespread. A balance between pro- and anticoagulant forces is required for normal circulatory function and in order to prevent spontaneous coagulation there are a variety of protective systems at play. Production of nitric oxide (NO) and prostaglandin I₂ by intact endothelial cells prevents inappropriate activation of platelets. Anticoagulant molecules (antithrombin III, protein C, thrombomodulin) which interfere with the coagulation cascade are expressed by endothelial cells. Endothelial cells also produce tissue plasminogen activator, which promotes enzymatic breakdown of polymerised fibrin from endovascular surfaces.

1.4.2 PLATELETS – STRUCTURE AND PHYSIOLOGY

Platelets (thrombocytes) are cytoplasmic fragments derived from bone marrow megakaryocytes. When unactivated they are lenticular discs, 2–3μm in diameter, and they lack nuclei. On a May-Grunwald-Giemsa (MGG) stained smear of peripheral blood they appear as dark purple spots (see image). Although first identified in the first half of the 19th century James Homer Wright coined the term “platelet” in 1910 (Wright 1910).
Figure 1.5: Platelets and erythrocytes, MGG, 60x. Individual platelets are visible at centre and upper right. Clumps of platelets are also present at bottom left (this specimen is derived from a thyroid FNA procedure and some platelet aggregation has occurred in the short time between the needle being inserted into the thyroid and its contents being expelled onto a glass slide).

Platelet function in haemostasis can be divided into adhesion, secretion and aggregation (Mitchell 2005). Platelets first adhere to components of extracellular matrix via von Willebrand factor (vWF), which joins platelet surface molecules such as glycoprotein Ib to collagen fibres with sufficient strength to overcome the shear forces of flowing blood. Adhesion is followed by secretion by platelets of alpha granules (which contain fibrinogen, fibronectin, factors V and VII PF4, platelet derived growth factor [PDGF] and transforming growth factor beta [TGFβ]) and dense bodies (which contain histamine, serotonin, adrenaline, calcium and adenosine di/triphosphate [ADP/ATP]). Calcium helps the coagulation cascade to proceed and ADP both mediates subsequent platelet aggregation and increases dense granule release from adjacent platelets in a positive feedback loop. Platelet aggregation is mediated by ADP and thromboxane A2 (also synthesised by platelets). ADP allows the platelet surface glycoprotein Ib-IIIa receptor to bind fibrinogen, linking together adjacent platelets. In addition, fibrinogen is converted to fibrin...
by thrombin at the end of the coagulation cascade, which forms an insoluble net of fibres which tie the platelet mass together.

1.4.3 PLATELETS AND CANCER

Trousseau first described “phlegmasia alba dolens” in 1865 – lower limb ischaemia as a consequence of deep vein thrombosis in the setting of gastric cancer (Trousseau 1865) – and his observation was later confirmed by Osler (1900). This was an early clinical observation of the tendency towards hypercoagulability in cancer patients. Further autopsy and clinical studies confirmed the association, and the first suggestion that the underlying cause might be an increased tendency towards platelet adhesion was made in 1949 (Moolten and Vroman 1949). Thrombocytosis in cancer patients (as distinct from increased adhesiveness of platelets) was described in the 1960s (Levin and Conley 1964). 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. Ovarian cancer patients who have thrombocytosis and elevated levels of interleukin 6 (IL-6) and thrombopoietin have more advanced disease and worse outcomes (Stone et al 2012). In tumour-bearing mice and ovarian cancer patients anti-IL6 antibody treatment reduced platelet counts, suggesting that tumour-derived IL6 may play a role. Platelets interact with carcinoma cells in the intermediate CTC phase of metastasis through a variety of mechanisms, which will be explored in more detail in section 5.7.1.
1.5 THE IMMUNE SYSTEM

1.5.1 ADAPTIVE IMMUNITY.

The immune system has evolved over millions of years in an ongoing effort to protect the body from a variety of threats, both external (viral, bacterial and fungal infections) and internal (malignancies). It comprises a complex series of subsystems which can be conceptually divided into innate and adaptive immunity. Adaptive immunity requires antibody-mediated recognition of non-self antigens, and their destruction by a number of lymphocyte-mediated mechanisms, both cellular and humoral. It is extremely powerful in combating infection and by means of plasma cells and memory cells can provide ongoing immunity to an infectious agent following an episode of acute infection (Abbas 2005).

1.5.2 INNATE IMMUNITY

The innate immune system is not organism-specific in its anti-infective mechanisms and can be considered analogous to a first responder to an emergency rather than the well-equipped hospital emergency department to which a casualty is subsequently transported. The innate, pre-existing defences against infection include the mechanical barriers which prevent ingress or proliferation of micro-organisms into the body (e.g. an intact layer of skin or gastrointestinal mucosa, functional cilia-driven clearance of entrapped micro-organisms from respiratory mucus, complete emptying of the bladder following micturition), the malfunctioning of which can predispose to infection. In addition phagocytic cells such as neutrophils (the effector cells of the acute inflammatory reponse) and macrophages can ingest and destroy micro-organisms. Circulating complement proteins can provide a humoral but non-antibody-mediated weapon with which to lyse micro-organisms – these proteins can also be activated as part of the adaptive immune response. The component of the innate immune response with which the current study is primarily concerned is the natural killer (NK) cell.

1.5.3 LYMPHOCYTES

Lymphocytes, which are derived from common lymphoid progenitors known as lymphoblasts, are the main cells involved in the adaptive immune system. They are present in the primary / central lymphoid tissues (bone marrow and thymus, where maturation takes place), in the secondary / peripheral lymphoid tissues (the spleen, lymph nodes and mucosa-associated lymphoid tissues) and as circulating cells in the blood. They are divided into T-lymphocytes (which mediate cellular adaptive immunity), B-lymphocytes (which mediate humoral adaptive immunity) and NK-cells (which are part of innate immunity). In general circulating lymphocytes are small cells with scant
cytoplasm, easily recognised on Giemsa staining. Plasma cells have more abundant amphophilic cytoplasm with a paranuclear pale zone or “hoff”, corresponding to an expanded smooth endoplasmic reticulum. NK-cells are a little larger than circulating B- and T-lymphocytes, have more cytoplasm and contain azurophilic granules, hence the haematological term “large granular lymphocyte”. During acute infections reactive lymphocytes with enlarged nuclei and increased cytoplasm can be seen on a blood smear. Large B-lymphocytes (such as marginal zone lymphocytes with more abundant cytoplasm, or germinal centre B-lymphocytes which can have convoluted or large nucleolated nuclei) can be found within the lymphoid tissues, but tend to be scanty in the peripheral blood.

1.5.3.1 T-lymphocytes
Each T-lymphocyte bears a T-cell receptor (TCR), composed of either α and β or γ and δ polypeptide chains. The TCR allows recognition by the T-lymphocyte of a specific antigen, which involves major histocompatibility complex (MHC)-mediated binding to a peptide in the case of αβ T-lymphocytes and non-MHC-mediated binding to peptides and lipids in the case of γδ T-lymphocytes. Rearrangements of the genes coding for the TCR occur during the process of T-lymphocyte maturation, resulting in the formation of TCRs which are specific for a huge variety of potential antigens, and the presence of a TCR gene rearrangement in a lymphocyte indicates that it is a T-lymphocyte. Part of signal transduction from the TCR into the T-lymphocyte nucleus following antigen binding is mediated by the CD3 molecule, whose presence as determined by immunophenotyping (immunohistochemistry or flow cytometry) is a useful T-lineage specific marker for identification of T-lymphocytes. Other accessory functional molecules such as CD7 and CD2 are also helpful in this regard, and CD4 and CD8 expression divides αβ T-lymphocytes into CD3+CD4+T-helper cells and CD3+CD8+ cytotoxic T-lymphocytes. T-helper cells, whose CD4 molecule binds to MHC class II molecules during T-lymphocyte activation, are primarily responsible for secreting cytokines which modulate the activation and function of other cellular and humoral components of the immune system. T-helper-1 cells secrete interleukin-2 and interferon gamma, and T-helper-2 cells secrete interleukins 4, 5 and 13. Cytotoxic T-lymphocytes, whose CD8 molecule binds only to MHC class I molecules, can secrete T-helper-1 cytokines but mainly function as cytotoxic killers, analogous to the NK-cells of the innate immune system.

1.5.3.2 B-lymphocytes
By analogy to T-lymphocytes, B-lymphocytes recognise antigen by means of immunoglobulins (antibodies) expressed at their surface. Similar to the TCR, the immunoglobulin genes are rearranged during lymphopoiesis in order to maximise the diversity of antigens which can be
bound. A rearranged immunoglobulin gene in a lymphocyte indicates that it is a B-lymphocyte, and detection by polymerase chain reaction of monoclonal immunoglobulin gene rearrangements in a B-lymphocyte population is a useful indicator in clinical practice of neoplastic transformation. Following antigen binding and activation B-lymphocytes can transform into plasma cells and secrete immunoglobulins which, when circulating, form the essential component of adaptive humoral immunity. Useful markers of mature B-lymphocytes include the cell-surface proteins CD19 and CD20. Immature and mature B-lymphocytes express CD79a and plasma cells express CD38 and CD138. Nuclear transcription markers also make attractive targets for immunohistochemistry, with PAX5 and OCT2 expressed in mature B-lymphocytes and MUM1 expressed in plasma cells.

1.5.3.3 Natural killer cells

NK-cells, which constitute 10-15% of peripheral blood lymphocytes, are larger than most circulating T- and B-lymphocytes. They do not have rearranged TCR or immunoglobulin genes and are negative for surface CD3 (although they can express CD3 subchains in their cytoplasm) and for B-lineage specific markers. They can bind and lyse cells which have been opsonised with IgG molecules, derived from the adaptive humoral immune system, but they do not themselves require activation by an intrinsic antigen-antibody reaction in order to undertake this “antibody-dependent cell mediated cytotoxicity”. NK-cell killing is mediated by perforins, molecules which when released close to a targeted cell form channels in the target cell membrane, allowing penetration by proteases such as granzyme, leading to apoptosis or lysis. NK-cells avoid killing cells which express MHC class I molecules (i.e. all normal “self” cells), but viral infection or neoplastic change can reduce MHC class I expression and facilitate NK-cell mediated killing, a process which is assisted by activating receptors such as NCR. NK-cells also secrete cytokines (interferon gamma [IFNγ], tumour necrosis factor alpha [TNFα]) and are activated by cytokines (IL-2, IL-12, IL-15) (Abbas 2015). CD56 (also known as neural cell adhesion molecule) is widely used for immunohistochemical identification of NK-cells, but it is not specific, being also expressed on γδ T-lymphocyte, CD8-positive cytotoxic T-lymphocytes, dendritic cells and a variety of non-lymphoid cells such as neurones and neuroendocrine epithelial cells.

NK-cell numbers in blood and in solid organs, together with their cytotoxicity and cytokine secretion, are reduced in obesity. Obese compared to non-obese patients have fewer circulating NK-cells (Lynch et al 2009), and those with hypertension, raised fasting glucose and unfavourable lipid profiles have less NK-cells than “metabolically healthy” obese patients. Obese subjects have fewer hepatic NK-cells and leptin receptor-positive cells compared with normal weight subjects.
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(Lautenback et al 2011). The NK-cell fraction of blood is sensitive to exercise (reviewed Timmons et al 2008), and five-fold increases in NK-cell concentrations following exercise have been noted. Brief exercise upregulates molecular pathways in circulating NK-cells associated with cancer and cell communication (Radom-Azik et al 2013). In healthy young men, hypoxic exercise training leads to enhanced in vitro NK-cell cytotoxicity (Wang et al 2011).

Obesity has substantial negative quantitative and qualitative effects on NK-cells, the effector cells for killing CTCs. In addition, evidence suggests that interactions between platelets and CTCs can dramatically impair NK-cell mediated immune editing. Recent research (Michelet et al 2018) demonstrates that obesity induces lipid accumulation in NK-cells, driven by peroxisome proliferator-activated receptor, reducing their cytotoxicity and inhibiting their antitumour activity, an exciting discovery into the link between obesity and poor cancer outcomes.
1.6 OBESITY

1.6.1 DEFINITION

Obesity in adults is defined by the World Health Organisation (WHO - World Health Organisation 2018) on the basis of body mass index (BMI), measured as height (in meters) divided by the square of weight (in kilograms – see table 1.1). In children, obesity is defined by WHO according to standard deviations from the mean for a given age and sex or by the Centres for Disease Control according to percentiles. While useful as a population-based metric, BMI is less suitable for indiivdualised risk assessment, as it takes no account of body constituents – for example, a professional rugby player 1.78m in height weighing 98kg would have an obese-range BMI. Other measure such as waist-to-hip ratio, waist circumference and waist-to-height ratio may provide more individualised risk assessment for the complications of obesity (Lee et al 2008).

<table>
<thead>
<tr>
<th>CLASSIFICATION</th>
<th>BMI (KG/M$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>&lt;18.5</td>
</tr>
<tr>
<td>Normal weight</td>
<td>18.5-24.9</td>
</tr>
<tr>
<td>Overweight</td>
<td>25.0-29.9</td>
</tr>
<tr>
<td>Obese Class I</td>
<td>30.0-34.9</td>
</tr>
<tr>
<td>Obese Class II</td>
<td>35.0-39.9</td>
</tr>
<tr>
<td>Obese Class III</td>
<td>≥40</td>
</tr>
</tbody>
</table>

*Table 1.1: Definition of obesity and overweight*

MS is a constellation of cardiovascular disease risk factors, including hypertriglyceridemia, hypertension and low high density lipoprotein (HDL) -cholesterol, with central adiposity and insulin resistance the most important components. Various diagnostic criteria have been proposed (summarised in Grundy et al 2005). The American Heart Association definition is widely accepted (Grundy et al 2004 and 2005) but varies from other internationally-employed definitions. In particular the earlier WHO definition (Alberti and Zimmet, 1998) required the demonstration of insulin resistance as a diagnostic criterion. A consensus statement attempting to reconcile the various definitions from a number of international groups provides a useful illustration (Alberti et al 2009). By this consensus, the presence of any three of the five criteria listed in table 1.2 suffice to diagnose MS.
Table 1.2: Criteria for diagnosis of metabolic syndrome

Despite the close association of obesity with MS, the WHO definition of obesity does not figure in the diagnostic criteria for MS. Rather it is central adiposity which appears to confer a great degree of the cardiovascular risk in obesity, and hence the inclusion of waist circumference as a surrogate marker for this. In European populations a waist circumference of ≥102cm in men or ≥88cm in women is recommended by the European Cardiovascular Societies as the diagnostic threshold for abdominal obesity (Graham et al 2007).

1.6.2 EPIDEMIOLOGY

The late twentieth century saw a dramatic increase in the prevalence of overweight and obesity worldwide. Age-standardised prevalence of obesity increased from 3.2% in 1975 to 10.8% in 2014 in men, and from 6.4% to 14.9% in women (NCD Risk Factor Collaboration 2016). In the USA more than 30% of adults are obese (Ogden at al 2014). Between 1990 and 2002, obesity in Irish men increased from 8% to 20%, with a further 47% of men overweight (McCarthy et al 2002). Obesity and its complications exact a heavy toll, both in terms of medical and social problems at the level of the individual patient, and in health economic terms (Bomberg et al 2017). These costs are progressively increasing - the cost to the NHS in the United Kingdom of obesity and its complications is expected to continue to rise by at least £1.9bn per year until 2030, and the increase in cost in the USA is expected to be at least $48bn per year (Wang et al 2011).
1.6.3 AETIOLOGY

Obesity and overweight are fundamentally a consequence of an imbalance in caloric intake on the one hand and energy expenditure on the other. Individual choices and activities clearly play a role in the development of obesity. However individuals can only act and make choices within the environment they inhabit and public policy can therefore play an important role in tackling obesity at a population level. For example the wide availability of energy-dense foods rich in refined carbohydrates and saturated fats, and the relative lack of availability of fresh fruit and vegetables in many areas (Larsen et al 2009) contribute to obesity. The increasingly sedentary nature of work and leisure time and decreases in routine exercise are long-term societal shifts which make obesity a problem which requires increasingly active measures to combat. At the individual level there are many recognised risk factors for obesity. Modifiable risk factors include lack of physical activity, unhealthy eating behaviours, sleep deprivation and psychological stress. Exposure to obesogenic chemicals (including cigarette smoke, air pollution and bisphenol A in childhood) may be avoidable. Non-modifiable risk factors include family history, certain genetic conditions (e.g. Praeder-Willi syndrome), race (obesity in America is most prevalent among black and Hispanic people) and age.

1.6.4 COMPLICATIONS

The health consequences of obesity are numerous (reviewed, Segula 2014, Kinlen at al 2018). They include diabetes mellitus, hypertension, dyslipidaemia, coronary artery disease and cerebrovascular disease, heart failure, dementia and a variety of cancers. A population-based American study estimated that 14% of all male cancer deaths and 20% of all female cancer deaths between 1982 and 1998 were caused by overweight and obesity (Calle et al 2003). Respiratory diseases such as obstructive sleep apnoea and asthma are closely linked with obesity. Disordered regulation of the immune system leads to increased infections and may also be associated with increased autoimmune diseases (Versini et al 2014). Obesity has surpassed alcohol and viral infection as the most common cause of chronic liver disease globally, and can progress to cirrhosis with its attendant complications. Gallstones, pancreatitis and gastro-oesophageal reflux disease are all closely linked with obesity. Fertility problems are frequent in obese men (reduced sperm count and erectile dysfunction) and obese women (e.g. polycystic ovarian syndrome). Other complications of obesity include osteoarthritis, gout and various psychological disorders including depression.
1.6.5 ASSOCIATIONS WITH SYSTEMIC INFLAMMATION

Obesity is well-recognised to be associated with an inflammatory state in metabolic tissues (reviewed by Gregor and Hotamisligil 2011). The distinction of “insulin-dependent” from “non-insulin-dependent” patients with diabetes mellitus led to a recognition that while some patients lacked insulin entirely, others were resistant to its effects. This latter group were often overweight or obese and were hyperinsulinaemic (Yalow et al 1965). Patients with infections or other states causing hypercortisolaemia sometimes developed insulin resistance, which was presumed to be related to hypothetical circulating insulin antagonist molecules. In a seminal paper, TNFα was shown to be constitutively expressed in adipose tissue and hyperexpressed in obesity (Hotamisligil et al 1993), as are a number of other inflammatory mediators (e.g. IL-6, IL-1β, CCL2). In addition to increased cytokine expression in adipose tissue obese individuals also have increased numbers of inflammatory cells (particularly pro-inflammatory M1 macrophages, NK-cells and mast cells) in these tissues, both in mouse models (Liu et al 2009, Ohmura et al 2010) and humans (Harman-Boehm et al 2007). The T-lymphocyte populations also alter, with a decreased CD4:CD8 ratio in adipose tissue. These cellular and molecular inflammatory changes in adipose tissue are chronic in duration rather than acute, and do not seem to lead to the resolution and tissue remodelling and fibrosis which is the typical sequela of chronic inflammation.

The systemic inflammation induced by obesity has effects on many different tissues. For example in the adipose tissue TNFα induces insulin resistance in adipocytes (Hotamisligil et al 1994). Signalling abnormalities are also seen in the liver, with steatosis and (if the inflammatory process escalates) steatohepatitis. In muscle tissue inflammatory cytokines can induce insulin resistance (Plomgaard et al 2005), reducing glucose uptake by this metabolically active tissue and tending towards hyperglycaemia and the diabetic paradigm of “starvation in the midst of plenty”. The pancreas also shows increased pro-inflammatory cytokine and macrophage numbers during the development of obesity-induced insulin resistance (Ehses et al 2007). Alterations in gut microbiota with associated inflammatory changes are also seen (Gregor and Hotamisligil 2011).

The mechanism by which obesity initiates these inflammatory changes is unclear, and in particular it is difficult to identify the initiating factor (the equivalent of a foreign body or tissue trauma in acute inflammation, for example). The theory of metabolic endotoxaemia is that obesity induces increased intestinal permeability. This leads to increased serum lipopolysaccharide following feeding in obese individuals, in turn leading to activation of the inflammatory cascade at a consistently higher level than in lean individuals, in whom the low-
level peaks of inflammation resolve when the nutrients have been metabolised (reviewed by Boutagy et al 2016). Some nutrients also appear to have an intrinsic ability to activate components of the immune system, suggesting that when consumed in excess (as in obesity) they may directly drive the inflammatory response to obesity. For example toll-like-receptor 4 (TLR4), the receptor for lipopolysaccharide (LPS) which is important in initiating the innate immune response of macrophage phagocytosis, appears to be activated by long-chain saturated fatty acids (lcSFAs). However TLR4 may not act directly as a receptor for fatty acids, rather “TLR4-dependent priming alters cellular metabolism, gene expression, lipid metabolic pathways, and membrane lipid composition, changes that are necessary for lcSFA-induced inflammation” (Lancaster et al 2018).

Apart from its metabolic effects, insulin mediates many other processes, including platelet inhibition by increasing NO expression, anti-inflammatory activity by decreasing nuclear factor kappa-light-chain-enhancer of activated B cells (NFκb) and C-reactive protein (CRP), and anti-thrombotic activity by reducing TF activity. In a rat model, rises in serum IL-6 and TNFα were suppressed by insulin (Dandona et al 2005). Insulin resistance is characteristic of MS. Therefore, obese men tend to be in a pro-inflammatory and pro-thrombotic state.

TF functions as the principal initiator of the coagulation cascade, and also as a pro-inflammatory mediator, triggering signaling through G-protein–coupled receptors. TF appears to have a critical role at the crossroads of obesity, inflammation and thrombosis (Badeanlou et al 2011). In mice, inhibition of TF-mediated signalling in haematopoietic cells reduced high-fat-diet-related insulin resistance (through downregulation of TNFα and IL-6 and upregulation of anti-inflammatory IL-10), without impact on body weight. In adipose tissue, inhibition of TF-mediated signaling led to reduced weight gain.

1.6.6 ADIPOKINES
The serum levels of adipokines, produced by adipose tissue and playing roles in appetite, energy balance, and insulin resistance, vary depending on adiposity. Adipokines have potentially pro-oncogenic roles in angiogenesis and cell proliferation. Adiponectin has anti-inflammatory effects and its serum concentration is inversely correlated with adiposity. It dose-dependently reduces platelet aggregation (Restituto et al 2010) and suppresses inactivation of NO, an inhibitor of platelet activation. Resistin is associated with insulin resistance through adenosine monophosphate (AMP) kinase down-regulation. It upregulates proinflammatory cytokines (IL-6, TNFα) which act via the NFκb pathway to increase pro-proliferative, pro-inflammatory and anti-
apoptotic protein transcription. Nuclear expression of NFkB is associated with nodal metastasis in PrCa (Ismail et al 2004).

1.6.7 THE ROLE OF SKELETAL MUSCLE IN OBESITY

Skeletal muscle plays an important role in counteracting the pro-inflammatory effects of obesity. Contracting skeletal muscles release myokines which act as antagonists to the generally pro-inflammatory adipokines (Reviewed Pedersen et al 2011). For example, although muscles produce IL-6, a pro-inflammatory mediator which contributes to the deleterious effects of MS when chronically elevated, they do so in a TNFα-independent manner. Physically active people have low basal levels of IL-6. High basal levels are associated with MS. This suggests a role for muscle-derived IL-6 in metabolism rather than inflammation, and IL-6 stimulates insulin-mediated glucose uptake in muscle cells in-vitro. Other myokines include IL-15, which decreases lipid deposition in preadipocytes, and myostatin, whose expression is reduced by aerobic and strength exercise. The overall effects of the skeletal muscle secretome involve muscle hypertrophy, adipose tissue oxidation, increasing insulin sensitivity, increasing osteogenesis, reducing inflammation, increasing antitumour activity and increasing pancreatic function. Obese men are characterised by sarcopenic obesity, and their reduced muscle mass contributes substantially to insulin resistance and MS. Skeletal muscle-derived factors interact substantially with those derived from adipose tissue, and increasing skeletal muscle mass as well as reducing adiposity is likely to be of benefit in reducing platelet cloaking of CTCs. The sarcopenia caused by ADT may be responsive to antagonists of myostatin (Padhi et al 2014). Myostatin negatively regulates skeletal muscle growth and is a member of the TGFβ superfamily. Chronic myostatin exposure can cause apoptosis in cancer cells, through a shift from oxidative phosphorylation to glycolysis (Liu et al 2013).

1.6.8 OBESITY AND PROSTATE CANCER

Hypogonadism, due to ADT – an important treatment for advanced PrCa - is an independent risk factor for the components of MS (Smith et al 2001, Dockery et al 2003, Smith et al 2006, Keating et al 2010, Keating et al 2006). 50% of men undergoing longterm ADT have MS (Braga-Basaria et al 2006), possibly contributing to the excess non-cancer mortality in this population (Van Hemelrijck et al 2010). Obesity / MS is associated with progression but not incidence of PrCa (Smith et al 2008). Overweight and high plasma C-peptide levels predispose to cancer death in men subsequently diagnosed with PrCa (Ma et al 2008). However, evidence for MS as a risk factor in development of PrCa is inconclusive. One study of more than 1800 men found a relative risk for development of PrCa of 0.77 in men with MS, perhaps due to a reduction in bioavailable
testosterone [Tande et al 2006]. A separate study of 1880 men found a relative risk for PrCa development of 2 in overweight men and 3 in obese men [Laukkanen et al 2004]. However, the evidence that overweight and obesity confer a worse prognosis in PrCa is more definitive. One study of a cohort of 1554 men from the RTOG 92-02 trial found that 210 deaths were due to PrCa, and that overweight conferred a hazard ratio for PrCa-related death of 1.77 [Smith 2008].

IL-6 and TNFα are both raised in the serum of patients with metastatic carcinoma, compared to patients without metastases. Both are elevated in metastatic PrCa in direct proportion to disease stage, and increases occur at the time of biochemical (PSA) disease progression (Michalaki et al 2004). TNFα enhances the invasion of PrCa cell lines through synthesis of selectin ligands (Radhakrishnan et al 2011).

MS may promote PrCa aggressiveness through altered insulin-like growth factor (IGF) signalling. IGF1 binds to IGF1 receptor (IGF1R), and its function can be modulated by IGF binding proteins (IGFBPs). When activated IGF1R increases proliferative and anti-apoptotic activity via the RAF and phosphoinositide-3-kinase (PI3K) pathways (Aggarwal et al 2013). MS-associated hyperinsulinaemia appears to inhibit the modulatory function of IGFBP1, increasing the concentration of nonbound bioavailable IGF1 (Luo et al 1992). In addition to this systemic effect, the local tumour environment also leads to increased sensitivity to IGF1 through increased expression of the IGF1R receptor (Hellawell et al 2002, Ryan et al 2007).
1.7 EXERCISE THERAPY

1.7.1 FOR NON-NEOPLASTIC DISORDERS
Exercise has well-documented benefits in improving cardiovascular risk and bone mineral density and in treating obesity and diabetes (reviewed, Warburton et al 2006), the details of which are beyond the scope of this discussion. It also has proven benefits in treatment of a variety of chronic musculoskeletal, nervous, respiratory and cardiovascular diseases (Smidt et al 2005).

1.7.2 IN CANCERS
The diagnosis of cancer may provide a “teachable moment” at which time men are more open to undertaking lifestyle changes to improve their health. Exercise following the diagnosis of cancer can reduce cardiovascular risk factors (Ligibel et al 2008) and improve general QoL even without significant reduction in body weight. Observational studies in breast (McTiernan et al 2010) and colon cancer (Meyerhardt et al 2009) suggest that more physical activity is associated with lower cancer-specific mortality. A Cochrane review (Mishra et al 2012) found that exercise may have beneficial effects at varying follow-up periods on health-related QoL on patients with cancer during active treatment.

1.7.2.1 Primary prevention
Regular exercise is important for primary prevention of many cancers. It is associated with decreased risk of endometrial (Voskuil et al 2007), colon (Boyle et al 2012), bladder (Keimling et al 2014), renal (Behrens et al 2013), gastrooesophageal (Behrens et al 2014) and breast cancers, with a dose-effect relationship in the latter in some studies (Wu et al 2013). Evidence for exercise in reduction of haematological cancer risk is not convincing (Jochem et al 2014).

1.7.2.2 Secondary and tertiary prevention
The emerging evidence for improved cancer-specific overall and progression-free survival in exercise therapy is exciting. While the potential benefits should not be overstated, exercise is increasingly considered an anti-cancer therapy in its own right. A systematic review found improved outcomes in breast and colon cancer (Ballard-Barbash et al 2012). The evidence includes the benefits of post-diagnosis physical activity (Irwin et al 2011) as well as long-term exercise participation before breast cancer diagnosis (West-Wright et al 2009). Similarly in colon cancer there is evidence for both pre- (Haydon et al 2006) and post-diagnosis (Meyerhardt et al 2006 and 2009) exercise. A supervised exercise programme may be associated with improved progression-free survival in lymphoma (Courneya et al 2015).
1.7.2.3 Quality-of-Life

Exercise helps ameliorate non-specific cancer-related symptoms, reduces cardiovascular risk factors (Ligibel et al 2008) and improves general QoL, even without reduction in body weight. A meta-analysis (Tomlinson et al 2014) found moderately reduced cancer-related fatigue and improved symptoms of depression and sleep disturbance. A Cochrane review (Cramp et al 2012) found that “aerobic exercise can be regarded as beneficial for individuals with cancer-related fatigue during and post-cancer therapy”. A separate Cochrane review (Mishra et al 2012) found improvements in body image/self-esteem, sexuality, social functioning, anxiety and pain. These QoL indicators are particularly important in patients with advanced cancers for whom treatment with the aim of cure is inappropriate.

1.7.3 PROSTATE CANCER AND BENEFITS OF EXERCISE

PrCa is a heterogenous disease, and risk factor associations for total non-aggressive disease are different from aggressive / lethal disease (Giovannucci et al 2007). A retrospective questionnaire-based study of 988 cancer patients (T2 or greater) and 1063 controls found that vigorous physical activity and physical activity over the first 18 years of life decreased cancer risk (Friedenreich et al 2004). A large prospective study found no association between occupational or leisure time activity and PrCa incidence (Johnsen et al 2009), although occupational activity was associated with lower risks of advanced stage PrCa. In the Health Professionals Follow-up Study (Giovannucci et al 2005) there was a lower risk of advanced, high Gleason grade or fatal PrCa for men over 65 years of age undertaking the highest category of vigorous activity. There was no association with PrCa incidence for total, vigorous and nonvigor activity overall. Most other population-based studies show similar findings, with little effect of exercise on overall incidence but some association with reduced aggressive cancers (Patel et al 2006, Littman et al 2006, Nilsen et al 2006). Some studies find increased risk of PrCa in selected groups of men undertaking exercise (Wiklund et al 2008, Zeegers et al 2005), which underlines the complexity of the issues involved and the difficulty in controlling for quantity and type of exercise in large-scale observational studies.

For PrCa patients, in whom most exercise studies assess QoL indicators, there is solid evidence that exercise (especially group exercise) improves muscular and aerobic endurance, reduces fatigue and improves overall QoL (Keogh et al 2012). There is relatively strong evidence for improved health-related, social and physical QoL. Exercise has specific benefits in ameliorating PrCa treatment side-effects, such as improved muscular strength, cardiorespiratory fitness, lean
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body mass, and fatigue (Gardner et al 2014). Among 66 men undergoing radiotherapy there was significantly reduced rectal toxicity following 30 minutes of aerobic walking exercise, three times per week, for four weeks (Kapur et al 2010). Among 121 men receiving radiotherapy and / or androgen blockade, both resistance and aerobic exercise improved fatigue, and resistance exercise also improved QoL, strength, triglycerides and body fat (Segal et al 2009). Another study found significant changes in waist circumference following a 16-week intervention, although with high drop-out rates (Culos-Reed et al 2010).

Accumulating data suggest that exercise can modify the biology of PrCa, in addition to improving QoL-related parameters. In a follow-up study of 2705 men with non-metastatic PrCa who survived at least four years following diagnosis, vigorous exercise (cycling, swimming, jogging) for more than three hours weekly led to lower all-cause and cancer-specific mortality (Kenfield et al 2011). Brisk walking trended towards lower cancer-specific mortality without achieving statistical significance. Another study found 57% reduced progression rates among men with clinically localised PrCa who walked briskly for more than three hours weekly. Faster walking pace was associated with decreased risk of progression independent of duration (Richman et al 2011). Several more recent large follow-up studies provide further evidence for physical activity in reducing PrCa-specific mortality (Bonn et al 2015, Friedenreich et al 2016).

1.7.4 EXERCISE AND SYSTEMIC INFLAMMATION

In patients without malignant disease, physical activity is known to reduce the levels of systemic inflammatory markers (Ho et al 2012), such as TNFα, and so exercise may represent a useful means of modifying the negative pro-inflammatory effects of obesity. The anti-inflammatory effect of physical activity appears to be dependent on volume, intensity and type of exercise, and does not depend directly on weight loss (Balducci et al 2010). Published data also support the anti-inflammatory effect of exercise in cancer patients – for example, one study found decreased CRP levels following a 12-week programme of resistance and aerobic exercise (Galvao et al 2010), and preliminary data from the Promenadgruppen study undertaken among 48 PrCa patients from Örebro, Sweden found significant changes in the concentrations of adipokines and inflammatory markers in men who underwent a ten week walking intervention comprising a weekly one-hour walk in small groups, as compared to the control group [Unpublished data]. Both study and control groups showed similar weight loss but the study group showed a 12mmHg reduction in systolic blood pressure, a 33% increase in levels of HDL and a 60% reduction in levels of CRP. IL-6 and TNFα are both raised in the serum of patients with metastatic carcinoma, compared to patients without metastases. Both are elevated in metastatic PrCa in direct proportion to disease
stage, and increases occur at the time of biochemical disease progression (Michalaki et al. 2004). TNFα enhances the invasion of PrCa cell lines through synthesis of selectin ligands (Radhakrishnan et al. 2011).
1.8 RESEARCH TECHNIQUES USED IN THIS PROJECT

1.8.1 LIGHT MICROSCOPY

People have noted since prehistory the refraction of light as it passes through a curved surface into a substance of different density. The earliest lenses were probably made from polished quartz or other crystal and have been dated to the 8th century BCE in the case of the Nimrud-Layard lens (Layard 1853). Writings on optics by Ptolemy and Euclid dating from Greece in the 2nd and 3rd century CE established the basis of understanding of the properties of refracted light, and the theoretic basis of optics was greatly expanded by Islamic scholars between the 9th and 13th centuries CE. Simple magnifying glasses and eyeglasses were being used in northern Italy in the 13th century, but the much higher quality of lenses necessary for microscopic visualisation of organic tissue was such that the first detailed written account was not published until the 1640s, when Giambattista Odierna (whose primary interest was astronomy) described the microanatomy of the eye of a fly in his work “L’occhio della mosca”. Natural scientists in Italy and northern Europe further described the microscopic structure of organic tissue over the following decades, including Marcello Malpighi’s work on the lungs and skin (1685), Robert’s Hooke’s beautifully illustrated Micrographia in which the term “cell” was first coined (1665) and Antonie van Leeuwenhoek’s descriptions of red blood cells, spermatozoa and micro-organisms.

A modern microscope of the sort used in histology laboratories (see figure 1.6) is a compound transmission optical light microscope which passes visible light through the specimen slide and then through multiple lenses to offer a magnified image of the sample, which can be examined visually, photographed or subjected to computerised image analysis.
The light source in modern laboratory microscopes is usually a halogen or LED bulb, with Köhler illumination provided by a field diaphragm and condenser diaphragm and lens to provide even, homogenous illumination of the specimen. The slide to be examined is placed on the stage, which can be moved up and down by means of coarse and fine focus knobs to enable focusing of the image through the objective lenses. Most microscopes include multiple objective lenses (2x to 60x), mounted on a turret which allows for rapid switching between levels of magnification. The turret can be manually rotated or may be driven by an electric motor. Objective lenses of higher magnification usually have a higher numerical aperture, a measure which describes the light-gathering ability and resolution of the objective. The image from the objective is gathered and focused by the eyepiece into the eyes (or eye – the illustrated microscope is binocular rather than monocular). The most common magnification for an eyepiece lens is 10x, giving a total range of magnification for standard histology microscope of 20x – 600x.
1.8.2 HISTOLOGY

Histology is the study of the microscopic anatomy of normal cells, tissues and organs, whereas histopathology is the study of the specific microscopic changes in these structures when they are affected by disease (Young et al 2006). The light microscope (see section 1.8.1) is the essential tool in histology, but the electron microscope is also useful for examining cells at an ultrastructural level.

1.8.2.1 History of histology

By the late 19th century advances in optics and lens making had enabled detailed microscopic studies of many human cells and organs, but two related problems remained. First, human tissue is generally colourless and therefore little distinction was possible between different cell components under the microscope. Secondly organic material tends to undergo autolysis soon after removal from a living patient, and without techniques for tissue preservation examination of delicate structures such as the brain or the mucosa of the gastrointestinal tract was difficult. Haematoxylin, a naturally-occurring pigment derived from the logwood tree (Haemoxylon campechianum) has been used as a nuclear stain since the 1830s (Von Waldeyer 1863), but its combination with a mordant to facilitate binding to tissue components improved results (Bohmer 1865). It has also been used in textile production as a dye (Titford 2005). At alkaline pH it is blue and stains nuclei (which are rich in acidic material) a blue / purple colour. Combined with eosin, a red acidic dye which highlights cytoplasm and extracellular matrix components, the H&E technique used to this day was born in the 1870s (Busch 1878). Other stains such as Ziehl-Neelsen, Gram and the Romanowsky / Giemsa stains followed by the turn of the 20th century (Reviewed, Cook 1997). Tissue preservation and fixation using formalin, which is the most widely used fixative employed today, was first undertaken in 1893. Prior to this the use of paraffin wax to infiltrate the tissue and support it during sectioning had been developed in the mid-19th century. Early microtomes, machines which enabled the preparation of very thin sections of tissue suitable for transmission light microscopy, were used by Andrew Prichard in 1835 (Smith 1915) and by Czech physiologist Wilhelm His (Loukas et al 2008). The cryostat, which enables the preparation of frozen sections, was first employed in 1951 (Titford 2013)

1.8.2.2 Current techniques in histology

Currently, the standard technique for the preparation of H&E sections requires tissue fixation in 10% neutral buffered formalin. The tissue is then dehydrated by immersion in a sequence of baths of ethanol of progressively increased concentration. Xylene is used to remove the ethanol and molten paraffin wax is infiltrated into the tissue in order to make it sufficiently hard for
sectioning. The tissue fragment can then be embedded in a mold containing molten paraffin wax, producing a tissue block suitable for microtomy (see figure 1.7).

![Figure 1.7: Paraffin-embedded tissue blocks](image)

A microtome with a steel knife is used to cut thin sections (e.g. 4μm thick) which are then mounted on glass slides and stained with H&E according to standard protocols. The paraffin-embedded tissue blocks can be stored indefinitely at room temperature, and additional sections can be cut for examination with other histochemical stains (e.g. Elastic-Van Gieson, Masson Trichrome etc) or for immunohistochemistry (IHC). For electron microscopy glutaraldehyde is the tissue fixative of choice and epoxy resins are used for tissue embedding.

1.8.2.3 Immunohistochemistry

Since the first demonstration by Coons (1941) of coupling fluorescent anthracene isocyanate to antipneumococcus antibodies, IHC has expanded to become a crucial part of the diagnostic and research pathologist's armamentarium. It is a technique which employs the principle of specific binding of antibodies to antigens within target cells and tissues. The antibody is combined with a reporter molecule which allows visualisation under the light microscope. The reporter can be a fluorophore or a chromogenic molecule, in which case the enzyme substrates most often used are alkaline phosphatase and horseradish peroxidise. A counterstain (e.g. haematoxylin) can then be applied to ensure that the background cellular constituents which do not bind the antibody are visible in the section. This allows for accurate localisation of IHC staining within cells. Apart from its numerous applications in research, IHC in diagnostic practice aids in categorisation of malignant tumours, determination of site of origin of metastases, and can detect molecules of prognostic or therapeutic significance (e.g. Her2 in breast cancer).
1.8.3 CYTOPATHOLOGY

Cytopathology is the microscopic examination of individual or grouped cells or tissue fragments for the purposes of diagnosis of disease. Unlike in histopathology, in which large intact tissue fragments are cut and sectioned intact, cytology is less effective at visualisation of tissue architecture and the relationships between cells, but allows for greater precision in the examination of the morphological features of individual cells.

1.8.3.1 History of cytopathology

Early examples of cytological diagnosis include an illustrated manuscript showing cells consistent with keratinising squamous cell carcinoma scraped from the surface of a pharyngeal tumour at post-mortem (Beale 1861). Papanicolaou’s seminal work on vaginal smears in carcinoma of the uterus (1941) began an era of expansion of scholarship in cytological diagnosis of cancer in various sites, driven predominantly by American researchers. Despite early scepticism the publication of Papanicolaou’s Atlas of Exfoliative Cytology (1954) and Koss’ Diagnostic Cytology and its Histopathologic Bases (1961) consolidated the discipline of cytopathology and confirmed its widespread acceptance as a valuable diagnostic and screening modality (Ramzy and Herbert 2010).

1.8.3.2 Specimen acquisition and preparation

Specimens for cytological analysis can comprise body fluid specimens (e.g. urine, pleural or peritoneal fluid, cerebrospinal fluid, sputum, blood), specimens derived from instrumented washing of mucosal surfaces (bronchoalveolar lavage, ureteric washings), specimens derived through brushing or scraping cells off a mucosal surface (biliary or bronchial brushing, uterine cervical smears) and FNA of mass lesions. FNA is more useful than NCB in many settings as it is less likely to lead to significant pain or haemorrhage and in many cases can acquire a greater volume of diagnostic material. In particular FNA is the preferred sampling technique for thyroid and salivary gland masses, and is often the first line investigation for lymphadenopathy. Endoscopic or endobronchial FNA allows sampling of lesions within the thoracic and abdominal cavities, and when combined with ultrasound the accuracy of targeting is enhanced. FNA allows the preparation of air-dried direct smears, and rinsing of the needle after specimen acquisition facilitates further examination with cytospin or ThinPrep techniques and the preparation of paraffin-embedded cell blocks for IHC or molecular analysis. Fluid specimens may be submitted to the laboratory neat or in a fixative such as CytoLyt. Direct smears are air-dried and then alcohol-fixed. Centrifugation (e.g. Cytospin) allows concentration of cells in a liquid specimen for transfer to a slide and staining. Liquid-based cytology techniques (e.g. ThinPrep) have
revolutionised sample acquisition and have allowed greater standardisation and automation, particularly in cervical screening cytology, with no loss of sensitivity (Ronco et al 2007).

1.8.3.3 Staining

Papanicolaou’s original staining technique (1942) was continuously updated and refined over the course of his career (Chantziantoniou et al 2017). As currently used, the Pap stain is a polychromatic stain including five dyes in three solutions: Haematoxylin (Harris’s formulation (1900) is usually used in cytology), which stains nuclei blue; Orange Green 6, which stains mature and keratinised squamous cells; and Eosin Azure, composed of Eosin Y (which stains cytoplasm, cilia, nucleoli and erythrocytes pink), Light Green SF (which stains cytoplasm of metabolically active cells blue / green), and Bismarck Brown Y. Pap staining allows for detailed assessment of nuclear morphology (see figures 1.8 and 1.9), as well as differential staining of cytoplasm in keratinising cells.

*Figure 1.8: Mesothelial cells stained with Papanicolaou preparation (60x)*
While Pap staining requires alcohol-fixed cells, Romanowsky-type stains, of which the MGG and Wright-Giemsa are most frequently used, are preferred for air-dried preparations. This makes them suitable for direct smears from FNA procedures, and the rapid staining available from techniques such as Diff-Quik allows for rapid on-site evaluation of slides for adequacy assessment or for diagnostic purposes (Silverman and Frable 1990). The MGG stain comprises a May-Grunwald stain incorporating eosin and methylene blue (which are acidic and basic respectively) and a Giemsa stain incorporating eosin and azure of methylene. The Giemsa stain induces metachromatic red staining for azurophil structures, on top of the orthochromatic May-Grunwald components, which tend to stain nuclei dark blue / purple (see figure 1.10). Compared to the Pap stain, Romanowsky techniques provide superior visualisation of cytoplasmic details and nuclear : cytoplasmic ratio, but cannot match the Pap stain in terms of fine nuclear detail (Krafts and Pambuccian 2011). Extracellular components such as extracellular matrix in salivary gland tumours and soft tissue tumours and colloid in thyroid aspirates are also better stained by Romanowsky techniques. Romanowsky staining has been used for detection of Plasmodia (Giemsa 1904), as well as Leishmania and Toxoplasma. It is also the technique of choice in haematology for assessment of haematological specimens, including blood smears and bone marrow aspirate smears, due to the exquisite detail of cytoplasmic structures and granules which can be seen. In clinical cytology it is crucial for assessment of lymph node FNA and for situations
where detection of neoplastic lymphoid cells is important, such as in cerebrospinal fluid. In practice, clinical specimens are often examined with both Romanowsky and Papanicolaou techniques in order to extract the maximum possible diagnostic information – for example, although MGG is preferred for morphological diagnosis of lymphoma on lymph node FNA, a Pap stain may be helpful in distinguishing centrocytes from centroblasts, as nucleoli are variably well seen on MGG.

Figure 1.10: Thyroid follicular epithelial cells, Diff-Quik, 40x. Note the red blood cells and neutrophils in the background

1.8.4 FLOW CYTOMETRY
Flow cytometry is a valuable multiparametric technique for immunophenotyping of haematopoietic cells, and is well-suited to fluid specimens (blood, cerebrospinal fluid, marrow aspirate) in which only small numbers of cells may be present for assessment (Stetler-Stevenson et al 2017). Following Wallace Coulter’s 1953 patent for an impedance-based system for counting particles suspended in a liquid (U.S. Patent 2,656,508), a cell sorter was developed and published in 1965 (Fulwyler). Processing of blood specimens requires their collection in an anticoagulant such as ethylene diamine tetra-acetic acid (EDTA). A stream of cells of interest are passed in single file through a series of lasers, scattering the light forwards at an angle proportionate to cell volume, and sideways at an angle proportionate to cell structural complexity. The alteration in
intensity of the laser light is measured by detectors. In addition cells can be complexed to antibodies conjugated to fluorescent molecules, specific for cellular molecules of interest. Multiple “colours” of antibody fluorochrome can be used simultaneously so that the expression by a single cell of up to ten different molecules can be assessed, both qualitatively and quantitatively. The more antibodies are used, the higher the diagnostic accuracy (sensitivity and specificity) of detection and characterisation of cells. The light emitted by the fluorochromes is sampled by the detectors and an associated computer system analyses the data (see figure 1.11), providing a powerful technique for research and clinical purposes.

Figure 1.11: Flow cytometer schematic diagram (By Kieran - Own work, CC BY 3.0, https://commons.wikimedia.org/w/index.php?curid=22102570)
1.9 SUMMARY OF PROJECT BACKGROUND

MS is characterised by low-level chronic systemic inflammation (reviewed Esser et al 2014), and evidence suggests that substantial crosstalk occurs between pathways involved in inflammation, coagulation and obesity (Zhang et al 2011). There are substantial preclinical experimental data, both in PrCa cell culture studies and murine models, to support the hypothesis that reductions in systemic inflammatory mediators (with consequent effects on platelet adhesion) may underlie exercise-associated improved outcomes. Incubation with serum from healthy male volunteers following exercise led to 31% inhibition of growth of LNCaP PrCa cell lines, and pre-incubation of LNCaP cells before injection into immunodeficient mice led to delayed tumour formation (Rundqvist et al 2013). Epidermal growth factor (EGF) and IGFBP1 may exert the inhibitory effect of post-exercise serum. A study similar to Rundqvist’s using serum from men undergoing a low-fat, high-fibre diet in addition to regular exercise reduced growth and increased apoptosis in LNCaP cells, associated with reductions in serum IGF1 (Soliman et al 2011). The cells also had increased p53 content (replicated elsewhere Leung et al 2004) and reduced NFκB activation. In a study of men with early stage, low grade PrCa who undertook comprehensive diet and lifestyle changes, it was found that their serum inhibited LNCaP cell growth more than did the serum of a comparison group of men who did not make these changes (Ornish et al 2005). Mice injected with PrCa cells and randomised to voluntary exercise tended towards reduced expression of prometastatic genes, with no change in primary tumour growth rate (Jones et al 2012). There was also improved tumour vascularisation, modulated through increased hypoxia-inducible factor (HIF)-1α and vascular endothelial growth factor (VEGF) concentrations leading to productive (non-pathological) angiogenesis. In a separate severe combined immune deficiency (SCID) mouse xenograft model, voluntary running wheel exercise led to suppressed PC-3 tumour growth in male mice, manifested by reduced mitosis and increased apoptosis. The regimen was associated with increased food and fluid consumption by the mice and led to reduced adiposity but no change in overall body weight (Zheng et al 2008). There are also intriguing clinically-derived data concerning the effects of exercise on systemic inflammation, as discussed in section 1.7.4.

Due to the combination of an aging population and rising levels of obesity, a “perfect storm” of increased incidence and aggressiveness of PrCa is imminent. Low cost interventions are required which can improve QoL and modify disease biology independent of the various surgical, radiological and pharmacological options already available. Although the mechanisms by which it does so are incompletely understood, the pro-inflammatory and pro-thrombotic milieu associated with obesity appears to be the pathway through which this lifestyle-modifiable
disease exerts its aggressive effects in PrCa. There is increasing observational and experimental evidence that exercise can modify the biology of PrCa and ameliorate some of the deleterious effects of obesity in these patients. This project attempts to elucidate some of the disease-modifying effects of exercise and to explore the useful underlying mechanisms.
Chapter 2 – Aims and Objectives

2. AIMS AND OBJECTIVES

2.1 GENERAL AIMS AND OBJECTIVES

The overall aim of this thesis is to explore the relationship between the inflammatory and immunological consequences of overweight / obesity in men with advanced PrCa and numbers of circulating tumour cells, taking into account the effects of an organised programme of exercise.
2.2 SPECIFIC AIMS AND OBJECTIVES

- To enumerate CTCs in blood samples from men participating in the ExPeCT trial, to determine whether they vary during the course of an exercise programme, and to compare numbers between overweight men and those of normal weight.

- To assess in detail the morphological features of CTCs in men with advanced PrCa.

- To identify platelet cloaking in CTCs and to compare between exercise and control groups, and between overweight men and those of normal weight.

- To assess the diagnostic biopsy material from the trial participants for the presence of atrophic lesions, to quantify benign prostate inflammation in the specimens, and to quantify intratumoural inflammatory cells.

- To quantify circulating lymphocyte populations in trial participants and to compare between exercise and control groups, and between overweight men and those of normal weight.

- To correlate CTC numbers with circulating lymphocyte populations, with platelet counts and with diagnostic biopsy inflammation, comparing exercise and control groups and overweight men and those of normal weight.
3. METHODS

3.1 STUDY DESIGN AND ACQUISITION OF FUNDING

In order to test the overarching hypothesis that enhanced platelet cloaking of CTCs in obese men with PrCa (due to increased systemic inflammation) is a mechanism underlying the worse prognosis of cancer in these patients, funding was sought from the World Cancer Research Fund to undertake a randomised multicentre international prospective trial. Funding of £250,000 was granted as part of the WCRF International Regular Grant Programme over a four year period, 2014-2018, under the project title “Evasion of immune editing by circulating tumour cells is an exercise-modifiable mechanism underlying aggressive behaviour in obese men with PrCa”. The trial was titled “ExPeCT” (Examining Exercise, Prostate Cancer and Circulating Tumour Cells), a name which was felt to have positive, optimistic emotive connotations in keeping with the active nature of the exercise intervention under assessment. The hypotheses to be tested were:

1. Platelet cloaking of circulating PrCa tumour cells is more prominent in men who are obese than those who are not
2. Regular exercise can ameliorate platelet cloaking
3. The degree of platelet cloaking varies relative to systemic and primary tumour inflammation and coagulability
4. Expression of an obesity-associated lethality gene signature leads to variation in platelet cloaking.

Participants in the ExPeCT study were to be divided into “exposed” and “non-exposed” groups based on body mass index (BMI ≥ 25 kg/m² or < 25kg/m²). They were randomised to a study group and a control group, with the study group participating in a six-month exercise programme, including a weekly group exercise class and a home-based exercise programme. The control group received usual care. Blood samples were taken, QoL questionnaires were completed and clinical data (including blood pressure, BMI, waist circumference etc) were gathered at the time of recruitment (T0) and after three (T3) and six (T6) months (see figure 3.1). Blood samples were also taken for full blood count (including platelet count) at each timepoint.
Chapter 3 – Methods

ExPeCT Trial Flowchart

Recruitment

- Patient identified in clinic
  - Obtain informed consent
  - Contact trial staff
  - Assign participant number

Are scientific staff available to filter blood sample?

- Yes
  - Take T0 blood sample
  - Complete T0 questionnaire
  - Complete T0 datasheet

- No
  - Trial staff will arrange T0 blood sample later

Randomize to control or intervention group

- Intervention group
  - Telephone call from trial staff to confirm participation ± arrange exercise intervention ± arrange T0 blood sample
  - First exercise class
    - 1. Distribute Polar heart rate monitors
    - 2. Plan exercise regimen
    - 3. Supervised exercise class
  - Daily home exercise regimen
  - Weekly exercise class
    - 1. Download heart rate monitor data
    - 2. Supervised exercise class
  - Complete T3 datasheet, complete T3 questionnaire and take T3 blood sample

- Control group
  - Usual care

Three months

- Daily home exercise regimen and monthly data download

Six months

- Complete T6 datasheet, complete T6 questionnaire and take T6 blood sample

End of participant’s involvement in trial

Figure 3.1: ExPeCT trial flow chart
Chapter 3 – Methods

In order to test the hypotheses listed above, the following four projects were undertaken (see figures 3.2-3.5):

1. Enumeration of CTCs in the T0 blood samples, quantification of adherent platelets and comparison between exposed and non-exposed groups

2. T3 and T6 blood samples assessed for CTC numbers and platelet cloaking and changes compared with the T0 samples, between exposed and non-exposed groups, and between the exercise and control groups.

3. Blood samples assessed for NK-cell numbers, markers of systemic inflammation, adipokines and serum factors related to platelet activation. Prostate NCBs examined morphologically and by immunohistochemistry for atrophy and inflammation, with particular reference to NK-cells, and correlated with platelet cloaking

4. Carcinoma in NCBs assessed for expression of an obesity-associated lethality gene signature (CXCR4, PLA2G7, PTGER1, AVPR2, and HTR2B, genes known to play a role in obesity or platelet aggregation and coagulation), and correlated with platelet cloaking of CTCs.

Figure 3.2: ExPeCT trial project 1 – Does cloaking of CTCs vary between exposed and non-exposed groups?
Figure 3.3: ExPeCT trial project 2 – Does six months of regular exercise reduce platelet cloaking?

Figure 3.4: ExPeCT trial project 3 – Does the degree of platelet cloaking vary relative to markers of systemic and localised tumour inflammation and coagulability?
Figure 3.5: ExPeCT trial project 4 – Is overexpression of certain lethality-associated genes, known to play a role in obesity or platelet aggregation and coagulation, associated with enhanced platelet cloaking?

The data presented in this thesis relate primarily to projects 1 and 2, and to the NCB and circulating NK-cell components of project 3. The author has contributed to publication of the study protocol (Sheill et al 2017), where the full details of the exercise programme and QoL questionnaire can be seen.

Men with metastatic PrCa were recruited from five Dublin hospitals (St James’s Hospital, Tallaght Hospital, St Luke’s Hospital, Mater Misericordiae University Hospital, Beaumont Hospital) as well as one London hospital (Guy’s and St Thomas’ NHS Foundation Trust).

The participant inclusion criteria were as follows:

1. Male patients aged at least 18 years
2. Pathologically confirmed diagnosis of prostate adenocarcinoma
3. Metastatic disease confirmed by CT/MRI or bone scan
4. Stable medical condition (no acute exacerbation of chronic illness, serious infection or major surgery within 28 days prior to randomisation)
5. Capable of participating safely in the proposed exercise intervention, as assessed by a medical practitioner

The exclusion criteria were

1. No history of radical prostatectomy
2. No previous diagnosis of any other malignant tumour (patients with non-melanoma skin cancer or carcinoma in situ of any type were not excluded provided they had undergone complete resection)

Participants were recruited at medical oncology clinics at the various study sites, and informed consent to participate was obtained according to the requirements of the International Conference on Harmonisation – Good Clinical Practice (ICH-GCP). Confirmation of medical fitness to participate was obtained from each participant’s treating clinician. Participants were identified within the study by means of unique Participant Identifier Numbers (PINs). Each participant was randomised to the intervention or comparison group through use of GraphPad software.
3.2 ETHICAL APPROVAL

Ethical approval was sought and obtained from the institutional research ethics committees of the various sites involved in the project.
3.3 CIRCULATING TUMOUR CELLS

3.3.1 ACQUISITION OF SPECIMENS

Blood samples were acquired from each participant for ScreenCell® filtration in 4ml K2EDTA tubes (Grenier Bio Ref:454023) using a 23-gauge, ¾” butterfly needle. Four tubes were acquired at each blood draw (yielding a total of 12-16ml on each occasion) and inverted at least ten times immediately afterwards. They were then stored at room temperature in order to avoid refrigeration-induced increases in viscosity, and filtered within four hours. Blood tubes were labelled with the ExPeCT PIN, timepoint and date of sampling.

3.3.2 FILTRATION OF BLOOD SAMPLES

ScreenCell® CTC enrichment depends on vacuum-assisted filtration through a microporous membrane filter to separate CTCs from other blood cells on the basis of size. Filtration of blood samples was undertaken in accordance with ScreenCell’s guidelines for use of ScreenCell® Cyto filters. The blood was inverted repeatedly prior to commencing filtration. Using a 5ml pipette, 3ml of blood was transferred to a 15ml conical tube, and 4ml of prepared ScreenCell® FC2 buffer was added. The tube was inverted five times and incubated for 8 minutes at room temperature. The 7ml of diluted blood was added to module A of the ScreenCell® Cyto filtration unit (see figure 3.6) using a 10ml pipette. The protective membrane at the bottom of module B was removed and module C was pushed fully into module B until filtration began. Once the blood level reached the yellow line of the filtration unit (see figure 3.6) 1.6ml of phosphate buffered saline was added using a 5ml pipette. Once all of the liquid had passed through the module the filter was released onto a piece of clean filter paper, ensuring that the filter serial code (and therefore the side with cells on it) was facing upwards. The filter was allowed to dry in darkness at room temperature overnight.
3.3.3 STAINING, STORAGE AND TRANSPORTATION OF FILTERS

Filters were stored in ScreenCell® filter storage units (see figure 3.7) prior to and after staining at 4°C. The filter storage unit was labelled with the participant’s ExPeCT PIN, timepoint and date of filtration. Filters were stained using the MGG technique, by immersion in a bath of May Grunwald at room temperature for 150 seconds with constant agitation, followed by a second bath of May-Grunwald diluted 1:1 with deionised water at pH 7 for 150 seconds. They were then immersed in a bath of Giemsa diluted 1:10 with deionised water at pH7 for 10 minutes. They were then rinsed clean of excess stain with deionised water at pH 7 for 1 minute, and air dried at room temperature for 20 minutes.

Stained filters were transported when necessary between clinical sites packaged in insulating material with frozen cold-packs, and stored on delivery in a refrigerator at 4°C. Filters were stored long-term at -20°C.

Figure 3.6: ScreenCell® Cyto filtration unit
3.3.4 MICROSCOPIC ASSESSMENT OF FILTERS

MGG-stained ScreenCell® filters were examined using an Olympus BX41 light microscope with 10x and 40x Olympus Plan Fluorite objective lenses and a 20x Olympus Plan Apo objective lens. Filters were screened at 20x with the condenser in-situ to enumerate CTCs. Groups of cells and cells with features suggestive of adherent platelets were examined at 40x, with and without the condenser. Illustrative photomicrographs were obtained at 20x and 40x with and without the condenser using a Tucsen Truechrome IIS camera and proprietary software. CTCs were counted with the aid of an electronic counter app for iOS (“Clicker Counter”, Rodskagg). The presence (and number) of CTCs and CTC clusters as well as the presence or absence of platelet cloaking and bare nuclei, were recorded per filter using a Microsoft Excel spreadsheet. Filters derived from London-based participants were also assessed for the presence of CTCs which retained their cytoplasm. The mean and maximum number of CTCs per filter was calculated for each blood draw episode. The microscopist was blinded as to whether the filter was from an exercise- or control-group participant, but not as to whether it was derived from a T0, T3 or T6 blood draw.

Figure 3.7: ScreenCell® Cyto filters and storage box
3.3.5 MORPHOLOGICAL DEFINITION OF CTCS, CLUSTERS AND PLATELET CLOAKING

CTCs were defined as cells in the same plane of focus as filter pores whose nucleus was at least twice the diameter of a filter pore, dark blue / purple in colour, generally of uniform staining intensity and with an outline which was well-defined around its entire circumference. Many CTCs were centred on filtration pores and had markedly irregular nuclear contours, but these were not considered definitional features. The presence of cytoplasm was not required to define a CTC as many CTCs lacked any cytoplasm and were considered “bare nuclei”.

*Figure 3.8: Circulating tumour cell bare nuclei on ScreenCell® filter (MGG, 40x)*
A CTC cluster was defined as a cohesive group of two or more cells which individually achieved the diagnostic criteria for CTCs. Flat sheets of large cells which did not show features of CTCs were not counted as CTC clusters.
Platelet cloaking of an individual CTC was defined as the presence of at least one platelet in direct contact with the edge of the CTC. In order to distinguish true platelet cloaking from procedure-related blood clot, platelet cloaking was only confirmed when a cloaked CTC was identified away from any areas of fibrin/platelet clot at the surface of the filter. Dense three-dimensional clusters of platelets were only considered to represent platelet cloaking if a definite CTC could be identified within.

![Figure 3.11: Circulating tumour cell with adherent platelets on ScreenCell® filter (MGG, 40x)](image)

3.3.6 DETAILED MORPHOLOGICAL ASSESSMENT OF SELECTED CTC-RICH FILTERS

The most CTC-rich filter from seventeen London-based participants were selected for detailed morphological examination of CTCs. For each CTC the following parameters were assessed: short axis diameter of nucleus (measured in multiples of filtration pore diameter \([PD]\)), regularity of nuclear membrane (smooth or indented), number of visible nuclear indentations, depth of deepest indentation (half, quarter or eighth of short axis diameter of nucleus), location of filtration pore relative to nucleus (overlying or not overlying pore), radiation of indentations from filtration pore, presence or absence of cytoplasm, short axis nuclear : cytoplasmic diameter ratio and location of nucleus relative to cytoplasm (central, eccentric or peripheral). A CTC nucleus was defined as having a smooth nucleus if it lacked indentations at least as deep as one eighth of the short axis nuclear diameter. For each indented CTC the depth of the largest indentation in PD was extrapolated.
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For each filter and for the sum total of all CTCs across all filters the following statistics were calculated: the proportion of CTCs overlying a filtration pore, the mean / median / maximum / minimum short-axis nuclear diameter, the proportion of CTCs with an indented nucleus, the mean number of indentations in indented nuclei, the mean depth of indentations relative to nuclear diameter, the mean absolute depth of deepest indentation in PD, the proportion of indented nuclei in which indentations were seen to radiate from a pore, the proportion of CTCs with cytoplasm, the mean nuclear:cytoplasmic ratio of CTCs with cytoplasm, and the proportion of such CTCs with central, eccentric and peripheral nuclei.
3.4 PERIPHERAL BLOOD FLOW CYTOMETRY

3.4.1 BLOOD SAMPLE ACQUISITION

At the time of each T0, T3 and T6 blood draw, a blood sample was acquired for flow cytometry from the Dublin-based trial participants. Blood was collected into a sterile K2-EDTA tube and transported promptly to the Department of Immunology at St James’s Hospital where analysis was undertaken.

3.4.2 FLOW CYTOMETRY METHOD

BD Multitest 6-color TBNK reagent (BD Biosciences, San Jose, CA, USA) is provided in 1 mL of buffered saline with 0.1% sodium azide. It contains: FITC-labeled CD3 (clone SK7); PE-labeled CD16 (clone B73.117-19); CD56 (clone NCAM16.2;20); PerCP-Cy™5.5–labeled CD45 (clone 2D1 (HLe-1);21); PE-Cy™7–labeled CD4 (clone SK3;22-24); APC-labeled CD19 (clone SJ25C1;25); APC-Cy7–labeled CD8 (clone SK1.22,23). Prompt processing of specimens is critical as some APC-Cy7 conjugates, and to a lesser extent PE-Cy7 conjugates, show changes in their emission spectra (and therefore artefactually reduced capture by the flow cytometer) with prolonged exposure to paraformaldehyde.

BD Trucount tubes (BD Biosciences, San Jose, CA, USA) include a lyophilised pellet which, on dissolution, releases a known number of fluorescent beads. During analysis comparison of cellular events with bead events allows calculation of the absolute number (cells/µL) of positive cells in the sample.

For each analysis, 20µL of BD Multitest 6-color TBNK reagent (including antibodies to CD3, CD4, CD8, CD19, CD45 and CD56) was pipetted into the bottom of a BD Trucount tube. 50µL of well-mixed EDTA-anticoagulated whole blood was then pipetted into the bottom of the tube. Care was taken to avoid smearing of blood down the side of the tube in order to ensure that the entire volume of blood was stained with the antibodies. The BD Trucount tube was capped, vortexed gently to mix, and incubated for 15 minutes in the dark at 25°C. 450µL of 1X BD FACS lysing solution was then pipetted into the tube which was then re-capped, vortexed and again incubated for 15 minutes in the dark at 25°C. The specimen was then ready to be analysed using the BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The principles of flow cytometry are discussed in section 1.8.4 above. The optics of the BD FACSCanto II consists of an excitation source of three lasers: blue (488-nm, air-cooled, 20-mW solid state), red (633-nm, 17-mW HeNe), and violet (405-nm, 30-mW solid state). The results were analysed using BS...
FACSCanto Clinical software (BD Biosciences, San Jose, CA, USA). An example of the results readout from the FACSCanto II is seen below (Figure 3.12)

![FACSCanto II flow cytometer readout example](image-url)
3.5 NEEDLE CORE BIOPSIES

3.5.1 ACQUISITION OF MATERIAL FROM DIFFERENT SITES

Biopsy material was acquired from Guy’s and St Thomas’ Foundation Trust in London and from all participating hospital sites in Dublin. Tissue blocks were acquired in all cases, and original H&E-stained slides were also acquired in some cases.

3.5.2 CUTTING AND STAINING

Sections for immunohistochemistry and for H&E staining (in those cases for which original H&E slides were not available) were cut using a HM 325 Rotary microtome at 4μm thickness, placed in a water bath at 48°C and placed on glass slides. The sections were heated at 48°C for 30 minutes, then at 62°C overnight and stored at room temperature.

H&E staining was undertaken at the Department of Histopathology, St James’s Hospital, according to standardised protocols, on an autostainer machine. Sections were immersed in a xylene bath and rehydrated with a series of washes in ethanol. They were then immersed in haematoxylin and eosin solutions for between 30 and 120 seconds and rinsed with de-ionised water. This was followed by sequential re-immersion in ethanol baths and xylene, application of a coverslip and drying.

Immunohistochemical staining with antibodies to CD3, CD56 and CD68 was undertaken at the Department of Histopathology, St James’s Hospital, according to standardised protocols.

3.5.3 MICROSCOPIC EXAMINATION

Inflammation within non-neoplastic benign background prostate tissue was assessed. The presence or absence of benign acute inflammation was recorded. For assessment of chronic inflammation in non-neoplastic prostate parenchyma the extent and grade of inflammation in the glandular, periglandular and stromal compartments was reported, a consensus definition for which was described by Nickel et al (2001 – see table 3.1).
<table>
<thead>
<tr>
<th>Anatomical location</th>
<th>Histological pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glandular</td>
<td>Inflammation within ductal / glandular epithelium or lumen</td>
</tr>
<tr>
<td>Periglandular</td>
<td>Inflammation within stroma, centred on glands / ducts, and within 50μm of them</td>
</tr>
<tr>
<td>Stromal</td>
<td>Inflammation within stroma, not centred on gland / ducts, and more than 50μm from them</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Extent</th>
<th>Tissue area involved by inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focal</td>
<td>&lt; 10%</td>
</tr>
<tr>
<td>Multifocal</td>
<td>10-50%</td>
</tr>
<tr>
<td>Diffuse</td>
<td>&gt; 50%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade</th>
<th>Morphological description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/mild</td>
<td>Individual inflammatory cells, separated by distinct spaces (&lt;100 cells / mm²)</td>
</tr>
<tr>
<td>2/moderate</td>
<td>Confluent sheets of cells with no tissue destruction or lymphoid nodule / follicle formation (100-500 cells / mm²)</td>
</tr>
<tr>
<td>3/severe</td>
<td>Confluent sheets of cells with tissue destruction or nodule / follicle formation (&gt; 500 cells / mm²)</td>
</tr>
</tbody>
</table>

Table 3.1: Definition of chronic inflammation in non-neoplastic prostate tissue (Nickel et al 2001)

Figure 3.13: Grade 3 stromal inflammation in benign prostate tissue (H&E, 20x).
Chapter 3 – Methods

The presence or absence was recorded of various types of inflammation-associated atrophy in the background benign prostate tissue, as categorised previously (De Marzo et al 2006, Davidsson et al 2011 – see table 3.2).

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Simple Atrophy (SA)</th>
<th>Postatrophic Hyperplasia (PAH)</th>
<th>Simple Atrophy with Cyst</th>
<th>Partial atrophy (PA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amount of cytoplasm</strong></td>
<td>Abundant</td>
<td>Little, variable</td>
<td>Little, variable</td>
<td>Little</td>
<td>Less than normal, more than other atrophic lesions</td>
</tr>
<tr>
<td><strong>Colour of cytoplasm</strong></td>
<td>Clear</td>
<td>Often dark</td>
<td>Often dark</td>
<td>Often clear</td>
<td>Clear</td>
</tr>
<tr>
<td><strong>Papillae</strong></td>
<td>Abundant</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>Gland size</strong></td>
<td>Medium/large</td>
<td>Similar to normal, but more variable</td>
<td>Small</td>
<td>Medium to large</td>
<td>Small to medium</td>
</tr>
<tr>
<td><strong>Gland shape</strong></td>
<td>Compound tubuloalveolar</td>
<td>Similar to normal, less complex</td>
<td>Mostly round</td>
<td>Round</td>
<td>Variable</td>
</tr>
<tr>
<td><strong>Gland packing</strong></td>
<td>Well-spaced</td>
<td>Similar to normal</td>
<td>Close</td>
<td>Close</td>
<td>Variable</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td>Absent</td>
<td>Usually present</td>
<td>Usually present</td>
<td>Usually absent</td>
<td>Usually absent</td>
</tr>
</tbody>
</table>

*Table 3.2: Inflammatory / Atrophic Lesions Definition (De Marzo et al 2006, Davidsson et al 2011)*

For H&E-based morphological assessment of tumour inflammation a summary “tumour chronic inflammation grade” was recorded, similar to that described by Banez et al (2010). Grade 0 inflammation was defined as no significant inflammation (figure 3.14), grade 1 as inflammatory cells infiltrating less than 10% of the tumour area (figure 3.15), and grade 2 as inflammatory cells infiltrating more than 10% of the tumour area (figure 3.16).
Figure 3.14: Grade 0 tumour chronic inflammation (H&E, 20x)

Figure 3.15: Grade 1 tumour chronic inflammation (H&E, 10x)
Figure 3.16: Grade 2 tumour chronic inflammation (H&E, 10x)

For IHC assessment of tumour inflammation the maximum number of CD3-positive T-lymphocytes, CD56-positive NK-cells and CD68-positive macrophages was counted per 40x high power field (HPF) in the area of greatest density of inflammation, following initial scanning of the slide at 10x magnification. The 40x microscopic field had a diameter of 0.6mm, giving a field area of 0.282mm$^2$. This method was based on that described by Ziegler-Johnson et al. (2016), but unlike in that paper the specimens for assessment were NCBs rather than prostatectomies. For this reason only the single most dense field was counted, rather than averaging the count over four HPFs. As there was some background staining noted on both CD56 and CD68-stained slides, only definite nucleated cells were counted. Macrophages within cysts or glandular lumina were not counted and only those within stroma or epithelium were enumerated. CD3-positive T-lymphocyte numbers were approximated to the nearest 10.

It was found that the Ziegler-Johnson method was suitable for enumeration of NK-cells and macrophages, as they tend to be evenly distributed throughout the biopsies. The marked regional aggregation of T-lymphocytes however made this method insufficient for meaningful assessment of T-lymphocyte numbers, as the absolute number of positive cells in any one 40x field was too great to accurately count. For this reason T-lymphocyte inflammation was also assessed semiquantitatively using a novel system, estimating the proportion of tumour area in which there was grade 0 CD3-positive T-lymphocyte infiltration (no T-lymphocytes at all – figure 3.17), the proportion of tumour area with grade 1 CD3-positive T-lymphocyte infiltration
(individual T-lymphocytes infiltrating the tumour, separated by well-defined spaces – figure 3.18) and the proportion with grade 2 CD3-positive T-lymphocyte infiltration (nodules or aggregates of T-lymphocytes – figure 3.19).

Figure 3.17: Area with Grade 0 CD3+ T-lymphocytes (CD3, 10x)

Figure 3.18: Area with Grade 1 CD3+ T-lymphocytes (CD3, 10x)
Chapter 3 – Methods

For statistical purposes the grade 0 and 1 areas were added together and compared to give a percentage of tumour with non-high grade inflammation, and grade 1 and 2 were added together to give a percentage of tumour with any inflammation.

In addition to the two methods described above for enumerating T-lymphocytes, a “low / high grade” score based on the 2010 Banez et al paper was also provided, analogous to the overall chronic inflammation grade.

All results were recorded in a Microsoft Excel spreadsheet.
3.6 STATISTICAL METHODS

GraphPad InStat Version 3.10 (© 1992-2009 by GraphPad Software, Inc) was used for statistical analysis.

Contingency tables with two rows and columns were analysed using Fisher’s exact test with a two-tailed p-value. Larger contingency tables were examined using the Chi square test for independence, with Chi square test for trend reported in cases with p<0.05.

For comparison of means between two groups the unpaired t-test with two-tailed p-value was employed when the standard deviations of the groups were not significantly different and the data was distributed in a Gaussian fashion as determined by the Kolmogorov and Mirnov test. The Mann-Whitney test with two-tailed p-value was used for data which failed the Kolmogorov and Mirnov normality test. For comparison of means in more than two groups the Kruskall-Wallis test (nonparametric analysis-of-variance, ANOVA) was employed when Bartlett’s test determined that there was a significant difference between the standard deviations of the groups. When p<0.05 by the Kruskall-Wallis test, Dunn’s multiple comparison test was undertaken to determine the level of significance of differences between individual groups.

For comparison of means in more than two groups with Gaussian distribution and no significant difference in standard deviations, one-way analysis of variance (ANOVA) was undertaken. When the values in each row were matched (for example, assessment of a given variable across the three timepoints), repeated measures ANOVA was performed, and both F-statistic and p-value were reported. This technique required the exclusion of any records which lacked complete data, such as lack of blood draw at a given timepoint for an individual participant.

For distributions which were not assumed to be normally distributed, or when the numbers of samples were low, the Friedman test (nonparametric repeated measures ANOVA) was employed, with reporting of the two-tailed p-value and the Friedman statistic (Fr).

Regression analysis was employed to test for the presence of a relationship between a dependent continuous variable and a single explanatory variable (linear regression), or multiple explanatory variables (multiple regression).
4. RESULTS

4.1 ACCRUAL AND RANDOMIZATION OF PARTICIPANTS

67 participants were recruited to the trial, 30 from centres in Dublin and 37 from the London centre. Six withdrew or were excluded prior to the first (T0) blood sample being acquired, leaving a total of 61 participants who were randomized to the exercise or control group and from whom at least an initial blood sample was acquired for assessment. A further two participants died and nine withdrew from the trial during its progress, leaving 50 participants from whom blood samples at all three time points (T0, T3 and T6) were available for filtration.

30 participants were randomized to the exercise group, 31 to the control group. Of the 50 participants who completed the six month trial, 22 were in the exercise group and 28 in the control group, a completion rate of 73.3% in the exercise group and 90.3% in the control group (p=0.1056, Fisher’s exact test). One participant had died during the trial in both the exercise and control groups.
4.2 BASELINE CLINICOPATHOLOGICAL FEATURES OF TRIAL PARTICIPANTS

The mean age of the 61 participants was 69.84 years (range 51-86 years), and randomization occurred a mean of 33.67 months following the participant’s PrCa diagnosis (range 4-128 months). The majority of participants had a Gleason score of 8 (32.8%) or 9 (42.6%). 11.4% had Gleason 7 carcinoma. The Gleason score was unknown in the remaining 13% of participants. Serum PSA at baseline among trial participants ranged from less than 0.03 to 1438ng/mL (mean 50.24, median 5.36ng/mL).

![Figure 4.1: BMI categories of ExPeCT trial participants](image)

The mean height of trial participants was 169.25cm, and the mean weight was 86.39kg (ranges 151-191.5cm and 59.7-125.4kg) at baseline. BMI among trial participants ranged from 21 to 38.9 (see figure 4.1), with a mean of 29.23 and median 28.5. Waist circumference ranged from 72 to 126cm (mean 102.33, median 101). Blood pressure ranged from 104-179mmHg systolic (mean 138.6mmHg) and 63-123mmHg diastolic (mean 78.5mmHg). On initial blood tests the mean haemoglobin was 12.9g/dL (standard deviation 1.58, range 7.1-15.9g/dL), the mean white cell count was 7.69 x 10^9/L (standard deviation 4.97, range 2.1-37.5 x 10^9/L) and the mean platelet count was 218.48 x 10^9/L (standard deviation 55.97, range 67-356 x 10^9/L – see figure 4.2).
Eleven participants (18.03%) had a BMI of less than 25 – these were considered the “non-exposed” group. The remaining 50 participants (81.97%) with BMI of 25 or greater were considered the “exposed” group (see figure 4.1). When participants with unknown Gleason grade were excluded, the non-exposed group had a greater tendency to have Gleason 7 or 8 carcinoma (87.5%) rather than Gleason 9 carcinoma (12.5%). The reverse was the case in the exposed group (Gleason 7/8 – 44.44%, Gleason 9 55.55%), but the difference did not quite reach statistical significance (p = 0.0504, Fishers exact test, two-sided p-value).
Chapter 4 – Results

4.3 CIRCULATING TUMOUR CELLS

4.3.1 GENERAL FEATURES OF FILTERS AND CTCs

598 ScreenCell® filters were prepared from 161 blood draws from 61 participants. 60 blood
draws were at T0 (220 filters), 52 blood draws were at T3 (197 filters) and 49 blood draws were at
T6 (181 filters). The number of filters examined per blood draw per participant ranged from 1 to
7, and did not vary significantly between timepoints (T0 mean 3.666 filters, T3 3.788 filters, T6
3.694 filters, p=0.8255, Kruskall-Wallis test).

308 (51.51%) filters were derived from control group participants, 290 from the exercise group
(48.49%). 491 (82.11%) filters were from the exposed group participants and 107 (17.89%) from
the non-exposed. Dublin-based participants contributed 271 filters (45.32%) and London
participants contributed 327 filters (54.68%). The proportions of blood draw episodes from these
groups were similar to the absolute numbers of derived filters: control group 83 (51.55%),
exercise group 78 (48.45%); exposed group 132 (81.99%), non-exposed group 29 (18.01%); Dublin
78 (48.45%), London 83 (51.55%).

33 of the filters (5.5%) showed microscopic features of suboptimal preparation. This included
filters which were bent or perforated, those with extensive blood clot or precipitate at the
surface, those with pale or overly dark staining, and some cases with extensive cytolytic artefacts.
Despite the suboptimal preparation these filters were included in the analysis as the filters could
still be subjected to microscopic screening, although with some difficulty.

CTCs were present in 567 filters (94.8%), in which 9182 CTCs were enumerated in total. Every
blood draw episode yielded at least one CTC-positive filter (161, 100%). Bare CTC nuclei were
present in almost all of these CTC-positive filters (565/567, 99.65%). The presence or absence of
CTCs with attached cytoplasm was recorded in the 327 filters from London-based participants
only - 46.48% of these filters contained at least one CTC with attached cytoplasm (see figure 4.3),
but the majority of individual CTCs lacked cytoplasm in detailed analysis of a small number of
filters (see section 4.3.4 below).
4.3.2 CTC ENUMERATION PER FILTER

4.3.2.1 Overall and subgroup analysis

A mean of 15.355 CTCs were identified on each of the 598 filters (standard deviation 13.539, range 0-99, median 12). There was a non-significant trend towards exposed group participant filters having fewer CTCs than the non-exposed group (mean 15.033 vs 16.832, p=0.0695, Mann-Whitney test). There was also a non-significant trend towards control group participant filters having fewer CTCs than the exercise group (mean 15.127 vs 15.597, p=0.0637, Mann-Whitney test).

4.3.2.2 Timepoint analysis

Overall there was a significant variation in median CTC numbers per filter between the timepoints, as determined by non-parametric ANOVA (see table 4.1). Median CTC numbers at T0 were 13.5 per filter, but 10 at T3 and 12 at T6. The difference between T0 and T3 was statistically significant. Subgroup analysis demonstrated that the difference lies in the control group rather than the exercise group, and the exposed group rather than the non-exposed group. Further analysis of the exposed exercise and exposed control groups did not reveal statistically significant differences in CTC numbers.
## Results

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T3</th>
<th>T6</th>
<th>p-value</th>
<th>Test Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overall</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>220</td>
<td>197</td>
<td>181</td>
<td>0.0185</td>
<td>Kruskal-Wallis test</td>
</tr>
<tr>
<td>Median</td>
<td>13.5</td>
<td>10.0</td>
<td>12.0</td>
<td></td>
<td>Dunn's multiple comparison test: T0 vs T3, p&lt;0.05 T0 vs T6, p&gt;0.05 T3 vs T6, p&gt;0.05</td>
</tr>
<tr>
<td>Range</td>
<td>0-99</td>
<td>0-69</td>
<td>0-83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>17.154</td>
<td>13.923</td>
<td>14.723</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Exercise group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>112</td>
<td>94</td>
<td>84</td>
<td>0.4948</td>
<td>Kruskal-Wallis test</td>
</tr>
<tr>
<td>Median</td>
<td>13.5</td>
<td>13.0</td>
<td>12.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0-68</td>
<td>0-69</td>
<td>0-40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>16.482</td>
<td>15.563</td>
<td>14.452</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Control group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>108</td>
<td>103</td>
<td>97</td>
<td>0.0449</td>
<td>Kruskal-Wallis test</td>
</tr>
<tr>
<td>Median</td>
<td>13.5</td>
<td>9.0</td>
<td>11.0</td>
<td></td>
<td>Dunn's multiple comparison test: T0 vs T3, p&lt;0.05 T0 vs T6, p&gt;0.05 T3 vs T6, p&gt;0.05</td>
</tr>
<tr>
<td>Range</td>
<td>0-99</td>
<td>0-48</td>
<td>0-83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>17.852</td>
<td>12.427</td>
<td>14.959</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Exposed group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>181</td>
<td>164</td>
<td>146</td>
<td>0.0132</td>
<td>Kruskal-Wallis test</td>
</tr>
<tr>
<td>Median</td>
<td>13.0</td>
<td>9.0</td>
<td>11.5</td>
<td></td>
<td>Dunn's multiple comparison test: T0 vs T3, p&lt;0.05 T0 vs T6, p&gt;0.05 T3 vs T6, p&gt;0.05</td>
</tr>
<tr>
<td>Range</td>
<td>0-99</td>
<td>0-69</td>
<td>0-83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>17.215</td>
<td>13.341</td>
<td>14.226</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Non-exposed group</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.9120</td>
<td>Kruskal-Wallis test</td>
</tr>
<tr>
<td>n</td>
<td>39</td>
<td>33</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>14.0</td>
<td>13.0</td>
<td>14.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>2-65</td>
<td>2-61</td>
<td>1-40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>16.872</td>
<td>16.818</td>
<td>16.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td>14.991</td>
<td>13.122</td>
<td>11.852</td>
<td></td>
<td>T3 vs T6, p&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>12.442</td>
<td>14.442</td>
<td>9.524</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.274</td>
<td>11.683</td>
<td>13.595</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.353</td>
<td>12.8</td>
<td>11.967</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.362</td>
<td>14.484</td>
<td>11.287</td>
<td></td>
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</tr>
</tbody>
</table>
### Chapter 4 – Results

#### Table 4.1: CTC numbers per filter, timepoint analysis (non-parametric ANOVA)

<table>
<thead>
<tr>
<th></th>
<th>Exposed Exercise group</th>
<th>Exposed Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>85</td>
<td>96</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>0-68</td>
<td>0-99</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>15.565</td>
<td>18.677</td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td>11.712</td>
<td>17.908</td>
</tr>
</tbody>
</table>

- **p=0.1713** (Kruskall-Wallis test)
- **p=0.0539** (Kruskall-Wallis test)

#### 4.3.3 CTC ENUMERATION PER BLOOD DRAW

**4.3.3.1 Maximum CTC numbers per blood draw – overall and subgroup analysis**

When the maximum number of CTCs on any one filter during each blood draw episode was considered, the mean number of CTCs was 21.44 (standard deviation 15.436, range 1-99, median 18). There was no significant difference in maximum CTC number per blood draw between the exposed (mean 21.182) and non-exposed (mean 22.621) groups (p=0.6174, Mann-Whitney test). There was also no significant difference in maximum CTC number per blood draw between the control (mean 21.241) and exercise (mean 21.654) groups (p=0.4003, Mann-Whitney test).

**4.3.3.2 Mean CTC numbers per blood draw - overall and subgroup analysis**

When the mean number of CTCs on all filters prepared from each blood draw episode was considered, the mean number of CTCs was 15.511 (standard deviation 12.781, range 0.5-87.666, median 12.666). There was no significant difference in mean CTC number per blood draw between the exposed (mean 15.143) and non-exposed (mean 17.187) groups (p=0.3117, Mann-Whitney test). There was also no significant difference in mean CTC number per blood draw between the control (mean 15.2) and exercise (mean 15.842) groups (p=0.1917, Mann-Whitney test).
4.3.3.3 Timepoint analysis

There were 46 participants for whom a complete set of T0, T3 and T6 blood draw data were available, including 9 non-exposed and 37 exposed group participants. 23 participants were in both exercise and control randomised groups. The mean number of CTCs per filter was compared at each timepoint, as was the maximum number of CTCs identified on any individual filter at each timepoint, using Friedman nonparametric repeated measures ANOVA. Taken as a whole, the 37 exposed participants had a significant reduction in median maximum CTC numbers per blood draw between T0 and T6 (p=0.0147, see table 4.2). Further subgroup analysis demonstrated a non-significant trend towards this being driven by the exercise group exposed participants (p=0.0622).

<table>
<thead>
<tr>
<th>Group</th>
<th>n=</th>
<th>p=</th>
<th>Fr=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>46</td>
<td>0.213</td>
<td>3.093</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0745</td>
<td>5.193</td>
</tr>
<tr>
<td>Exercise group</td>
<td>23</td>
<td>0.3679</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5501</td>
<td>1.195</td>
</tr>
<tr>
<td>Control group</td>
<td>23</td>
<td>0.4987</td>
<td>1.391</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1045</td>
<td>4.517</td>
</tr>
<tr>
<td>Exposed group</td>
<td>37</td>
<td>0.0663</td>
<td>5.429</td>
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<tr>
<td></td>
<td></td>
<td><strong>0.0147</strong></td>
<td>8.434</td>
</tr>
<tr>
<td>Non-exposed group</td>
<td>9</td>
<td>0.6854</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>0.6744</td>
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</tr>
<tr>
<td>Exposed exercise group</td>
<td>16</td>
<td>0.0622</td>
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</tr>
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<td></td>
<td></td>
<td>0.131</td>
<td>4.066</td>
</tr>
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<td>Exposed control group</td>
<td>16</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>0.11</td>
<td>4.415</td>
</tr>
</tbody>
</table>

*Table 4.2: CTC numbers per blood draw, timepoint analysis (repeated measures ANOVA)*

4.3.4 PLATELET CLOAKING

Platelet cloaking was identified in 33 filters (5.5%), involving a similar proportion of filters across all timepoints (T0 5%, T3 5.08%, T6 6.63% - p=0.7349, Chi square test for independence). It was
more frequently identified in filters from exposed (6.11%) than non-exposed (2.8%) participants, but the difference was not statistically significant (p=0.2423, Fisher’s exact test). Platelet cloaking was also more often encountered in filters from control group (6.82%) than exercise group (4.14%) participants, but again the difference did not reach the threshold of significance (p=0.2092, Fisher’s exact test).

Platelet cloaking was present in at least one filter on 24 of 161 blood draw occasions (14.91%). There was a statistically non-significant trend towards platelet cloaking becoming more frequently encountered in later blood draws (T0 11.67%, T3 15.38%, T6 18.37%, p=0.6161, Chi square test for independence). When subgroups were considered, platelet cloaking was identified in 16.67% of blood draws from exposed participants, and 6.9% of blood draws from non-exposed participants (p=0.2534, Fisher’s exact test). Platelet cloaking was significantly more frequently seen in blood draws from control group (20.48%) than exercise group participants (8.97%, p=0.0476, Fisher’s exact test). However when this was broken down by timepoint, there was no significant difference in the control group (p=0.4374, Chi-square test for independence). Chi square results were not valid for the exercise group due to low numbers of blood draws positive for platelet cloaking at each time point, but when the T0 and T6 figures were combined there was no significant difference identified (p=1, Fisher’s exact test).

4.3.5 CTC CLUSTERS
28 filters (4.68%) contained at least one CTC cluster, with a mean of 2.07 clusters on these filters. There was no significant difference in the number of filters with CTC clusters between the exposed (4.48%) and non-exposed (5.6%) groups (p=0.6143, Fisher’s exact test). There was also no significant difference in the number of filters with CTC clusters between the control (5.51%) and exercise (3.79%) groups (p=0.3397, Fisher’s exact test).

4.3.6 DETAILED CTC MORPHOLOGY IN SELECTED LONDON FILTERS
The morphology of 431 CTCs from 17 distinct London-based participant filters was examined in detail. This represented 6.175% of the 9182 total CTCs identified in the complete cohort of filters. 423 (98.14%) of CTCs were seen to be overlying a filtration pore. The mean short-axis nuclear diameter was 2.838 PD, with a range from 2 to 6 PD (see figure 4.4). 276 CTCs (64.03%) had one or more nuclear indentations. The mean number of indentations was 2.167, and the majority (78.99%) of indentations did not exceed 1/8th the short axis nuclear diameter. The mean relative depth of each CTC’s deepest indentation was 0.154 of the short axis nuclear diameter, corresponding to 0.444 PD. In 222 indented CTCs (80.43%) the indentations appeared to radiate...
from the overlain filtration pore (see figure 4.5). Only 31 CTCs (7.19%) had visible cytoplasm, in which 38.71% had centrally-located nuclei, 45.16% had eccentric nuclei and 16.13% had peripheral nuclei. The mean nuclear : cytoplasmic ratio was 0.71.

Figure 4.4: Nuclear diameter of CTCs in selected CTC-rich filters
There was substantial variation in the morphological features of CTCs between individual filters (table 4.3). For example the proportion of indented nuclei per filter ranged from 11.11% to 81.25% (mean 61.38%, SD +/- 16.61%), and the mean number of indentations per CTC nucleus per filter ranged from 1 to 3 (mean 2.135, SD +/- 0.436. The relative depth of indentations per CTC nucleus per filter was more uniform (mean 0.155 of nuclear diameter, SD +/- 0.02.
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<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of CTCs per filter</td>
<td>25.35</td>
<td>+/- 13.05</td>
<td>5</td>
<td>48</td>
</tr>
<tr>
<td>Proportion CTCs overlying pore</td>
<td>97.45%</td>
<td>+/- 5.27%</td>
<td>80%</td>
<td>100%</td>
</tr>
<tr>
<td>Nuclear size (short axis, PD)</td>
<td>2.804</td>
<td>+/- 0.155</td>
<td>2.556</td>
<td>3.113</td>
</tr>
<tr>
<td>Proportion indented nuclei</td>
<td>61.37%</td>
<td>+/- 16.6%</td>
<td>11.1%</td>
<td>81.25%</td>
</tr>
<tr>
<td>Mean number indentations</td>
<td>2.135</td>
<td>+/- 0.436</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Mean relative depth indents</td>
<td>0.155</td>
<td>+/- 0.02</td>
<td>0.125</td>
<td>0.208</td>
</tr>
<tr>
<td>Mean absolute depth indents (PD)</td>
<td>0.441</td>
<td>+/- 0.052</td>
<td>0.335</td>
<td>0.542</td>
</tr>
<tr>
<td>Proportion radiating indentations</td>
<td>81.53%</td>
<td>+/- 17.88%</td>
<td>36.36%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 4.3: Morphological features of CTCs in selected CTC-rich filters

The morphological features of the 31 CTCs which had cytoplasm were compared with the total, and were found to be similar. CTCs with cytoplasm were slightly less likely to overlie a pore (93.55% vs 98.14%) and to have nuclear indentations (58.06% vs 64.04%). Their mean nuclear size was slightly smaller (2.774 vs 2.838 pore diameters) and their mean number of indentations was lower (1.722 vs 2.166 indentations), but those indentations tended to be slightly deeper (mean 0.1875 vs 0.154 nuclear diameters).
Chapter 4 – Results

4.4 PERIPHERAL BLOOD FLOW CYTOMETRY

4.4.1 OVERALL FINDINGS
Ten participants from the Dublin cohort of patients had blood drawn for flow cytometric immunophenotyping of circulating lymphoid subsets at each timepoint. There were five participants each in the exercise and control groups, and eight in the exposed and two in the non-exposed groups. One control-group, exposed-group participant did not have blood drawn at the T3 timepoint, so the total number of blood samples subjected to flow cytometry was 29.

Across all groups and timepoints, CD3+ T-lymphocytes constituted a mean of 71.34% of lymphoid cells in the blood samples. CD4+ and CD8+ cells constituted means of 45.34% and 25.45% respectively. Smaller proportions of lymphocytes were CD19+ B-lymphocytes (9.9%) or CD56+ NK-cells (17.52%). The mean absolute total lymphocyte count was 1800.93, including means of 1304.21 CD3+ T-lymphocytes, 838.48 CD4+ T-lymphocytes, 455.62 CD8+ T-lymphocytes, 174.59 CD19+ B-lymphocytes, and 300.28 CD56+ NK-cells.

4.4.2 TIMEPOINT ANALYSIS
When the single participant lacking a T3 blood draw was excluded, the lymphoid cell numbers were subjected to analysis-of-variance analysis to compare levels across timepoints. No significant variance was identified between the T0, T3 and T6 samples in any lymphocyte group (see table 4.4).
4.4.3 SUBGROUP ANALYSIS

When subgroups were considered, there was no significant difference in percentage of circulating CD56+ NK-cell numbers between the exposed (17.65%) and non-exposed (17%) groups (p = 0.7743, unpaired t-test). Comparison of absolute NK-cell numbers also did not demonstrate a significant difference (exposed 299.7, non-exposed 302.5, p = 0.5536, Mann Whitney test). The number of blood samples in the non-exposed group was not sufficient for further analysis of variation at various timepoints.

When the exercise and control-groups were compared across all timepoints, the exercise group was found to have significantly higher mean proportions of CD3-positive T-lymphocytes and significantly lower proportions of CD19-positive B-lymphocytes and CD56-positive NK-cells than the control group (see table 4.5).

Table 4.4: ANOVA of flow-cytometry derived lymphocyte counts per timepoint

<table>
<thead>
<tr>
<th></th>
<th>P=</th>
<th>F= (Fr=)</th>
<th>Effective matching, p=</th>
<th>F=</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD3</td>
<td>0.9131</td>
<td>0.182 (Fr)</td>
<td>0.0003</td>
<td>7.504</td>
<td>Friedman test</td>
</tr>
<tr>
<td>% CD4</td>
<td>0.8485</td>
<td>0.166</td>
<td>&lt;0.0001</td>
<td>19.483</td>
<td>Repeated measures ANOVA</td>
</tr>
<tr>
<td>% CD8</td>
<td>0.1814</td>
<td>1.903</td>
<td>&lt;0.0001</td>
<td>1.903</td>
<td>Repeated measures ANOVA</td>
</tr>
<tr>
<td>% CD19</td>
<td>0.5109</td>
<td>0.7005</td>
<td>&lt;0.0001</td>
<td>10.51</td>
<td>Repeated measures ANOVA</td>
</tr>
<tr>
<td>% CD56</td>
<td>0.9466</td>
<td>0.05503</td>
<td>0.0137</td>
<td>3.62</td>
<td>Repeated measures ANOVA</td>
</tr>
<tr>
<td>Absolute CD3</td>
<td>0.569</td>
<td>1.556 (Fr)</td>
<td></td>
<td></td>
<td>Friedman test</td>
</tr>
<tr>
<td>Absolute CD4</td>
<td>0.6801</td>
<td>0.395</td>
<td>&lt;0.0001</td>
<td>16.104</td>
<td>Repeated measures ANOVA</td>
</tr>
<tr>
<td>Absolute CD8</td>
<td>0.6854</td>
<td>0.889 (Fr)</td>
<td></td>
<td></td>
<td>Friedman test</td>
</tr>
<tr>
<td>Absolute CD19</td>
<td>0.8716</td>
<td>0.1386</td>
<td>0.0269</td>
<td>3.068</td>
<td>Repeated measures ANOVA</td>
</tr>
<tr>
<td>Absolute CD56</td>
<td>0.898</td>
<td></td>
<td></td>
<td></td>
<td>Ordinary ANOVA</td>
</tr>
<tr>
<td>Total lymphocytes</td>
<td>0.9712</td>
<td>0.222 (Fr)</td>
<td></td>
<td></td>
<td>Friedman test</td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td>0.4481</td>
<td>0.8443</td>
<td>&lt;0.0001</td>
<td>77.735</td>
<td>Repeated measures ANOVA</td>
</tr>
</tbody>
</table>
## Chapter 4 – Results

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
<th>p=</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD3</td>
<td>67%</td>
<td>75.4%</td>
<td>0.0003</td>
<td>Unpaired t</td>
</tr>
<tr>
<td>% CD4</td>
<td>43.5%</td>
<td>47.07%</td>
<td>0.2747</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>% CD8</td>
<td>22.86%</td>
<td>27.87%</td>
<td>0.1401</td>
<td>Unpaired t</td>
</tr>
<tr>
<td>% CD19</td>
<td>11.93%</td>
<td>8%</td>
<td>0.0264</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>% CD56</td>
<td>19.71%</td>
<td>15.47%</td>
<td>0.015</td>
<td>Unpaired t</td>
</tr>
<tr>
<td>Absolute CD3</td>
<td>987.57</td>
<td>1599.73</td>
<td>0.0013</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>Absolute CD4</td>
<td>680</td>
<td>986.4</td>
<td>0.0094</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>Absolute CD8</td>
<td>293.71</td>
<td>606.73</td>
<td>0.002</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>Absolute CD19</td>
<td>182.36</td>
<td>167.33</td>
<td>0.6625</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>Absolute CD56</td>
<td>280.71</td>
<td>318.53</td>
<td>0.0521</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>Total lymphocytes</td>
<td>1468.14</td>
<td>2111.53</td>
<td>0.0027</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td>2.272</td>
<td>2.075</td>
<td>0.6467</td>
<td>Mann-Whitney</td>
</tr>
</tbody>
</table>

*Table 4.5: Comparison of lymphocyte counts between exercise and control groups*

The numbers of specimens were insufficient for comparisons to be drawn between groups at the T0, T3 and T6 timepoints.
4.5 PROSTATE BIOPSY INFLAMMATION AND ATROPHY

4.5.1 SPECIMENS FOR REVIEW

Material from 48 diagnostic biopsy specimens was received for review, from 47 participants, including 44 NCBs, 3 transurethral resection specimens and 1 bone reaming specimen. Both NCB and transurethral resection specimens were available for review from one participant – in this case the NCB specimen was used for analysis. The bone reaming specimen was not included as there was no prostate tissue present for assessment of inflammatory / atrophic changes. In two cases no tissue was present for assessment on the IHC-stained slides – one from the exposed group and one from the non-exposed group – such that 44 specimens were included in the immunohistochemistry analysis (see section 3.4.4). Of the 46 prostate specimens reviewed, 37 (80.43%) were from the exposed group and 9 (19.57%) from the non-exposed group.

4.5.2 PROSTATE ATROPHIC LESIONS

The H&E slides from 46 prostate specimens (44 NCBs and 2 TURPs) were examined for the presence of atrophic lesions. Atrophic lesions were present in 20 (43.48%) of the specimens, and in a higher proportion of exposed (48.65%) than non-exposed (22.22%) participants, but the difference was not statistically significant (two-tailed p-value 0.2621, Fisher’s exact test).

PIA, manifested by either SA or PAH, was present in all cases in which an atrophic lesion was identified. PIA was represented almost entirely by SA, which was present in all PIA cases. A single case in the non-exposed group also had PAH. Non-PIA (SACF, PA) was present in 3 cases (6.52%) overall, all of which were in the exposed group (8.11% of this group).

4.5.3 MORPHOLOGICAL ASSESSMENT OF INFLAMMATION IN BENIGN PROSTATE

Acute inflammation was present in only one case (2.17%), which was in the exposed group. A chronic inflammatory infiltrate was seen in the background benign prostate tissue in 30 specimens (65.22%), with no significant difference between exposed (67.57%) and non-exposed (55.56%) groups (2-tailed p-value 0.6982, Fisher’s exact test). When the anatomical location of this infiltrate was considered, 7 specimens (23.33%) showed chronic inflammation within the glandular compartment, 14 showed periglandular inflammation (46.67%) and 22 (73.33%) showed stromal inflammation. Grade 1 intensity chronic inflammation was most frequently encountered in all anatomical locations, and it was usually focal in extent (see table 4.6).
Table 4.6: Intensity and distribution of chronic inflammation in benign regions of prostate NCBs

<table>
<thead>
<tr>
<th></th>
<th>Glandular</th>
<th>Periglandular</th>
<th>Stromal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Grade 1</strong></td>
<td>7</td>
<td>100.00%</td>
<td>10</td>
</tr>
<tr>
<td><strong>Grade 2</strong></td>
<td>0</td>
<td>0.00%</td>
<td>2</td>
</tr>
<tr>
<td><strong>Grade 3</strong></td>
<td>0</td>
<td>0.00%</td>
<td>2</td>
</tr>
<tr>
<td><strong>Focal</strong></td>
<td>6</td>
<td>20.00%</td>
<td>12</td>
</tr>
<tr>
<td><strong>Multifocal</strong></td>
<td>1</td>
<td>3.33%</td>
<td>2</td>
</tr>
<tr>
<td><strong>Diffuse</strong></td>
<td>0</td>
<td>0.00%</td>
<td>0</td>
</tr>
</tbody>
</table>

There were no significant differences between the exposed and non-exposed groups relating to the presence of chronic inflammation in the various anatomical compartments, although there was a trend towards periglandular inflammation being seen more frequently in the exposed (52%) than the non-exposed group (20% - 2-tailed p-value 0.2259, Fisher’s exact test).

4.5.4 ASSESSMENT OF INFLAMMATION IN PROSTATE CANCER TISSUE

4.5.4.1 H&E tumour inflammation

Of the 46 biopsies, 5 (10.87%) contained no significant tumour inflammation, 34 (73.91%) contained grade 1 inflammation, and 7 (15.22%) contained grade 2 inflammation. There was a trend towards participants in the exposed group having tumours with more inflammation. 22.22% of non-exposed specimens lacked any tumour inflammation, but only 8.11% of exposed specimens did; no non-exposed specimens had grade 2 tumour inflammation whereas 18.92% of exposed specimens did. However these differences were not statistically significant either when grades were considered individually (p = 0.2174, Chi square test) or when grades 1 and 2 (2-tailed p-value 0.2484, Fisher’s exact test) or grades 0 and 1 (2-tailed p-value 0.3159) were combined.

4.5.4.2 T-lymphocytes

32 biopsies were considered to have low-grade CD3-positive T-lymphocyte inflammation (72.73%) and 12 (27.27%) high grade. There was a non-significant trend towards high-grade T-lymphocyte inflammation being more often seen in exposed than non-exposed group participants (30.56% vs 12.5%, 2-tailed p-value 0.4126, Fisher’s exact test).
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The mean maximum number of CD3-positive T-lymphocytes within a HPF of tumour was 235.91, with no significant difference between exposed (241.11) and non-exposed (212.5) groups (p = 0.6753, unpaired t-test).

The mean tumour area with grade 0 CD3-positive inflammation was 39.87%, that with grade 1 was 50.8% and that with grade 2 was 9.32%. Although the area with grade 2 inflammation was similar in the exposed (9.44%) and non-exposed (8.75%) groups, there was a trend towards exposed group participants having a lower area of grade 0 inflammation (37.36% vs 51.25%, p=0.2019, Mann-Whitney test) and a greater area of grade 1 inflammation (53.19% vs 40%, p=0.2459, unpaired t-test).

4.5.4.3 Natural killer cells

The mean maximum number of CD56-positive NK-cells per HPF was 3.659 (range 0-13 / HPF, standard deviation 3.213). There was no significant difference in numbers between the exposed group (3.722 / HPF) and the non-exposed group (3.375 / HPF, p=0.9754, Mann-Whitney test).

4.5.4.4 Macrophages

The mean maximum number of CD68-positive macrophages per HPF was 21 (range 5-63 / HPF, standard deviation 12.784). There was a non-significant trend towards higher numbers of macrophages in the exposed group (22.361 / HPF) than in the non-exposed group (14.875 / HPF, p=0.0907, Mann-Whitney test).
4.6 CORRELATIONS

4.6.1 CORRELATION BETWEEN CTC NUMBERS AND CIRCULATING LYMPHOCYTES

The CTC numbers from the 29 blood draw episodes which were subjected to flow cytometry analysis (see section 3.4) were compared with the mean number of CTCs per filter identified at each blood draw episode. Linear regression analysis was employed for each variable (see table 4.7).

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>r squared</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lymphocyte count</td>
<td>0.4134</td>
<td>0.1709</td>
<td>5.566</td>
<td>0.0258</td>
</tr>
<tr>
<td>Absolute CD3 count</td>
<td>0.3122</td>
<td>0.09745</td>
<td>2.915</td>
<td>0.0992</td>
</tr>
<tr>
<td>Absolute CD19 count</td>
<td>0.2292</td>
<td>0.05254</td>
<td>1.497</td>
<td>0.2317</td>
</tr>
<tr>
<td>Absolute CD56 count</td>
<td>0.707</td>
<td>0.4999</td>
<td>26.987</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Absolute CD4 count</td>
<td>0.2751</td>
<td>0.07566</td>
<td>2.21</td>
<td>0.1487</td>
</tr>
<tr>
<td>Absolute CD8 count</td>
<td>0.2372</td>
<td>0.05628</td>
<td>1.61</td>
<td>0.2153</td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td>-0.00515</td>
<td>0.0000265</td>
<td>0.000715</td>
<td>0.9789</td>
</tr>
</tbody>
</table>

Table 4.7: CTC number / absolute lymphocyte count correlations (linear regression analysis)

There was a significant correlation between mean CTCs identified per blood draw and both total lymphocyte count and NK-cell count (see figures 4.6 and 4.7).
Figure 4.6: Correlation between total lymphocyte count and mean CTC count at a given timepoint
Figure 4.7: Correlation between absolute NK-cell count and mean CTC count at a given timepoint
Figure 4.8: No correlation between CD4:CD8 ratio and mean CTC count at a given timepoint

Analysis was also performed relating to the percentage rather than absolute numbers of each lymphocyte subgroup. No significant correlation was seen between any lymphocyte percentage and the mean CTC count at that blood draw (see table 4.8)

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>r squared</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD3</td>
<td>-0.1893</td>
<td>0.03582</td>
<td>0.1587</td>
<td>0.6935</td>
</tr>
<tr>
<td>% CD4</td>
<td>-0.1749</td>
<td>0.03058</td>
<td>0.8518</td>
<td>0.3642</td>
</tr>
<tr>
<td>% CD8</td>
<td>0.09642</td>
<td>0.009296</td>
<td>0.2533</td>
<td>0.6188</td>
</tr>
<tr>
<td>% CD19</td>
<td>-0.07644</td>
<td>0.005842</td>
<td>0.1587</td>
<td>0.6935</td>
</tr>
<tr>
<td>% CD56</td>
<td>0.2478</td>
<td>0.06143</td>
<td>1.767</td>
<td>0.1949</td>
</tr>
</tbody>
</table>

*Table 4.8: CTC number / lymphocyte fraction correlations (linear regression analysis)*
4.6.2 CORRELATION BETWEEN CTC NUMBERS AND PLATELET COUNTS

There were 150 matched blood draw CTC count / platelet values subjected to analysis. There was a significant correlation between platelet count and mean CTC count at a given blood draw episode ($r=0.3070$, $F=15.403$, $p=0.0001$ – see figure 4.9).

![Figure 4.9: Correlation between platelet count and mean CTC count at a given timepoint in all groups](image)

This correlation persisted when the 79 measurements from the control group were analysed ($r=0.3642$, $F=11.776$, $p=0.001$ – see figure 4.10). There was a trend towards a correlation in the 71 measurements from the exercise group, but the finding did not reach statistical significance ($r=0.2166$, $F=3.397$, $p=0.0696$).
There was a similar correlation between platelet count and mean CTC count at a given blood draw episode within the 123 data pairs in the exposed group ($r=0.3474$, $F=16.611, p<0.0001$). There was no such identifiable correlation in the non-exposed group ($r=0.05369$, $F=0.07226$, $p=0.7903$ – see figure 4.11).
Similarly, when the presence of platelet cloaking on any filter from a given blood draw was used to define subgroups, a linear correlation was present in the absence of platelet cloaking ($r=0.3528$, $F=17.916$, $p<0.0001$, 128 data points - see figure 4.12). The correlation was absent in the 22 blood draws in which platelet cloaking had been identified ($r=-0.03812$, $F=0.02911$, $p=0.8662$ – see figure 4.13).

Figure 4.11: No correlation between platelet count and mean CTC count at a given timepoint in non-exposed group
Figure 4.12: Correlation between platelet count and mean CTC count at a given timepoint in blood draws which lacked platelet-cloaking
4.6.3 CORRELATION BETWEEN CTC NUMBERS, LYMPHOCYTE SUBGROUPS AND PLATELET COUNTS

A statistical model was constructed to determine the relationships between CTC numbers on the one hand and flow-cytometry-derived lymphocyte subsets and platelet counts on the other hand. There were 27 complete sets of data subjected to analysis. The absolute number of CD4-positive T-lymphocytes, NK-cells and platelets were all independently correlated with mean CTC count per blood draw. The CD8-positive T-lymphocyte count and B-lymphocyte count were not (see table 4.9).
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#### Table 4.9: Multiple regression analysis of mean CTC count per blood draw against absolute lymphocyte and platelet counts

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SE</th>
<th>95% CI lower</th>
<th>95% CI upper</th>
<th>t ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>(constant)</td>
<td>-26.445</td>
<td>12.256</td>
<td>-51.937</td>
<td>-0.9525</td>
<td>2.158</td>
<td>0.0427</td>
</tr>
<tr>
<td>CD4</td>
<td>-0.02426</td>
<td>0.01002</td>
<td>-0.0451</td>
<td>-0.003425</td>
<td>2.422</td>
<td>0.0246</td>
</tr>
<tr>
<td>CD8</td>
<td>0.00635</td>
<td>0.01089</td>
<td>-0.0163</td>
<td>0.029</td>
<td>0.5683</td>
<td>0.5661</td>
</tr>
<tr>
<td>CD19</td>
<td>0.03094</td>
<td>0.03044</td>
<td>-0.03237</td>
<td>0.09424</td>
<td>1.016</td>
<td>0.321</td>
</tr>
<tr>
<td>CD56</td>
<td>0.09942</td>
<td>0.02726</td>
<td>0.04273</td>
<td>0.1561</td>
<td>3.648</td>
<td>0.0015</td>
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<tr>
<td>Platelets</td>
<td>0.1555</td>
<td>0.07365</td>
<td>0.002259</td>
<td>0.3086</td>
<td>2.111</td>
<td>0.047</td>
</tr>
</tbody>
</table>

R squared = 0.6386, adjusted R squared = 0.5526, Multiple R = 0.7992, F = 7.4229

Degrees of freedom = 21

\( P = 0.0004 \)

#### 4.6.4 CORRELATION BETWEEN CTC NUMBERS AT T0 AND NCB INFLAMMATORY CELLS

The mean CTC numbers per filter from the 44 T0 blood draw episodes for which a corresponding NCB with sufficient tissue for immunohistochemistry analysis was available, were compared with the maximum number of subsets of inflammatory cells seen per HPF. Linear regression analysis was employed for each variable (see table 4.10).

#### Table 4.10: Linear regression analysis of mean CTC count per blood draw against maximum numbers of inflammatory cells per HPF in NCB specimens

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>r squared</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum CD3-positive cells per HPF</td>
<td>0.07232</td>
<td>0.005230</td>
<td>0.2208</td>
<td>0.6408</td>
</tr>
<tr>
<td>Maximum CD56-positive cells per HPF</td>
<td>0.1402</td>
<td>0.01965</td>
<td>0.8419</td>
<td>0.3641</td>
</tr>
<tr>
<td>Maximum CD68-positive cells per HPF</td>
<td>-1.033</td>
<td>0.01066</td>
<td>0.4527</td>
<td>0.5048</td>
</tr>
</tbody>
</table>

No significant relationships between CTC numbers and numbers of tumour-infiltrating inflammatory cells were identified. In particular, there was no demonstrable relationship with intratumoral CD56-positive cells (see figure 4.14).
Figure 4.14: No correlation between intratumoral NK-cell count and mean CTC count at T0
Chapter 5 – Discussion

5. DISCUSSION

5.1 MAIN FINDINGS

The main findings of this study are summarised as follows:

- There was a significant reduction in CTC numbers between T0 and T3, with the change driven by differences in the control group and the exposed group.

- Platelet cloaking was significantly more frequently seen in blood draws from the control group than the exercise group participants.

- There was a higher circulating fraction of CD3-positive T-lymphocytes and a lower circulating fraction of B-lymphocytes and NK-cells in the exercise group when compared with the control group.

- Linear correlations were identified between CTC numbers on the one hand and platelet count, total lymphocyte count, CD4-positive T-lymphocyte count and NK-cell count on the other hand, relationships which were independent of one another by multiple regression analysis.

- There was no correlation between CTC numbers and the density of inflammatory cell subsets in core biopsy tissue.
5.2 PARTICIPANT ACCRUAL

Participant accrual to the ExPeCT study was slower than anticipated. A total of 67 participants were recruited, whereas grant funding had been sought on the basis of 200 participants. In general the quality of clinical evidence for exercise programmes in advanced cancer is known to be limited by difficult accrual of participants to trials. Individual studies should be assessed critically, as the ability to undertake exercise may act as a surrogate for better performance status, which may in turn act as confounding factor. Supervised exercise programmes are feasible in colon (Sellar et al 2014) and even post-treatment lung cancer (Peddle-McIntyre et al 2012). The PEACH trial (Broderick et al 2013) recruited a heterogeneous sample of cancer survivors for a supervised exercise intervention - 186 patients were available for recruitment, 43 (23%) of whom were randomised into the programme. A common reason for exclusion was a potential participant residing too far from the study site, suggesting that recruitment from multiple clinical sites may help to achieve accrual targets.

Many PEACH trial participants were breast cancer survivors, and had a younger age profile than would be expected for a PrCa study. PrCa patients might be less inclined to participate in moderate-to-vigorous intensity exercise than younger breast cancer patients. However, Richman’s study (2011) included many participants who engaged in regular walking at a brisk pace (34.5%). Kenfield’s study (2011) shows improved mortality in those who exercised for >9MET-h/week, with approximately 75% engaging in this level of activity. The Richman et al study (2011) supports the hypotheses that brisk walking may elicit the same physiologic responses as more vigorous exercise does in a different man, and that a moderate to brisk intensity walking intervention may be acceptable to a considerable proportion of PrCa patients.

Several participants were excluded prior to randomisation, and during the trial there was a greater rate of non-completion among the exercise group (73.3%) as compared to the control group (90.3%). Numbers of deaths were equal in both groups. It is possible that the exercise intervention was found to be excessively strenuous for a proportion of the participants, which might explain the different completion rates in the two groups. The difference did not reach statistical significance however and may simply be coincidence.
5.3 BASELINE CLINICOPATHOLOGICAL FEATURES OF PARTICIPANTS

The mean participant age of 69.84 was typical of PrCa patients. The fact that participants were recruited up to 12 years following their initial diagnosis emphasises the fact that for many men PrCa is a chronic disease, and one in which lifestyle changes targeted at improving both QoL and non-cancer-specific mortality are very useful. Most of the participants had at least Gleason 8 carcinoma, in keeping with the relatively aggressive course of their disease thus far.

The vast majority of the participants were overweight at baseline, with only 11 of them having a BMI of less than 25. An additional 31 had a BMI between 25 and 29.99, considered overweight but not obese by the WHO definition. 22 had a BMI more than 30, considered obese. The exposed and non-exposed groups were defined based on a BMI cutoff of 25, i.e. between normal weight and overweight. It is evident from this study that overweight is very prevalent among men of this age. Had a cutoff value of 30 kg/m$^2$ been selected, the exposed and non-exposed groups would have been more numerically balanced. However the choice of 25kg/m$^2$ was made in advance, as it was anticipated that using either the formal WHO criteria for metabolic syndrome or a 30kg/m$^2$ cutoff would excessively restrict the numbers in the exposed group. There is evidence that BMI 25kg/m$^2$ defines an important breakpoint in PrCa prognosis (Efstathiou et al 2007), and so it was felt that this choice of cutoff value was justified. In retrospect 30kg/m$^2$ would probably have been more suitable but post-hoc alteration of the cutoff criterion would not be methodologically acceptable.

There was a trend towards exposed / overweight participants having higher Gleason score carcinoma than non-exposed participants. This is in line with previously published studies, in which obesity is reported as increasing the risk for high-grade PrCa (Vidal et al 2014, Perez-Cornago et al 2017). In longitudinal studies the risk for development of high grade PrCa is greater than that for low grade PrCa among men who gain weight over time (Wang et al 2018), and weight loss can mitigate this risk (Rodriguez et al 2007). Overweight is associated with increased lethality in PrCa, and some molecular genetic evidence suggests that abnormal chromatin regulation may partly explain the connection (Ebot et al 2017).
5.4 CIRCULATING TUMOUR CELLS

5.4.1 SCREENCELL® CYTO FILTERS

Several different technologies are available for detection of CTCs in patients with cancer, several of which rely on cytometric or immunological characteristics. The CellSearch® system for example employs bead-conjugated antibodies against EpCAM, but a potential drawback of such a system is failure to detect EpCAM-negative CTCs, as well as false positives due to detection of benign epithelial cells. ScreenCell® Cyto filters use a filtration membrane which allows selection of CTCs on the basis of size, preserving the cells for morphological assessment, and showing good recapture rates of cells following in-vitro spiking experiments (Desitter et al 2011). ScreenCell® has been shown to have a relatively better sensitivity for CTC detection than EpCAM-based techniques in certain settings, such as in colorectal cancer (Nicolazzo et al 2017). ISET, another filtration-based system, has been shown to detect greater numbers of CTCs than CellSearch® technology in non-small cell lung cancer (Krebs et al 2012). ScreenCell® Cyto filters have been employed in the indentification of CTCs in patients with lung cancer (Chudasama et al 2017), with moderate interobserver agreement (Freidin et al 2014). In some settings ScreenCell-detected CTC quantitation has been shown to provide prognostic information (Coco et al 2017). Not all ScreenCell-detected circulating epithelial cells (CECs) are carcinoma cells however – one study found no difference in the cytological features of CECs between patients with pancreatic adenocarcinoma, pancreatic neuroendocrine tumour and chronic pancreatitis (Rosenbaum et al 2017). Blood samples from healthy control patients with no pancreatic disease did not contain detectable CECs (Kulemann et al 2015, Cauley et al 2015, Rosenbaum et al 2017). The presence of epithelial cells in the bloodstream may therefore be an indicator of organ damage rather than a finding specific to cancer patients.

Morphological diagnostic criteria for CTCs in general and on ScreenCell® filters in particular are not well established, and vary between tumour types. The pancreatic study described above (Rosenbaum et al 2017) defined CECs as “enlarged (>2 times the pore size), with either irregular hyperchromatic nuclei and scant cytoplasm or clusters of cells with round-to-oval nuclei with occasional grooves and visible cytoplasm. Suspicious CECs were epithelioid but fell short of the criteria listed above or lacked clear cytoplasm”. In a study of non-small cell lung cancer patients (Hofman et al 2012), cells meeting at least two of the following criteria were defined as circulating malignant cells, and as uncertain malignant cells when less than two criteria were present: anisonucleosis (ratio > 0.5), nuclei larger than three PD (i.e > 24um), irregular nuclei, high nuclear:cytoplasmic ratio, presence of three-dimensional cell groups. Cells without visible
cytoplasm were not included in the study. Among 10 cytopathologists there was excellent interobserver agreement for the detection of circulating malignant cells. In the current study the nuclear:cytoplasmic ratio was generally not assessable, as most cells lacked cytoplasm. Another study in lung cancer patients used similar criteria, except for accepting nuclei greater than 16 µm rather than 24um, and required the presence of four criteria of the five criteria (Mascalchi et al 2016). In lung cancer the CTCs sometimes retain the morphological features of the cells in the primary tumour, as in the reported case of a single patient with well-differentiated lung adenocarcinoma (Marinucci et al 2009).

A combination of ScreenCell® filtration and morphometry was used to assess nuclear features of CTCs in one study of PrCa patients (defined as cells which expressed CK8, CK18 and CK19 and which were CD45 negative)(Awe et al 2016). The average nuclear diameter was found to vary from 9.1 to 15.6um, with significant variation in size, similar to that seen in the current study (in which nuclear diameter ranged from 2 to 6 pore diameters). The cells also had high nuclear cytoplasmic ratios, similar to the current study.

Occasional filters in the current study contained flat sheets of bland cells with monotonous features. As a general principle in diagnostic cytology benign cell groups are often flat (e.g. mesothelial cells in peritoneal washings, biliary epithelial cells in bile duct brushings specimens). On the other hand malignant cell groups are often three-dimensional, as cell proliferation is spatially disorganised and individual cells lack polarity. While the flat sheets in this study may represent CTCs lacking cytological features of malignancy, it is hypothesised that in many cases they represent endothelial cells. Circulating endothelial cells are often seen in patients with advanced cancers (Mancuso et al 2001), and are thought to derived from damaged vasculature in the context of carcinoma-induced neoangiogenesis. There is also research interest in circulating endothelial cells in the context of vascular disease. Most studies rely on flow cytometry for enumeration of circulating endothelial cells (e.g. Almici C et al 2017, Najjar F et al 2017). Morphological studies of circulating endothelial cells are rare (Samsel et al 2013) but they are often mentioned as potential confounders in morphological CTC studies. Apart from being a potential biomarker relating to angio-angiogenic cancer therapy (reviewed Strijbos et al 2008) they may present a confounder for the identification of CECs by morphological methods, as in this study. In one ScreenCell-based study of patients with renal cell carcinoma, most clusters of cells with benign morphological features and 33% of putative singly-arranged CTCs were positive for CD31. This vascular endothelial marker is not often seen in epithelial cells, and its finding
suggested that morphological criteria may not be sufficient to distinguish circulating endothelial cells from CTCs (El-Heliebi et al 2013).

5.4.2 MICROSCOPIC ASSESSMENT OF CTCS
Abundant material was present on the ScreenCell® filters, most of which did not represent CTCs. Granular precipitate, dust, coagulated red blood cells, endothelial cells, contaminant squamous cells, fibrin and platelets, and abundant white blood cells were present on many filters (see figures 5.1-5.5). In order to avoid overinterpreting this material the morphological criteria for identification of CTCs were defined in a stringent fashion, and only objects on the filter which achieved the criteria were considered to represent a CTC. By adopting such stringent criteria it is possible that some CTCs present on the filters were not identified as such in the course of the study, particularly in the minority of filters in which staining was suboptimal.

*Figure 5.1: Fungal organisms on ScreenCell® filter (MGG, 40x)*
Figure 5.2: Anucleate squamous cell on ScreenCell® filter (MGG, 40x)

Figure 5.3: Contaminant fibres on ScreenCell® filter (MGG, 20x)
Some filters were damaged during filtration or storage. In many cases the metal rim of the filter was bent, which meant that continuous adjustment of focus was required during microscopic screening in order to adequately examine the complete plane of filter pores. In other cases the transparent filter membrane was corrugated or perforated. This led to stretching of the
membrane adjacent to the perforation site with consequent morphological distortion of any adjacent CTCs.

Multiple ScreenCell® filters were obtained and stained from the blood sample taken each time a participant presented for a T0, T3 or T6 visit. The numbers of CTCs identified on the filters from a single blood draw varied somewhat. For comparison purposes with other timepoint-related variables (such as flow cytometry) it was necessary to determine a representative number of CTCs per blood draw episode. The use of either the mean or the maximum number of CTCs per filter per blood draw episode was considered. The mean figure would on first glance appear to be the most representative value, assuming some sampling variation in the number of CTCs per 4ml of blood drawn. However one would assume that CTCs are in fact very evenly distributed in the bloodstream and that any apparent variation in CTC numbers between filters taken at the same blood draw episode is more likely to be due to technical variation in filter preparation.

CTC clusters were identified on the filters less frequently than expected, but CTCs within clusters usually retained their cytoplasm, perhaps because a cohesive cluster of cells provides the constituent CTCs with a degree of protection from the shear forces in their vicinity during filtration.

5.4.3 COMPARISON OF CTC NUMBERS BETWEEN GROUPS AND ACROSS TIMEPOINTS
There were no significant differences in CTC numbers in filters derived from exposed and non-exposed group participants, or from exercise and control group participants. However the trend was towards overweight participants having slightly fewer CTCs per filter than their normal weight counterparts, and similarly for control group participants to have slightly fewer CTCs than the men who participated in the exercise intervention. Were they to be real these findings would be counterintuitive, but it must be emphasised that the findings did not reach the level of statistical significance, and so no firm conclusions can be drawn from them.

Significant differences in CTC numbers per filter were identified between timepoints, with a drop in CTC numbers between T0 and T3. This finding remained significant when the control group was analysed separately, and also when the exposed group was analysed, but not in the exercise or non-exposed groups. When the filters from the exposed members of the control group were analysed the finding were no longer significant (p=0.0539), perhaps because there were now too few datapoints for analysis. Non-exercise-related treatments undergone by the participants may influence circulating CTC numbers over these time periods.
Complete T0, T3 and T6 CTC number data were available for 46 participants, which enabled the use of Friedman nonparametric repeated measures ANOVA. Rather than comparing the overall mean for the T0, T3 and T6 timepoints, repeated measures ANOVA allows “matching” of results from individual participants, rendering the test more powerful. Both the maximum and mean number of CTCs per filter was used to provide a CTC reading per blood draw episode. The only significant finding was of the exposed group having a significant reduction in maximum CTC number per blood draw episode between T0 and T6. Similar to the per-filter analysis discussed above, the exposed group appears to have had the most impressive response to CTC numbers of the course of the study. Whether this is due to the exercise intervention or not remains unproven.

5.4.4 PLATELET CLOAKING
There is increasing evidence that subpopulations of CTCs are extensively covered in platelets, and these otherwise hard-to-identify cells can be detected using a microfluidic platform (Jiang et al 2017). Platelet cloaking proved to be a rarely-identified event in the current study, present in only 5.5% of filters, and usually only involving a tiny minority of CTCs when it was identified. It is likely that the relative rarity of platelet cloaking in the clinical samples is due to the artefactual removal of the cytoplasm and any attached platelets during filtration, which has proved to be a significant limitation of this morphological technique for the identification of CTC platelet cloaking. Where platelet cloaking was identified it was confined to CTCs which had retained their cytoplasm. Occasional filters exhibited dense clusters of platelets within which no definite CTC could be identified. As these could represent a non-CTC related phenomenon they were not counted as platelet cloaking. Because the number of filters with platelet cloaking was so small it was difficult to demonstrate significant differences between groups. Due to its rarity it was considered appropriate to measure platelet cloaking as a positive-or-negative variable per blood draw episode, rather than per individual filter, and to analyse the data in that fashion. Perhaps a blood draw which does in fact contain abundant cloaked CTCs would yield several filters in which all cloaked CTCs are disrupted by the filtration process, but one filter from that blood draw might contain a representative identifiable cloaked CTC. When analysed in this fashion there was a significantly greater proportion of blood draws with identifiable platelet cloaking in the control than the exercise group. This would be consistent with the hypothesis that exercise reduces platelet cloaking, which in turn may be a mechanism by which exercise improves survival in men with advanced PrCa. It is recognised that this method for identification of platelet cloaking is
flawed, however, and further investigation is required. Even if there is a true correlation the relationship may not be causative.

5.4.5 DETAILED CTC MORPHOLOGY

In order to critically assess the morphology of CTCs in general, a representative sample of filters was examined, all from London-based participants, with a single filter assessed per participant. The most CTC-rich filter from a given blood draw was selected for examination. As these filters were randomly selected it was hoped that the CTCs present on the filters, which represented approximately 6% of the total number of CTCs identified in the entire study, would provide a good representative sample.

ScreenCell® filters capture CTCs in part by virtue of the relatively large diameter of the cells, as well their lack of deformability. In addition, this study used size as part of the diagnostic criteria for microscopic identification of CTCs, requiring a candidate CTC to be at least two PD in diameter before it could be included. It is unsurprising therefore that the CTCs measured in this part of the study had a mean short-axis nuclear diameter of 2.838 PD. A clear majority of CTCs had a diameter between 2 and 3 PD, but some were more than 4 PD.

The vast majority of CTCs were identified overlying a filtration pore, and only 7% had visible cytoplasm, most being represented as “bare nuclei”. Almost two thirds of them showed variably prominent nuclear indentation, to a much greater degree than is generally seen in prostate carcinoma biopsy specimens. In many cases (80%), indentations were seen to be radially orientated inwards towards the filtration pore. These findings suggest that absence of cytoplasm and nuclear indentation may be artefacts introduced during filtration, as cytoplasm and nucleus are partly sucked into the pore by the filtration vacuum forces. It is possible that the process of filtration may traumatis these relatively delicate cells and strip them of their cytoplasm. The lack of cytoplasm was much less obvious in the original cell line spiking experiments in which most of the cells retained their cytoplasm (Desitter et al 2011).

There was substantial variation in morphological features of individual CTCs between filters (and therefore between participants). This suggests that the degree of pleomorphism in an individual participant’s CTCs is variable, and that there is no such thing as a morphologically “typical” population of PrCa CTCs. For example the mean nuclear diameter was 2.556 PD on one participant’s filter, but 3.113 on another’s. On one filter only 11% of nuclei showed indentation, but on another it was 81% - perhaps due individual cancers’ variability in expression of
cytoskeletal proteins and therefore varied resistance to filtration-induced deformation. Cytopathologists and researchers should be aware of the varied morphology of CTCs when employing morphological methods for CTC identification.
Chapter 5 – Discussion

5.5 PERIPHERAL BLOOD FLOW CYTOMETRY

The sample of participants whose blood samples were subjected to flow cytometry was relatively small, but nonetheless some interesting findings emerged. Although no significance variance between timepoints was detected by ANOVA analysis, there were significant differences between the exercise and control group in several lymphoid parameters.

The exercise group had significantly more circulating total lymphocytes than the control group. The proportion of CD3-positive T-lymphocytes in the peripheral blood was higher in the exercise than control group, and the absolute number of CD3-positive T-lymphocytes was also higher. This included higher absolute numbers of both CD4-positive T-helper cells and CD8-positive cytotoxic T-lymphocytes, although the CD4:CD8 ratio was similar between the two groups. The proportion of circulating B-lymphocytes and NK-cells was correspondingly lower in the exercise group. It appears that the exercise group participants had an absolute increase in circulating lymphocytes, driven predominantly by a proportionate increase in CD4-positive and CD8-positive T-lymphocytes. The absence of a significant corresponding increase in B-lymphocytes and NK-cells caused the proportions of these populations relative to T-lymphocytes to decline significantly.

There is a large amount of published data relating to alterations in circulating lymphocyte counts during and after exercise. In the acute setting it has long been recognised that strenuous exercise in healthy subjects produces a transient lymphocytosis (Robertson et al 1981, Soppi et al 1982), which includes increased absolute numbers of CD4 and CD8-positive T-lymphocytes, CD19-positive B-lymphocytes and NK-cells (Pedersen and Toft 2000). However, NK-cell function is enhanced in those physically fit persons who engage in chronic exercise training (Pedersen et al 1989). In acute exercise NK-cell numbers increase rapidly in the blood (within two minutes), reach maximum within 30 minutes, and remain elevated during exercise for up to three hours (Idorn and Hojman 2016). Prolonged strenuous exercise can temporarily reduce NK and B-lymphocyte function during the recovery period. Given the established role of NK-cell mediated cytotoxicity in cancers, it is attractive to consider the evidence for NK-cell recruitment as a likely mechanism whereby exercise can improve cancer outcomes (see section 1.7.2). In a mouse model exercise with associated IL6-mediated NK-cell recruitment is associated with reduced tumour growth (Pedersen L et al 2016). In clinical studies NK-cell numbers increase post-exercise in breast cancer survivors, although to a lesser extent than in healthy controls (Evans et al 2015), but definitive correlation with clinical outcomes is still lacking. It seems that exercise must be of sufficient intensity to increase circulating catecholamine levels in order to elicit an NK-cell
mobilising response (Idorn and Hojman 2016), suggesting that low-intensity exercise may not be sufficient. Skeletal-muscle-derived myokines are also likely to play a role.

In the current study blood samples were taken at clinic visits rather than during or after exercise episodes, and so any acute increases in circulating NK-cell numbers would be expected to return to normal by the time of sample acquisition. In chronic exercise many studies of health young controls show increased cytotoxic activity of circulating NK-cells, but generally no change in absolute numbers. The effect of increased cytotoxicity in older adults is less pronounced (Zimmer et al 2017).
5.6 NEEDLE CORE BIOPSIES

5.6.1 ACUTE INFLAMMATION
Acute inflammation was not a frequent finding in this biopsy series. PSA screening can lead to false-positive elevated PSA in the setting of acute prostatitis, leading to benign biopsy. Baseline acute and chronic inflammation in a benign biopsy appears to be associated with decreased risk of carcinoma in subsequent biopsies (Moreira et al 2014, Vavasada et al 2018). In addition, acute prostatitis may occur as a complication of the prostate NCB procedure. The participants in this trial all had a malignant diagnosis on their core biopsies and any patients whose elevated PSA was solely due to acute prostatitis would not have been included in the cohort.

5.6.2 PROSTATE ATROPHIC LESIONS
Atrophy was found in 43.48% of the specimens in this study. One series (Benedetti et al 2016) of 203 biopsies found a prevalence of 72.9%, predominantly PIA (as was the case in the current study). A series of negative biopsies in men who subsequently had a positive biopsy diagnosis of PrCa found at least focal prostate atrophy in 69.5% of specimens (Freitas et al 2017). Another similar study found SA in up to 94% of sextant biopsies (Postma et al 2005). These studies were of benign biopsies in patients subsequently diagnosed with cancer. While there are many studies of atrophy in benign and malignant prostatectomy specimens, in which atrophic changes are almost ubiquitous (Tomas et al 2007), there is limited published data on the prevalence of atrophic changes in malignant core biopsies. The volume of benign tissue in the cases in the current study was limited, as much of the tissue was replaced by tumour. This may explain the lower incidence of atrophy compared to benign biopsy series.

5.6.3 INFLAMMATION IN BENIGN PROSTATE TISSUE
The prevalence of chronic inflammation in benign areas of the NCBs (65.22%) was similar to other published studies, such as 86.2% in a case-control study of PrCa patients (Gurel et al 2014). In that study the presence of chronic inflammation was associated with increased odds of PrCa, particularly for high grade disease. Overall no significant differences were demonstrated between the exposed and non-exposed groups.

5.6.4 INFLAMMATION IN PROSTATE CANCER TISSUE
The proportion of cases with grade 0 tumour chronic inflammation as assessed by H&E was 10.87%, similar to that in the Banez et al paper (2010), in which 18% had no significant inflammation. Overall, men in the exposed group had tumours which showed a non-significant
trend towards a greater degree of intratumoural inflammation. For example, no patients in the non-exposed group had grade 2 tumour inflammation, whereas 18.92% of exposed group patients did. Using similar methods to the current study, Zeigler-Johnson et al (2016) found no significant differences in numbers of tumour infiltrating lymphocytes or macrophages in prostatectomy specimens from obese and non-obese men, although they found that CD68-positive macrophage numbers were associated with higher grade tumours. There was a non-significant trend in the current study towards exposed-group men having higher numbers of intratumoural macrophages (22.361 vs 14.875 per HPF). Tumour associated macrophages have been previously shown to be associated with poor outcomes in breast cancer (Tiainen et al 2015), perhaps via an obesity-mediated mechanism.

Only small numbers of CD56-positive NK-cells were seen within the tumours. A variety of non-haematological CD56-positive cells were identified however, including benign prostate epithelial cells (see figure 5.6) and nerves (see figure 5.7).

*Figure 5.6: CD56 expression in benign atrophic prostate epithelial cells (CD56, 20x)*
No significant differences in numbers of intratumoural CD56-positive NK-cells were seen between the exposed and non-exposed groups. Increased numbers of CD56-positive tumour-infiltrating lymphocytes are associated with a lower risk of progression in PrCa (Gannon et al 2009), in keeping with their known antitumour activity. However, Pasero et al (2016) showed that NK-cells tend to be present in both benign and malignant prostate tissue in only small numbers, and that those NK-cells which were present had an immature activated phenotype with little cytotoxic potential.
5.7 CORRELATIONS

5.7.1 CTC / PLATELETS
A strong direct correlation was identified between platelet count and mean CTC numbers per filter at a given blood draw. When subgroups were analysed the correlation persisted in the control group and the group who were overweight at randomization, and there was a non-significant trend towards the correlation in the exercise group. However there was no demonstrable correlation in the group who were of normal weight at randomization. This may be due to lack of statistical power, only 27 data pairs being available for assessment, but if this group truly does not show a correlation which is otherwise strongly demonstrated in this study then this is an important finding. It would suggest that whatever mechanism drives the correlation between platelets and CTC numbers in advanced PrCa patients as a group is attenuated among the (minority) subpopulation of patients of normal weight. Interestingly the platelet/CTC correlation was demonstrated in those blood draws where platelet cloaking had not been identified, but not in the 22 blood draws where at least one cloaked CTC was seen. It is tempting to conclude that overweight men have greater numbers of circulating platelets and a consequently greater tendency for platelets to cloak their CTCs. In truth it is difficult to draw any such firm conclusions from this however. While it has been difficult in the current study for technical reasons to directly demonstrate and measure platelet cloaking, the presence and absence of a direct correlation between CTCs and circulating platelet numbers in men who are overweight and of normal weight respectively does provide supportive evidence indicative of a central role in obesity in platelet-CTC interactions, and one which merits further investigation.

A study from Tibet compared platelet counts in CTC-positive and CTC-negative patients with lung cancer, and found significantly higher platelet counts in CTC-positive participants (Yang et al 2018). A study of oesophageal squamous cell carcinoma patients using both the ISET system (similar to ScreenCell) and CellSearch® , had similar findings (Li et al 2015). Although CTC enumeration was performed in the Li study for ISET/CellSearch® comparison purposes, correlation between platelet counts and CTC numbers as a continuous variable was not available in these studies.

A detailed study of 60 patients with gastric cancer (Zheng et al 2017) used filtration and cytological examination to identify CTCs, and then compared CTC numbers with a number of clinical inflammation-based indicators which are known to be associated with worse outcomes in many carcinomas. Several of these, the systemic immune-inflammation index (SII) and the
platelet lymphocyte ratio (PLR), include assessment of platelet counts. PLR has previously been shown to be a useful prognostic indicator in stage II colorectal cancer (Ozawa et al 2015) and following pancreatic cancer resection (Smith et al 2009, Shirai et al 2015). Zheng et al found significant correlations between SII and CTC count, and PLR and CTC count. The use of these systemic inflammation-based composite indicators is attractive as we hypothesise that platelet-CTC interactions are intimately associated with and dependent upon systemic inflammation, which can in turn be driven by the metabolic abnormalities found in obesity. A clinical trial attempting to assess the change in CTC numbers in breast cancer patients through inhibition of platelet function with clopidogrel and aspirin was unsuccessful (Roop et al 2013). This study was impaired by the low baseline level of CTCs in trial participants, and one might imagine that such a study might be more successful in the cohort of patients in the current study, in whom CTCs are abundant.

To our knowledge the current study is the first to establish a linear relationship between CTC numbers and platelet counts in patients with PrCa. There is no reason to assume that such a relationship would not exist in epithelial malignancies other than gastric and prostatic adenocarcinoma, but further research is required in this regard.

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 tumour dissemination. Evidence now shows that platelets support tumour metastasis by various mechanisms (Gay et al 2011). Platelets are involved in arrest of CTCs in the vasculature and through endothelial interactions enable their extravasation. Platelets also secrete various pro-oncogenic factors including PDGF and VEGF, and mediate pro-survival signals in ovarian cancer cells (Egan et al 2011). Platelets can adhere to CTCs and may thereby impair NK-cell mediated killing.

The interactions between CTCs and platelets are complex (reviewed Menter et al 2014), but overall tumour cell induced platelet aggregation (TCIPA) correlates with metastatic potential, and may be due to “cloaking” of tumour cells by adherent platelets. The interaction between CTC cloaking by platelets and their killing by NK-cells is incompletely understood. Thrombocytopenic mice exhibited reduced tumour metastatic burden when tumour cells were NK-cell sensitive, and in-vitro studies demonstrated reduced NK-cell tumourlytic activity when platelets aggregated around tumour cells (Nieswandt et al 1999). In a later mouse model, deficiency for Gαq, a G-protein critical for platelet activation, markedly decreased experimental and spontaneous
metastases (Palumbo et al 2005). This effect was eliminated in NK-cell deficient mice, further supporting the hypothesis that adherent platelets may obstruct the direct cell-cell contact required for NK-cell killing. Release of TGFβ by adherent platelets, which downregulates the NK-cell activating receptor NKG2D (Kopp et al 2009), may also inhibit NK-cell-tumour interactions. Platelets may enable evasion of NK-cell killing by conferring a “pseudonormal” phenotype on CTCs through high tumour cell surface expression of normal MHC class I antigen (Placke et al 2012).

TF expression by tumour cells and the presence of serum factor XIII transglutaminase support metastasis by impeding NK-cell killing of CTCs (Palumbo et al, 2007 and 2008). Of note, prostasomes secreted by benign and malignant prostate cells contain high concentrations of TF, with strong procoagulant effects in vitro (Babiker et al 2007), suggesting a link between TF activity and platelet cloaking of CTCs.

In a murine model, platelets activated by CTC interactions release adenine nucleotides which, acting upon the endothelial P2Y2 receptor, open the endothelial barrier and facilitate transendothelial migration (Schumacher et al 2013). An experimental antibody preferentially induces oxidative platelet fragmentation by targeting the β3 subunit of the platelet’s fibrinogen receptor, reducing metastasis (Zhang et al 2012, Ware et al 2012). Platelet-derived TGFβ and direct platelet-tumour cell contacts activate TGFβ/Smad and NF-κB cancer cell pathways, resulting in EMT and enhanced metastasis in mice (Labelle et al 2011). All of these mechanisms require close interaction of tumour cells with platelets, interactions which can be described and quantified as platelet cloaking.

Further research demonstrating 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 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.

5.7.2 CTC / LYMPHOCYTE SUBSETS
A correlation between CTC numbers and absolute circulating NK-cell count was detected in this study. There are surprisingly few published data relating correlations between lymphocyte subsets and circulating tumour cells. One study of patients with inflammatory breast cancer
Mego et al (2016) found no correlation between CTC numbers and total lymphocyte count, but did find that patients with more CTCs “had significantly lower percentages of CD3+ T cells and TCR-activated CD8+ T cells that synthesized TNF-α and IFN-γ, and a higher percentage of T-regulatory lymphocytes”. A study of 83 late-stage non-small cell lung cancer patients, which employed an antibody-dependent FISH methodology for enumerating CTCs, found that the percentages of CD3+, CD4+ and NK-cells were lower in CTC-positive than in CTC-negative patients (Ye et al 2017). Unlike in the current study, correlations with absolute numbers of the various lymphoid subpopulations were not reported. In treatment-naïve triple-negative breast cancer patients, there was a positive correlation between CTC status and peripheral NK-cell ratio, and in fact the combination of CTC count and NK-cell enumeration could predict progression free survival (Liu et al 2018). While absolute NK-cell numbers and percentages generally appear to be increased, the functional ability of circulating NK-cells to lyse CTCs may be impaired in advanced cancer. Using a chromium-51 percent specific lysis assay patients with breast cancer were found to have significantly decreased responses by their immune cells when they had more than 5 CTCs identified per 7.5ml of blood by the CellSearch® system (Green et al 2013). A study involving patients with breast, colorectal and PrCa (Santos et al 2014) found higher proportions of circulating NK-cells in the peripheral blood compared to healthy controls, and also that those patients with high numbers of CellSearch® -detected CTCs had impaired cytolytic activity compared to controls. Whether this is linked to the impairment of NK-cell function by platelets previously reported (Palumbo et al 2005) remains to be elucidated. However the direct correlations between CTC numbers, platelet numbers and NK-cells identified in this study provides supportive evidence.

It must be noted that most published studies have assessed lymphocyte populations as percentages of the overall lymphocyte count, rather than as absolute numbers. In the current study significant correlation was seen with absolute concentrations of lymphocytes, but not with percentages. The percentage NK-cells in a given blood draw varied from 9 to 28% in the current study, but the interquartile range was only 16-22%. 29 data points may not have provided sufficient statistical power to detect a true relationship over such a narrow range of percentages.

5.7.3 CTC / INFLAMMATION IN NCBS
There was no detectable correlation between numbers of CTCs at T0 and the degree of tumour inflammation in the NCB specimens. This is not surprising, considering that the biopsies represent in most cases the first diagnostic tissue obtained from the participants, months to many years in advance of their commencement of the trial (range 4-128 months, mean 33.67 months). CTC
numbers would be expected to change over time as each participant’s disease progresses. In many cases these biopsies would have been obtained when the patients were at an early stage of their disease, and may not represent the high-stage scenario in this study which was associated with high numbers of CTCs. Inflammation in the needle core biopsies may be a better surrogate for systemic inflammation and obesity, variables which are less likely to change dramatically over time, than for fluctuating CTC numbers.
6. CONCLUSIONS

This thesis describes a series of experiments on material derived from participants in the ExPeCT clinical trial who have advanced PrCa, particularly blood samples filtered using ScreenCell® Cyto filters for identification and enumeration of CTCs. The background to the project relates to the intersection of obesity, systemic inflammation and platelet-mediated hypercoagulability in advanced cancer. CTCs are a useful substrate for research in this regard as their interactions with the immune and coagulation systems in the bloodstream appear to be influenced by systemic inflammation, which can be driven by obesity.

It was found that CTCs are almost universally present in blood samples from ExPeCT trial participants, and that CTC numbers reduced significantly over the first three months of the trial – a change which remained statistically significant on subgroup analysis of overweight or obese participants, but not those of normal weight. Participation in an exercise intervention did not appear to affect CTC numbers, and in fact non-participants had a significant decrease in CTC numbers.

CTCs in samples derived from these men usually lacked cytoplasm and were therefore generally unsuitable for assessment of surface platelet adhesion (“platelet cloaking”), perhaps due to vacuum-filtration-related artefacts. However, blood draws from non-participants in the exercise intervention were significantly more likely to exhibit platelet cloaking than exercise programme participants. This finding is of questionable validity in view of the apparent artefactual stripping of cytoplasm for these cells in filtration, but does provide an interesting observation from a clinical population which would benefit from validation with a different CTC enrichment methodology.

Independent linear correlations were demonstrated between CTC numbers on the one hand and platelet count, total lymphocyte count, CD4-positive T-lymphocyte count and NK-cell counts. The demonstration of a relationship with platelet count is the first such report in men with PrCa. Although correlation does not imply causation it is abundantly clear from the literature that CTCs interact with platelets in various ways to enhance their metastatic potential, and this finding provides further evidence of a close association between the two. In PrCa patients, as in most cancers (with the exception of brain tumours and critically-located head and neck carcinomas), metastatic disease is the most frequent cause of cancer-related death. The therapeutic
implications are profound as if there is a way to break the partnership between the villainous CTC and its nefarious platelet sidekick then there is potential for a new type of anticancer drugs specifically targeted at this crucial step in the metastatic cascade. The correlations with various lymphocyte subsets are also interesting and emphasise the complex nature of interactions between CTCs and the innate and adaptive immune systems.

The findings relating to comparisons between participants who were overweight / obese and those of normal weight were overall disappointing. The study may not have been sufficiently powered to generate statistically significant differences between the two across many variables, and the selection of $25\text{kg/m}^2$ rather than $30\text{kg/m}^2$ as a cut-off point may have contributed to this.

Overall this study provides useful support for the hypothesis that metastasis, platelet function, systemic inflammation and hypercoagulability are closely linked in advanced cancer, and elucidates several potentially valuable directions for future research.
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