The Immune System: A Common Link Mediating Exercise Effects on the Tumour Microenvironment and Skeletal Muscle Mitochondria?

Linda Anita Buss

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Abstract

Exercise reduces the risk of developing a range of different cancers and has been associated with improved cancer patient survival. However, preclinical studies suggest that in some cases, exercise may be ineffective or even accelerate tumour growth. Understanding the mechanisms behind the exercise-cancer relationship may help us better identify which individuals are likely to benefit from exercise, and aid the development of new treatment strategies.

The overarching aim of this thesis was to investigate the mechanisms behind the effect of exercise on cancer outcomes, including its effects on the tumour microenvironment, tumour growth rate, cancer-associated muscle dysfunction and interaction with immunotherapy. Specifically, I investigated the response of the tumour and skeletal muscle to exercise (alone and in combination with anti-PD-1 immunotherapy) by characterising changes in tumour-infiltrating immune cells, tumour hypoxia and blood flow, and markers of muscle mitochondria in mice with transplanted melanoma or breast cancers. I hypothesised that exercise would enhance anti-tumour immune responses and improve tumour blood flow, thereby boosting the efficacy of concurrent immunotherapy. However, I also hypothesised that tumour burden would impair muscular adaptation to exercise, thereby potentially limiting exercise effects on the tumour.

Exercise, both alone and in combination with anti-PD-1 treatment, produced changes in the number and proportion of CD8+ tumour-infiltrating T cells. The direction of these changes varied with treatment and tumour type, but did not significantly affect tumour growth rate. Furthermore, breast cancer inhibited skeletal muscle mitochondrial adaptation to exercise. This impaired response was restored by anti-PD-1 treatment (in mice with either tumour type), suggesting that the immune system plays a critical role in effective muscular adaptation to exercise. Finally, I found that muscular COX-IV expression (as a proxy for mouse ‘fitness’ and an indicator of effective muscle response to exercise), was associated with slower tumour growth and reduced tumour hypoxia in hyperlipidaemic ApoE-/ but not wild-type mice.

Taken together, these results highlight the immune system as an important mediator of exercise effects on both the tumour and muscle tissue, and suggest that exercise may ‘normalise’ the negative effects of metabolic abnormalities on tumour progression.
Therefore, this work has provided mechanistic understanding of the exercise-cancer relationship and has generated the hypothesis that the combination of exercise and immunotherapy may be a potential novel therapeutic strategy for the prevention of cancer-associated muscle wasting.
Dedication

To my mother, Hendrikje, who was taken from us far too young. You may have only seen the beginning of my scientific journey, but you are the reason I came to UOC and as such have played an integral role in the scientist I have become. I would not be where I am today, professionally or personally, without your unwavering support. I see so much of you in me, and I know you will continue to guide me through everything you taught me: about life, about love and about finding joy in discovery.
Acknowledgements

First and foremost, I would like to extend a huge thank you to my primary supervisor, Associate Professor Gabi Dachs. I could not have asked for a better mentor and I will be eternally grateful for your role in moulding me into the scientist I am today. In particular, your support during the first year of my PhD made an incredibly stressful time in my personal life immeasurably easier.

Of course, I would also like to acknowledge my co-supervisors: Dr Abel Ang, Professor Bridget Robinson, Dr Barry Hock and Associate Professor Margaret Currie for always being available with words of advice and wisdom.

To the other members of the Mackenzie Cancer Research Group, both past and present: not everyone is lucky enough to work with people they even like, and yet somehow I had the good fortune to spend 4 years working with people who became like family to me. I don’t know what I would have done without your constant support and love.

To everyone who gave me scientific guidance who is not one of my supervisors: thank you. I would specifically like to mention Dr Troy Merry, without whose expertise and advice a key part of this project would not have happened, and Dr Elisabeth Phillips, who is an absolute goldmine of knowledge for experimental techniques.

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## Abbreviations

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<tbody>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HRQoL</td>
<td>Health-related quality of life</td>
</tr>
<tr>
<td>ICB</td>
<td>Immune checkpoint blockade</td>
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<tr>
<td>ICI</td>
<td>Immune checkpoint inhibition/inhibitors</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>ITSM</td>
<td>Immunoreceptor tyrosine-based switch motif</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid derived suppressor cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Non-obese diabetic, severe combined immunodeficient</td>
</tr>
<tr>
<td>Oxphos</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient-derived xenograft</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised controlled trial</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>TAM</td>
<td>Tumour-associated macrophage</td>
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### General abbreviations

<table>
<thead>
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<tr>
<td>TH cell</td>
<td>T helper cell</td>
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<tr>
<td>TME</td>
<td>Tumour Microenvironment</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple-negative breast cancer</td>
</tr>
<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt; cell</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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### Gene and protein abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMPK</td>
<td>AMP kinase</td>
</tr>
<tr>
<td>CA-IX</td>
<td>Carbonic anhydrase IX</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C motif chemokine ligand 2</td>
</tr>
<tr>
<td>CDX</td>
<td>Cluster of differentiation (number X)</td>
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<tr>
<td>CI-V</td>
<td>Oxidative phosphorylation complexes I-V</td>
</tr>
<tr>
<td>COX-IV</td>
<td>Cytochrome c oxidase subunit 4</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box protein 3</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>ILX</td>
<td>Interleukin X</td>
</tr>
<tr>
<td>LIGHT/TNFSF14</td>
<td>Homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor</td>
</tr>
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<td>Gene and protein abbreviations</td>
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<tr>
<td>expressed on T lymphocytes/Tumour necrosis factor superfamily member 14</td>
<td></td>
</tr>
<tr>
<td>MHC Major histocompatibility complex</td>
<td></td>
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<tr>
<td>NF1 Neurofibromin 1</td>
<td></td>
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<tr>
<td>PD-1 Programmed death protein 1</td>
<td></td>
</tr>
<tr>
<td>PD-L1 Programmed death protein ligand 1</td>
<td></td>
</tr>
<tr>
<td>PGC-1α Peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
<td></td>
</tr>
<tr>
<td>pHH3 Phospho-histone H3</td>
<td></td>
</tr>
<tr>
<td>PI3K Phosphatidylinositol-3-kinase</td>
<td></td>
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<tr>
<td>PR Progesterone receptor</td>
<td></td>
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<tr>
<td>PTX-3 Pentraxin 3</td>
<td></td>
</tr>
<tr>
<td>SHP Src-homology domain protein</td>
<td></td>
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<tr>
<td>SPARC Secreted protein acidic and rich in cysteine</td>
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<tr>
<td>TCR T cell receptor</td>
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<tr>
<td>TCR T cell receptor</td>
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<tr>
<td>VEGF Vascular endothelial growth factor</td>
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### Definitions

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>Acute exercise</td>
<td>A single exercise bout, used in a research setting to study the acute effects of exercise.</td>
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<tr>
<td>Chronic exercise/training</td>
<td>Regular exercise, which induces physiological adaptations over time.</td>
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<tr>
<td>Endurance exercise</td>
<td>Low intensity, long duration exercise, aimed at increasing aerobic endurance.</td>
</tr>
<tr>
<td>Exercise adaptability/exercise responsiveness</td>
<td>The capacity for an individual to adapt to regular exercise.</td>
</tr>
<tr>
<td>Exercise capacity</td>
<td>The maximum amount of physical exertion an individual can sustain, the gold standard measurement for which is VO$_2$ max (maximal oxygen consumption). Colloquially referred to as ‘fitness’.</td>
</tr>
<tr>
<td>Exercise volume</td>
<td>The total amount of exercise undertaken in a given period. Usually refers to distance run (most common for rodent studies, e.g. 8 km/day) or time spent exercising (more common for clinical/epidemiological studies, e.g. 2.5 h/week).</td>
</tr>
<tr>
<td>Immunogenic cell death</td>
<td>Cell death which induces an immune response</td>
</tr>
<tr>
<td>Lymphocytosis</td>
<td>A high circulating lymphocyte count.</td>
</tr>
<tr>
<td>Myokine</td>
<td>Muscle-derived cytokine.</td>
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<tr>
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<tr>
<td>Resistance exercise</td>
<td>High intensity, short duration exercise, generally aimed at increasing muscle mass and strength.</td>
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Publications


Buss*, Linda A; Mandani*, Anishah; Phillips, Elisabeth; Scott, Nicola J A; Currie, Margaret J; Dachs, Gabi U. Characterisation of a Mouse Model of Breast Cancer with Metabolic Syndrome. In vivo (Athens, Greece) 2018: 32(5); 1071-1080.

*joint first authors


1 Introduction

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1.1 Overview

Gone are the days in which cancer patients are treated to the adage “rest is best”. A wealth of epidemiological studies over the past two decades have provided evidence that physical activity or exercise reduces the risk of developing a range of different cancers (such as breast, colorectal and lung) and is even associated with improved survival outcomes in breast and colorectal cancer patients [1,2]. Furthermore, exercise exhibits a remarkable safety profile compared with cancer therapeutics – it is not associated with any toxicities of its own and may even reduce the rate or severity of treatment-associated adverse events [3]. In addition to survival benefits, exercise has been shown to improve cognitive and physical functioning, reduce anxious and depressive symptoms, and improve health-related quality of life in cancer patients (reviewed in [3]).

However, there are indications that exercise is not universally beneficial for all cancer patients. Occasional preclinical studies have suggested that, in some situations, exercise may not affect tumour growth rate at all – or even increase tumour growth rate [4,5]. It is unclear what the determinants of an exercise-responsive versus non-responsive tumour are, or how to identify which patients will benefit from exercise. The key to this may lie in how exercise affects the tumour microenvironment (TME).

The TME is the local cellular, physical, chemical and humoral environment that tumour cells inhabit. More specifically, it includes a range of cell types other than cancer cells, such as endothelial cells, immune cells, fibroblasts and adipocytes. Physical, chemical and humoral factors include the stiffness of the extracellular matrix, pH, oxygen tension, cytokines and growth factors. It is now well-known that tumour growth characteristics and metastatic capacity are in large part defined by the make-up of the TME. Therefore,
understanding the effects of exercise on the TME is integral to understanding how exercise exerts its beneficial effects (or lack thereof).

Cancer cachexia is a debilitating syndrome that often accompanies advanced cancer. It is characterised by involuntary weight loss, loss of muscle mass and strength, and fatigue, combining to generate a poorer quality of life and increased risk of death [6]. Given that exercise can enhance muscle mass and strength, it is possible that it may be able to attenuate cachexia [7]. However, it is unknown whether cachectic (or pre-cachectic) muscle responds to exercise in the same way as healthy muscle, and understanding this is essential to identifying and optimising viable exercise-based treatment or prevention strategies for cancer cachexia.

This chapter will summarise and synthesise available preclinical (and clinical, where available) data on the effects of exercise on the TME, and briefly summarise preclinical and epidemiological evidence on how exercise affects tumour growth/patient survival. The main focus of this chapter will be to discuss the finer details of how exercise affects the TME, including the role of muscle-derived factors, tumour vasculature/perfusion, hypoxia and immunity. In addition, I will describe the role of exercise in preventing cachexia and the factors affecting individual exercise capacity and exercise adaptability.

1.2 Mouse Models of Exercise

The three main exercise modalities used for preclinical research in mice are voluntary wheel running, treadmill running and swimming, each with advantages and disadvantages.

Wheel running has the advantage that mice exercise spontaneously, allowing for their natural preference for short, high intensity bouts of activity [8]. However, controlling the exercise ‘dose’ is not possible, resulting in large variation between running distances of individual mice, even those of the same strain [8].

Treadmill exercise (enforced by electric shock or gentle prodding) and swimming allow for better control of ‘dose’ (frequency and intensity), but are inherently stressful due to forcing the mice to exercise [9]. It is possible that this stress response might confound exercise effects. In addition, the distances run are typically much less than those seen in voluntary wheel running [10].
It should also be noted that there are some differences in exercise adaptation using treadmill compared with wheel exercise. Kim et al. found that although fat mass gain was attenuated to a similar degree by treadmill exercise and wheel running, only wheel running resulted in a significant improvement in grip strength [10]. In addition, expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α, the master regulator of the muscle response to exercise) was increased to a greater degree in the soleus muscle by wheel running, but treadmill exercise was more effective at inducing its expression in the gastrocnemius muscle [10]. This illustrates that although there are some parallels, different mouse exercise modalities can have subtly different effects on the body.

Encouragingly, the major physiological responses to both acute and chronic exercise appear to be very similar in mice and humans [11]. Both mice and humans show improvements in maximal aerobic exercise capacity (VO2 max) and muscle strength with training, as well as increases in mitochondrial content and function [11,12]. In addition, acute exercise-induced lymphocytosis (increased circulating lymphocyte count) is seen in both rodents and humans [13,14]. Furthermore, mouse skeletal muscle respiratory capacity and control is similar to that seen in human skeletal muscle [15].

1.3 Effect of Exercise on Tumour Growth/Patient Survival

In this section I will give only a brief overview regarding the effect of exercise on tumour growth/progression and patient survival, as the main focus of this thesis is on the physiological adaptations of the tumour microenvironment (TME) and muscle to exercise. I refer the interested reader to the many comprehensive reviews on the effect of exercise on cancer patient survival [2,3,16,17] and the effect of exercise as a sole intervention in preclinical studies [3,18–21].

1.3.1 Clinical Studies

The majority of clinical studies investigating the effect of exercise or physical activity on cancer patient survival have been observational studies. There is evidence that meeting the World Health Organisation (WHO) guidelines of 150 minutes of moderate intensity or 75 minutes of vigorous intensity exercise per week confers a significant survival benefit for cancer patients, and for some cancer types, such as colorectal cancer, a dose-response relationship between exercise volume and survival has been reported [2].
Current evidence supports a 40-50% reduction in all-cause mortality for breast, colorectal and prostate cancer survivors engaging in high levels of physical activity; other cancers have not yet been sufficiently studied in this context [17]. However, there is a large possibility of reverse causation for the relationship between cancer survival and physical activity level (patients who are less well may exercise less, rather than high activity levels causing an improved outcome) [17], which emphasises the importance of conducting randomised controlled intervention trials to fully investigate the role of exercise in improving patient survival. Two large intervention studies (the CHALLENGE trial and the INTERVAL trial) are ongoing to address this and will shed more light on whether a targeted exercise intervention can improve survival [22,23].

1.3.2 Preclinical Studies

The effect of exercise on tumour growth in preclinical studies is less clear than in epidemiological studies. This is likely to be largely due to heterogeneity in study design. Key factors that influence outcomes of preclinical studies are the rodent strain used (different strains have different inherent exercise capacity and adaptability), immunocompetency of the rodent strain, timing of exercise initiation (pre vs post-‘diagnosis’), tumour type and anatomical location, exercise modality (forced: swimming or treadmill, voluntary: wheel running), tumour burden and study endpoint (predetermined time after tumour initiation or ethically determined by tumour size). Very few studies have more than a few of these factors in common, making comparisons difficult. Comprehensive discussion of these parameters is beyond the scope of this chapter (refer to [19] for comparison of the effects of some of these parameters on tumour growth), but I will briefly discuss the role of pre- versus post-implantation (mimicking pre- vs post-diagnosis) exercise on tumour growth.

In many preclinical studies, post-implantation exercise either only marginally affects tumour growth rate [24–27], or does not affect tumour growth rate at all [28–32]. In agreement with this, only a “small to moderate” effect size of exercise on final tumour size was observed in a recent systematic review [19]. Of those studies included in the analysis that did find a statistically significant difference in final tumour size, 4/8 had a ‘probably high’ risk of bias (as determined by the authors of the systematic review, mainly due to missing data) and one even showed an increased tumour size with exercise. Together with the observation that many studies find a statistically but not clinically
significant result, this suggests that exercise as a sole intervention (monotherapy) is minimally effective at slowing primary tumour growth rate.

The documented effect of pre-implantation exercise is more consistent than that of post-implantation exercise. In the above-mentioned systematic review [19], studies in which exercise was performed both pre- and post-implant had a larger effect size for exercise to reduce tumour growth than studies in which exercise was performed only after tumour implant [19]. In addition, Pedersen et al. found that growth rate of B16-F10 melanoma was slowed with pre-implantation or pre-and post-implantation exercise, but not post-implantation exercise only [29]. Similarly, a number of studies using carcinogen-induced models (which typically start exercise after carcinogen administration but before tumours become detectable) have found a reduction in malignant tumour incidence and/or overall tumour burden (by number of tumours per animal or combined weight of tumours) [29,33]. This suggests that while exercise monotherapy may not be very effective (as discussed above), exercise pre-conditioning may be important both for prevention and slower growth of cancer once it has arisen. In a clinical setting, this may also translate to reduced rates of recurrence after tumour control following treatment, although this remains speculative.

It seems clear that exercise can reduce tumour growth rate, but likely only to a significant extent in a pre-implantation setting or possibly in combination with cancer therapies. However, some studies have found differences in the exercise-responsiveness of different tumours, using the same exercise protocol, with some tumours (of the same subtype) exhibiting either no change in growth rate or even an increased growth rate with exercise [4,5]. Specifically, Glass et al. found that three different claudin-low breast cancer models showed diverse responses to post-implant exercise, with EO771 tumour growth being reduced with exercise training, 4TO7 growth showing no change and C3(1)SV40Tag-p16-luc tumours showing accelerated growth [4]. Moreover, a second study found that the growth of 3/6 colorectal patient-derived xenograft (PDX) tumours was slowed by exercise, while the remaining 3/6 showed unchanged growth rates [5]. This is important, as it indicates that exercise treatment is far from a one-size fits all approach and some patients may not benefit from exercise.
1.3.3 The Effect of Exercise on Metastasis

Metastasis is the process whereby tumour cells migrate from the site of the primary tumour to establish secondary tumours in different tissues. For many cancer types, it is the emergence of secondary tumours in vital tissues which ultimately causes death.

Before cancer cells can seed in secondary sites, they must survive transport through the circulation. Regmi et al. have shown that high shear stresses (such as those present in the vasculature during intense exercise) can kill circulating tumour cells using an in vitro microfluidic system [34]. However, most of the time points used were not clinically relevant. Cells were circulated under high shear stress for up to 18 hours – this mimics the scenario of vigorous exercise for 18 hours, which is highly unrealistic for the vast majority of the healthy population, let alone cancer patients. In one experiment, increased lactate dehydrogenase (LDH) release (a proxy for necrotic cell death) was seen after one hour of circulating under high shear stress, which represents a more achievable length of exercise time. Much longer than this is not realistically achievable in a clinical setting. It would be prudent to repeat these experiments with shorter time-points that more accurately mimic the exercise behaviour of the average population (and specifically, cancer patients).

As with preclinical data examining the effect of exercise on primary tumour growth, it is unclear how exercise affects metastasis due to varying results. However, there are several studies describing a reduced number or mass of metastases with both spontaneous [31,35,36] and experimental metastasis [24,29,37–39], with fewer studies reporting no change [38,40] or an increase in the number/mass of metastases [24,41], suggesting that in many situations, exercise can inhibit metastatic tumour formation. Stress may play a role in how exercise affects metastasis. Zhang et al. found that swimming for 8 min/day (which mice performed without added encouragement) reduced the relative size of experimental lung metastases, whereas when mice were forced to swim for 16 or 32 min/day, the relative size of metastases was increased [24]. More mechanistic studies are required to help delineate how exercise affects different aspects of the metastatic cascade.

1.4 Myokines and Other Circulating Factors

During exercise, muscle tissue releases a vast array of factors into the circulation, collectively termed ‘myokines’ (from ‘muscle-derived cytokines’). These myokines have
known effects on peripheral tissues, such as skeletal muscle remodelling in response to exercise and improvements in cognitive function (reviewed in [42]). It is thought that the action of myokines (and other factors) is directly (affecting cancer cells) and/or indirectly (affecting other cells of the TME) responsible for many of the changes seen in the TME with exercise.

A number of in vitro studies have found that post-exercise serum (serum harvested from humans or animals following an acute exercise bout) can directly inhibit cancer cell proliferation, viability or survival when supplemented into the cell culture media [43–47]. This has been attributed to a few different myokines, including secreted protein acidic and rich in cysteine (SPARC) [48], irisin [49] and oncostatin M [46].

Direct in vivo data indicating the effect of select myokines on tumour growth is still largely lacking. However, Aoi et al. found that the protective effect of exercise against azoxymethane-induced colon tumourigenesis was nullified in SPARC knock-out mice, and SPARC was able to induce colon cancer cell apoptosis in vitro [48]. This suggests that the myokine SPARC plays an important role in the protective effect of exercise on cancer development and highlights the value of further investigating the effects of exercise-induced myokines on tumour growth.

Research by Hojman and colleagues indicates that exercise-induced catecholamines (epinephrine and norepinephrine) have both an indirect and a direct effect resulting in the reduction of tumour growth rate [29,44]. Human breast tumour cells preconditioned with exercise serum were less able to form xenograft tumours in mice, and this effect was completely abolished when the beta-blocker propranolol was also added to the pretreatment [44]. In addition, daily injections of epinephrine or norepinephrine were able to significantly slow MCF-7 and MDA-MB-231 xenograft growth rate [44]. Indirectly, catecholamines (and IL-6) were essential for the exercise-induced mobilisation of natural killer (NK) cells to the tumour site, which were themselves essential for the exercise-induced delay in tumour growth seen in that study [29].

A third factor that has been linked to an exercise-associated delay in tumour progression is dopamine. Zhang et al. found that moderate swimming exercise reduced the tumour weight of subcutaneous and pulmonary hepatomas in mice, and this was mirrored by an increase in dopamine levels in the prefrontal cortex, serum and tumour tissue [24]. Moreover, dopamine treatment was able to reduce tumour weight to the same extent as
swimming, and a dopamine receptor 2 antagonist (domperidone) abolished the tumour growth inhibitory effect of both dopamine and swimming exercise [24].

Although there is still a scarcity of data investigating the effects of exercise factors on tumour growth and the tumour microenvironment, those studies that have been done indicate that exercise-induced systemic factors may mediate the effects of exercise on the tumour microenvironment.

1.5 Effect of Exercise on Tumour Vascularity, Hypoxia and Perfusion

1.5.1 Effect of Exercise on Normal Vasculature

Acute exercise modulates blood flow to different organ systems, with some receiving increased (such as skeletal muscle) and some receiving decreased (such as skin) blood flow during exercise [50]. This enables the body to cope with the stress of acute exercise by providing those tissues directly involved in exercise with more oxygen and nutrients. Meanwhile, chronic exercise can induce vascular remodelling [51]. Skeletal muscle is the tissue most affected by these changes, but many tissue types are affected to some degree, including the brain, heart and bone [52–54].

It has been shown in vitro and in vivo that exercise can directly affect endothelial cell behaviour. Schadler et al. transplanted Matrigel plugs (an artificial matrix containing gelatine and basement membrane proteins) containing primary mouse endothelial cells into mice and found that those implanted into exercising mice were better perfused and showed elongated vessels compared with those implanted into non-exercising mice [30]. In addition, endothelial cells exposed to exercise-conditioned serum in a microfluidic system showed reduced sprouting (i.e. less angiogenesis), as did those exposed to high shear stress (mimicking that present during exercise) [30]. This seems counterintuitive, but the authors argue that this reflects increased vascular maturity which is ultimately conducive to more stable vascular networks. Interestingly, exercise may also reduce age-associated venous endothelial cell senescence in humans [55].

Given that exercise can affect endothelial cell behaviour and induce vascular remodelling in a variety of normal tissues, it is possible that tumour vasculature may also be affected by exercise. A number of preclinical studies have investigated how exercise affects
tumour hypoxia, perfusion and vascularity in various tumour types and locations (results summarised in Figure 1.2).

![Diagram of acute and chronic exercise effects on tumour vascular characteristics and hypoxia]

**Figure 1.1: Effects of acute and chronic exercise on tumour vascular characteristics and hypoxia.**

Acute exercise increases blood flow to tumours located in tissues that receive constant or increased blood flow during exercise, while decreasing blood flow to tumours located in tissues that are poorly perfused during exercise. This may alter levels of hypoxia and affect drug delivery in the indicated directions. Vascular contractility and dilatory responsiveness are impaired and unchanged by acute exercise, respectively. On the other hand, long-term training (chronic exercise) may improve vascular maturation by improving responsiveness and pericyte coverage, which may improve perfusion, oxygen extraction and drug delivery, and reduce hypoxia. Areas in need of further research are indicated with ?. Figure reused from [56] under the Springer Nature author licensing permissions.

### 1.5.2 Effect of Exercise on Tumour Hypoxia

Just like any other tissue, tumours require blood flow delivering oxygen and nutrients in order to survive. However, tumours are not evenly perfused and are characterised by disorganised, dysfunctional vasculature and regions of hypoxia [57]. This leads to the activation of hypoxia factors such as the hypoxia-inducible factors (HIFs), which stimulate the transcription of a large array of genes which help the cell adapt to low oxygen conditions. These include genes central to angiogenesis, cell metabolism and metastasis [58]. In normal tissue, this results in improved vascular coverage and subsequent improved perfusion and alleviation of hypoxia. However, in tumours, the hypoxic response does not improve perfusion as the new blood vessels formed are often
immature and may be leaky or lack proper haemodynamic control [59,60]. This perpetuates tumour hypoxia rather than alleviating it.

One study found that hypoxia in orthotopic prostate tumours was reduced during exercise [61], and this was also evident in tumours from trained rats [32]. Furthermore, Betof et al. observed that tumour hypoxia was decreased in orthotopic 4T1 breast tumours from exercising mice [27].

Levels of the hypoxic response protein HIF-1α were decreased with exercise in an orthotopic breast cancer model [4]. In contrast, HIF-1α levels were increased with exercise in human-derived breast xenografts in athymic mice [62], and in orthotopic breast and prostate cancer in immunocompetent mice [4,31]. In subcutaneous Ewing sarcoma xenografts, HIF-1α and carbonic anhydrase IX (CA-IX, a HIF-1 target gene) mRNA were decreased with exercise in one of two tumour cell lines used [63]. It is unclear why this disparity exists between measured hypoxia and levels of HIF-1α, given that HIF-1α protein stability is strongly dependent on oxygen levels. However, it can partially be regulated independently of hypoxia (for example by oxidative stress), which may explain the above-described results [58,64].

To my knowledge, hypoxia in tumours from mice starting exercise prior to tumour cell inoculation has not yet been investigated and nor has tumour hypoxia in exercising cancer patients. It is also unknown whether the reduction in tumour hypoxia occurs in tumour types other than breast and prostate cancer.

**1.5.3 Tumour Perfusion and Vessel Density**

Initial anti-cancer strategies targeting tumour blood vessels focussed on inhibiting angiogenesis, as tumour cells will die if completely deprived of oxygen and nutrients. However, as with all cancer therapies, many tumours develop resistance to anti-angiogenic agents. An alternative strategy is vascular normalisation [59], which aims to promote the normal development of tumour vessels to form a functional, evenly perfused network which more closely resembles that of normal tissue. This would reduce hypoxia, thereby reducing metastatic potential and enhancing radiosensitivity. In addition, drug delivery throughout the tumour would be improved.
1.5.3.1 Preclinical studies

The effect of exercise on tumour vascularity and perfusion remains unclear. Studies by various groups have demonstrated an increase in perfusion homogeneity, the level of perfusion and/or vessel density or $Cd31$ mRNA levels (breast, prostate and pancreatic cancer) [27,30,31,65–67], but others have found no change in the mean level of perfusion (15,19) or vessel density in breast and/or prostate cancer (112). Further studies even found reduced numbers of blood vessels in breast tumours or lymphomas from exercising mice [69,70].

McCullough et al. observed increased blood flow to orthotopic prostate tumours during acute exercise (but not with exercise training) which was associated with a reduction in tumour hypoxia, which suggests that levels of tumour perfusion may change with acute exercise but this is not necessarily maintained after exercise cessation [61].

A potential reason for the differing results observed in different studies may be the method used to detect tumour perfusion. McCullough et al. used IV injection of Hoechst 33342 prior to euthanasia to label perfused blood vessels [32], while other studies used MRI to generate a perfusion map of the entire tumour while the animal was still alive [27,31].

A further important consideration is the impact of anatomical location of the tumour on blood flow responses to exercise. Garcia et al. elegantly demonstrated that blood flow during exercise is increased to orthotopic prostate tumours in rats, but decreased to subcutaneous tumours of the same type [50]. They further measured blood flow to different organs during exercise, including the bladder, prostate (location of the orthotopic tumour), soleus muscle, kidneys, skin, subcutaneous adipose (location of the ectopic tumour) and visceral adipose tissue. Blood flow to the bladder and prostate was unchanged, but increased to the soleus muscle and decreased to the kidneys, skin, subcutaneous and visceral adipose tissue [50]. This suggests that host tissue haemodynamics in response to exercise also play a role in regulating blood flow to the tumour. As such, tumours located in tissues that become less well perfused during exercise may not benefit from the increased perfusion seen in prostate tumours, and may even become more poorly perfused during exercise, which could exacerbate tumour hypoxia. This has yet to be further investigated in different tumour models and is likely
to also be affected by the arrangement of the tumour microvessel network with regards to that of the normal tissue and supplying arterioles, as outlined in Figure 1.2.

![Diagram showing effects of acute exercise on tumour blood flow and hypoxia.](image)

**Figure 1.2: Hypothetical effects of acute exercise on tumour blood flow and hypoxia.**

Acute exercise increases blood flow to tissues whose energy demand increases during exercise, such as working muscle, and decreases blood flow to other tissues not involved in the acute exercise response, such as the gut. This, in combination with the fact that tumour vessels can be arranged in parallel or series to normal tissue microvessels with regard to the supplying arterioles, likely means that exercise will differentially affect blood flow to tumours localised in different tissues. Tumours whose vessels are arranged in parallel to tissue that receives increased blood flow during exercise may themselves receive decreased blood flow during exercise due to the vascular steal effect. In this case, arterioles supplying the normal tissue dilate but those supplying the tumour do not (due to impaired vascular responsiveness to dilatory or contractile cues), thereby effectively pulling blood away from the tumour (a). This would likely result in increased levels of tumour hypoxia. The opposite would occur in tissues that receive reduced blood flow during exercise (in which the supplying arterioles contract), and the result would be a more highly perfused and oxygenated tumour (b). In the situation of tumour vessels being arranged in series to normal tissue vessels, the scenario would differ. In this case, tumours localised in tissues receiving increased blood flow during exercise would also receive increased blood flow (c), while those localised in tissues receiving decreased blood flow during exercise would also receive decreased blood flow (d). In reality, the tumour microvessel network would likely be arranged in a combination of series and parallel, making the blood flow response of particular tumours to exercise difficult to predict. The thickness of the arrows indicates the level of blood flow through the vessel.

Tissue blood flow is largely regulated by vascular contractility and myogenic tone. Due to their poor maturation (lack of smooth muscle cells and innervation), tumour vessels have poor contractile and dilatory responsiveness, which limits their ability to regulate blood flow [50,61,71]. Contractile responsiveness to norepinephrine does not change with exercise training, indicating that tumour vessel response to both acute and chronic exercise is impaired compared with normal tissue [50,61]. Due to this, tumour blood flow cannot be regulated to provide optimal conditions for oxygen extraction. Optimal oxygen extraction relies on complex haemodynamics, the most important factor being red blood cell flux (number of red blood cells flowing through a vessel per unit time) [64]. Red blood cell flux is determined by the orientation of microvessels within the network, the
diameter of the vessels (with higher flux to large diameter vessels) and intravascular oxygen concentration (low intravascular oxygen concentration increases red blood cell adherence, thereby increasing blood viscosity and slowing flow) [64]. In addition, tumour oxygenation can be reduced by shunt flow (whereby large diameter vessels shunt blood around the perimeter of the tumour, away from the tumour body), low vascular density and a high oxygen consumption rate [64]. As mentioned above, acute exercise does not alter tumour vessel contractility, but it is unknown whether long-term exercise could improve vascular maturation to a point where vessel contractile and dilatory responses are restored, thus providing improved regulation of red blood cell flux and oxygen extraction.

Part of the therapeutic appeal of vascular normalisation is the enhanced delivery of anti-cancer agents to the tumour. Two preclinical studies have investigated how exercise affects chemotherapy delivery to the tumour [30,63]. Schadler et al. found that although exercise alone did not reduce tumour growth rate of subcutaneous, pancreatic PDAC or B16-F10 melanoma, chemotherapy in combination with exercise significantly slowed tumour growth rate over and above the effect of chemotherapy alone [30]. Immunofluorescence analysis revealed that there was increased expression of the DNA damage marker γH2AX in PDAC tumours from mice receiving both chemotherapy and exercise compared with those only receiving chemotherapy, and higher levels of doxorubicin fluorescence in B16-F10 tumours from exercised mice receiving chemotherapy compared with those receiving chemotherapy only. This was only the case in tumours from trained mice; one acute exercise session was insufficient to enhance doxorubicin delivery to the tumour [30]. Furthermore, the authors demonstrated that pharmacologically increasing tumour blood velocity by the use of an anti-hypertensive agent (prazosin) also enhanced the growth inhibitory effect of gemcitabine on PDAC tumours [30]. Similarly, Morrel et al. found that exercise improved doxorubicin delivery to subcutaneous Ewing sarcomas and this was associated with a further reduced tumour growth rate compared with exercise or doxorubicin alone (although exercise alone also had a strong growth inhibitory effect) [63]. These data suggest that exercise can induce vascular changes leading to improved tumour blood flow even in tumours that are located in tissue that does not receive enhanced blood flow during acute exercise (i.e. subcutaneous adipose tissue), although this is yet to be corroborated by other groups and
it has not been investigated whether exercise affects systemic pharmacokinetics of chemotherapy drugs.

1.5.3.2 Clinical studies

Excitingly, the first study examining the effect of exercise on intratumoural vessel density in clinical samples was recently published [67]. In that study, patients with pancreatic adenocarcinoma were provided with a home-based exercise program for at least 6 weeks during neoadjuvant chemotherapy or chemoradiation, until surgical resection. Exercising patients were well-matched with historical controls in terms of age, sex, time to surgery, pre-operative treatment type and surgery type, but tended to have more ‘potentially resectable’ than ‘borderline resectable’ disease compared with controls (61% vs 23% and 30% vs 69%, respectively; p=0.07). The authors found that patients who had participated in the exercise program (n=23) had significantly higher numbers of CD31+ vessels (>20 vs approx. 5 vessels per 200x field) in their tumours compared with historical controls (n=15) [67]. Outcome data was not available, but this provides preliminary evidence that exercise may be able to enhance vascularisation in pancreatic tumours in humans, but does not indicate whether these vessels are functional. Future work should aim to determine whether the increased vessel density is accompanied by markers of vascular maturation (such as increased pericyte coverage) and reduced hypoxia, which may reduce tumour aggressiveness and improve drug delivery/efficacy.

1.5.4 Markers of Angiogenesis and Vascular Maturation

Further to the above-described effects of exercise on tumour hypoxia, vascularity and perfusion, a few studies have investigated markers of angiogenesis and vascular maturation in tumours following exercise.

Betof et al. found that voluntary wheel running not only increased CD31+ vessel density, but also enhanced pericyte coverage (a marker of vascular maturation) in orthotopic 4T1 breast tumours [27]. In addition, pericyte coverage was increased by exercise in two Ewing sarcoma models [63]. Conversely, Schadler et al. found that the α smooth muscle actin (α-SMA, a pericyte marker) to CD31 ratio did not change with exercise in subcutaneous B16-F10 tumours [30]. This discrepancy may simply be due to differing tumour models.
A few studies have investigated tumour levels of the angiogenic factor vascular endothelial growth factor (VEGF, a HIF-1 target) in mammary tumours. One study observed increased mRNA levels of Vegfa [27]. In agreement with this, another group found increased VEGFA protein expression in mammary tumours from exercised rats [65]. In contrast, two other studies found that VEGF protein expression was reduced in tumours from mice exercising after tumour implant [26,69].

1.5.5 Conclusions on Tumour Vascularity, Hypoxia and Perfusion

It seems clear that acute exercise can regulate tumour blood flow, either increasing or decreasing blood flow depending on tumour location [50]. However, it remains unclear whether or how long this persists after exercise cessation, and whether chronic exercise can remodel the TME in such a way as to normalise the vasculature to improve perfusion and oxygen extraction even at rest. These questions are central to future work in this area, as a thorough understanding of tumour blood flow and perfusion dynamics (with respect to acute and chronic exercise) is required to inform relevant intervention trials and subsequent clinical practice to achieve the greatest benefit from exercise together with standard cancer therapies.

1.6 Effect of Exercise on the Immune Microenvironment

1.6.1 Overview of the Immune System

The role of the immune system is to protect the host from disease caused by pathogens or by the organism’s own cells (cancer). In addition, it plays a major part in the healing of injuries. As such, the vertebrate immune system is a complex interplay of organ systems, cell types and humoral factors that work together to defend the host [72]. It can be divided into two major arms: the innate and the adaptive immune system.

All immune cells originate in the bone marrow, from haematopoietic stem cells [73]. Haematopoietic stem cells differentiate into lymphoid and myeloid progenitor cells, which further differentiate and mature into specific immune cell types outside of the bone marrow [73]. Cells belonging to the myeloid lineage include granulocytes (neutrophils, basophils and eosinophils), dendritic cells, mast cells and macrophages, while lymphoid cells include B cells, T cells and natural killer (NK) cells [72]. Basic interactions between these cells are shown in Figure 1.3.
Following an immunogenic stimulus, there is an initial inflammatory reaction in which the pathogen is removed or dead cells/debris are cleaned away (as in the case of injury) [73]. Then, the response switches to a more tolerogenic (immunosuppressive), anti-inflammatory response which promotes tissue repair. If this switch does not occur and inflammation continues unchecked, tissue damage can occur. Examples of this are autoimmune diseases, in which an inflammatory reaction to the host’s own tissues occurs, or allergies, in which an inflammatory reaction to an innocuous environmental antigen occurs [73].

Figure 1.3: Overview of the basic interactions between different immune cell types. Following an immunogenic stimulus, the innate immune system is activated, resulting in inflammatory effector functions such as phagocytosis of microbes by M1 macrophages or tumour cell lysis by natural killer (NK) cells. Dendritic cells (DC) take up antigens, travel to the lymph nodes and present these to naive T cells, which are then activated and mature into different effector cell subtypes depending on the cytokine milieu. Type 1 T helper (T\textsubscript{H}1) cells support cellular immunity by promoting M1 macrophage polarisation and cytotoxic T lymphocyte (CTL) function. T\textsubscript{H}2 cells support humoral immunity by activating B cells and promoting antibody production by plasma cells. Regulatory T (T\textsubscript{reg}) cells and M2 macrophages are immunosuppressive, inhibit effector cell functions, resolve inflammation and promote tissue repair.

1.6.1.1 Innate immunity

Innate immunity is the host’s initial line of defence and generates an immediate, non-specific response to a stimulus, with recruitment of the adaptive immune system once
activated. Key immune cells belonging to the innate immune system are NK cells, macrophages, granulocytes and dendritic cells [73].

NK cells are able to directly lyse target cells without recognition of a specific antigen, and instead recognise other hallmarks of aberrant cells such as downregulation of major histocompatibility complex (MHC)-I [74]. As such, their primary function is defence against virus-infected and cancerous cells.

Macrophages and granulocytes are phagocytes, which can engulf and destroy pathogens. In addition, these cells play an important role in wound healing and injury repair: in the inflammatory phase, they phagocytose cellular debris, and in the anti-inflammatory phase they promote angiogenesis and extracellular matrix production to induce tissue regeneration [73,75].

Dendritic cells are the link between the innate and the adaptive immune system. They take up antigens in the periphery, then mature and travel to the lymph nodes to present the antigens to naïve T cells [73].

1.6.1.2 Adaptive immunity

The adaptive immune system provides a delayed, but antigen-specific response to a stimulus and is capable of immunological memory – the phenomenon by which the host is protected from repeat infection. The two main cell types belonging to the adaptive immune system are T and B cells. During development their respective antigen recognition receptors are randomly rearranged at the genomic level, resulting in receptors with unique recognition sites which bind to a specific antigen [73]. Upon contact with this antigen, clonal expansion of the corresponding cell leads to a pathogen specific immune response with the goal of destroying the pathogen or infected cells.

B cells provide humoral immunity: upon activation, they mature into plasma cells and produce antibodies, which act by neutralising viruses and toxins, opsonisation of microbes (which aids phagocytosis) and antibody-dependent cell-mediated cytotoxicity (which aids NK-cell-induced apoptosis of the target cell) [76].

T cells either promote the response of other immune cells (CD4+ T helper cells) or destroy infected cells (CD8+ cytotoxic T cells). T cells recognise foreign antigens bound to MHC molecules on the surface of host cells via the T cell receptor (TCR) and the respective
co-receptor (CD4 or CD8). After activation in this manner, the effector function of the cell is initiated [77].

One such effector function is the targeted killing of infected cells by CD8+ cytotoxic T cells. CD8+ T cells recognise and bind foreign antigens presented on MHC I molecules and kill the infected cell by induction of apoptosis via the fas ligand and the secretion of granzymes and perforins [78].

T helper (TH) cells operate mainly in an indirect manner by the secretion of cytokines which induce or repress the effector function of other cell types, such as CD8+ T cells or macrophages. In addition, they can activate B cells by binding to antigen-MHC II complexes on the cell’s surface [73]. A number of different TH-cell subsets are known, each of which express characteristic transcription factors, surface markers and cytokines and are functionally distinct. To which effector subset a CD4+ T cell will differentiate depends on the cytokines present in the microenvironment, which induce a lineage-specific transcriptional program [79].

TH1 cells secrete pro-inflammatory cytokines such as interferon gamma (IFNγ), thereby promoting cellular immunity by supporting CD8+ T cell effector functions and an M1 (pro-inflammatory) macrophage phenotype [73].

In contrast, TH2 cells are primarily responsible for the activation of B cells, thereby supporting antibody production and humoral immunity [73].

Regulatory T cells (Treg cells) are immunosuppressive and as such promote tolerance to innocuous antigens. They play a key role in the prevention of autoimmunity [80]. They are characterised by the expression of the lineage-specific transcription factor Foxp3 high levels of the interleukin (IL)-2 receptor alpha chain (CD25) and the ability to suppress the inflammatory function of other immune cells [81].

1.6.1.3 The tumour immune microenvironment

The immune microenvironment of a tumour is largely immunosuppressive [82]. Tumour cells employ strategies such as the secretion of immunosuppressive cytokines, the expression of inhibitory molecules such as programmed death ligand 1 (PD-L1), and the downregulation of MHC-I molecules to evade immune recognition [82,83]. This promotes an immunosuppressive phenotype of infiltrating innate and adaptive immune cells, resulting in large proportions of M2 (anti-inflammatory) tumour-associated
macrophages (TAMs), myeloid derived suppressor cells (MDSCs, a heterogeneous population of myeloid-lineage cells with immunosuppressive functions) and T_{reg} cells [84]. These cell types in turn inhibit the cytotoxic capabilities of cells such as NK cells and CD8^{+} T cells [73]. In addition, prolonged antigen exposure results in T cell exhaustion, characterised by high expression of inhibitory receptors such as PD-1 and an impaired ability to execute effector functions [85].

1.6.2 Effect of Exercise on Immunity in Healthy Individuals

Acute exercise causes a rapid rise in the number of circulating immune cells; this includes an increase in numbers of all major subclasses (lymphocytes, monocytes and granulocytes) [86]. Lymphocytes, in particular NK cells, are among those that respond most strongly to acute exercise [87]. Following exercise cessation, lymphocyte counts in the blood rapidly decrease, falling below pre-exercise levels by one hour post-exercise [86]. This was previously thought to be due to lymphocyte apoptosis and attributed to an immunosuppressive effect of exercise, but based on evidence that lymphocyte apoptosis post-exercise only accounts for a small fraction of the observed lymphocytopenia, it seems more likely that the bulk of this is due to egress into peripheral tissues and may present a mechanism for heightened immune surveillance of tissues post-exercise [88]. Direct evidence for this is still lacking, but is supported by evidence that leukocyte subtypes that are preferentially mobilised by exercise tend to be cytotoxic subtypes and express markers associated with extravasation and tissue migration (such as integrins and chemokine receptors) [89–91].

Regular moderate intensity exercise has been linked with enhanced overall immunity, such as improved NK cell cytotoxic activity, increased lymphocyte proliferation, reduced T cell senescence and enhanced vaccine responses [92–94]. There is some controversy regarding the effect of intensive exercise on immunity, with the ‘open-window hypothesis’ stating that intense exercise is followed by a transient state of immune depression, which becomes chronic if regular intense exercise is performed [95]. This has recently been challenged by Campbell and Turner, who argue that the evidence supposedly supporting the open window hypothesis (increased frequency of upper respiratory tract infections, a fall in salivary IgA and lymphocytopenia following intense, acute exercise such as a marathon) has been largely misinterpreted [96]. They argue that the supposed increase in incidence of upper respiratory tract infections is either due to
symptoms of an infection but no actual infection (rather caused by airway irritation due to increased ventilation or non-specific inflammation) or an actual infection caused by factors not directly related to intense exercise such as increased exposure to pathogens due to a large accumulation of people. As discussed above, acute lymphocytopenia following exercise is now thought to be due to lymphocyte egress into peripheral tissues.

1.6.3 Effect of Exercise on Immunity in the TME

1.6.3.1 Peripheral Immunity in Cancer Survivors

Changes in circulating levels of immune cells with exercise may provide an indication of whole-body immunity, including effects on the tumour, in cancer patients. In some patients, the acute exercise-induced increase in circulating immune cells is attenuated or even abolished [97,98]. Lymphocytes seem to be most strongly affected by this, with two studies showing a nullified or attenuated lymphocytosis but intact neutrophil [98], granulocyte and monocyte response with acute exercise [97]. Another study has found an increase in both lymphocytes and granulocytes immediately following acute exercise in chronic myeloid leukaemia patients [99]. However, other studies were in patients with solid tumours [97,98], which may impact systemic immune responses differently. These results suggest that either tumour burden or treatment may negatively affect immune cell mobilisation in response to exercise, which may reduce immune surveillance of peripheral tissues.

Chronic exercise does not alter numbers of circulating immune cells in most studies [98,100–104]. However, occasionally some studies have found an increase in various immune cell types with chronic exercise, including granulocytes, leukocytes, lymphocytes and neutrophils (systematically reviewed in [103]). Others have reported a decrease in lymphocytes or monocytes [103]. In addition, exercise training was unable to prevent the chemotherapy-associated decline in immune cell numbers [104]. Taken together, this suggests that exercise training does not alter numbers of circulating immune cells in cancer patients and other factors may be responsible for the observed increases or decreases in certain components in some studies.

1.6.3.2 Ex Vivo Immunity

The effect of exercise on immune cell function is difficult to measure in vivo. However, a number of studies have isolated immune cells from either the spleen, tumour or peritoneum of exercising and non-exercising animals and compared their cytotoxic
capacity, phagocytic capacity or cytokine production \textit{in vitro}. Preclinical exercise studies reporting on \textit{ex vivo} or intratumoural immunity are summarised in Figure 1.4.

**Figure 1.4: Effects of exercise on peripheral immunity and the immune TME.**

Exercise increases cytotoxicity of peripheral, IL-2 activated NK cells and macrophages against tumour targets and enhance phagocytic activity of macrophages \textit{ex vivo}. In addition, exercise may increase recruitment of cytotoxic lymphocytes (NK cells and CTLs) to the tumour site, while decreasing number and/or changing phenotype of myeloid cells such as neutrophils and macrophages to an anti- (M1/N1) rather than pro-tumour (M2/N2) state. CTL: cytotoxic T lymphocyte, NK cell: natural killer cell, IL-2: interleukin 2. Figure reused from [56] under the Springer Nature author licensing permissions.

The first studies investigating \textit{ex vivo} immune function against tumour targets were conducted by MacNeil and Hoffman-Goetz in the early 1990s. Splenic NK cells isolated from healthy mice immediately following an acute exercise session had higher activity when stimulated with IL-2 than those from non-exercised mice [105]. In addition, splenic NK cells isolated from tumour-bearing mice performing chronic exercise beginning prior to tumour implant exhibited increased activity against tumour targets [106–109]. In contrast, Pedersen \textit{et al}. found no change in the cytotoxic activity of splenic NK cells isolated from trained compared with non-exercised mice bearing B16-F10 melanoma [29]. This discrepancy may be due to the activation status of the NK cells. In a few of the
above mentioned early studies, the authors showed that only IL-2-activated but not unactivated NK cells from exercised mice had increased cytotoxicity against tumour targets [105,107]. In addition, unactivated NK cells are poorly effective against lysis-resistant tumour cell lines, but are able to achieve up to ~60% lysis when pre-stimulated with IL-2 and IL-12 [110]. This suggests that the in vivo anti-tumour activity of NK cells may be dependent on the intratumoural milieu. In support of this, exercise prior to tumour implant causes significantly slower growth of B16-F10 tumours, and these tumours show higher mRNA expression of IL-2 and other NK-cell activating factors [29].

Macrophage phagocytosis and phenotype has also been reported to change with exercise in tumour-bearing rodents. Peritoneal macrophages from exercised mice produce more IFN-γ, IL-12, TNF-α and IL-4 than those from non-exercised mice, and less of the immunosuppressive cytokines TGF-β and IL-10, suggesting a polarisation towards an anti-tumour M1 phenotype [111]. Furthermore, peritoneal macrophages from exercised, tumour-bearing rats are more phagocytic than those from non-exercising rats [112] and macrophages isolated from healthy, trained mice are able to induce higher cytolysis of tumour targets compared with those from non-exercised mice [113]. Finally, phagocytes isolated from subcutaneous breast tumours in moderately exercised mice have higher phagocytic activity against Staphylococcus aureus than those from non-exercised (or exhaustively exercised) mice [114].

In humans, it has been found that both acute [115,116] and chronic [94,117] exercise can improve cytotoxic activity of peripheral blood NK cells from healthy individuals against tumour targets, although one study found no change in NK cell cytotoxicity following exercise training [118] and another found decreased activity [119]. In cancer patients, chronic exercise has also been shown to increase NK cell cytotoxicity ex vivo [103,120,121]. In addition, ex vivo lymphocyte proliferation and phagocytic activity of monocytes is increased post-exercise training, while neutrophil oxidative burst is unchanged [103].

Taken together, ex vivo immune functionality data from both human and animal studies suggest that exercise can improve anti-tumour cytotoxicity.

1.6.3.3 Intratumoural Immunity

Given the hypothesis that the transient lymphocytopenia following exercise is due to cytotoxic lymphocyte egress and surveillance of peripheral tissues, it follows that
exercise may also redistribute these cells to the tumour. This is indirectly supported by work showing that NK cell and T cell numbers were increased in subcutaneous B16-F10 tumours following 6 weeks of exercise training [29] and that Cd8 gene expression was increased in mucosal scrapings from exercised compared with non-exercised Apc\textsuperscript{Min/+} mice [122]. In addition, Zielinski et al. found increased intratumoural lymphocyte density in subcutaneous EL-4 tumours following exhaustive exercise training compared with non-exercise mice [70]. Conversely, Bianco et al. found no change in numbers of tumour-infiltrating T cells into 4T1 tumours with post-implant exercise [123]. This may be due to the length of exercise, timing of exercise initiation or tumour model used. Pedersen et al. began exercise 4 weeks prior to tumour implant, whereas Bianco et al. started exercise at tumour implant [29,36,123]. Although Zielinski et al. also had a short exercise period of approximately 2 weeks, they used a tumour model which spontaneously regresses (EL-4 lymphoma), indicating that this tumour cell line induces a strong anti-tumour immune response \textit{in vivo}, which is enhanced by exercise [70]. Just one study has investigated B cell numbers, and found that they were unchanged in the tumour with chronic exercise [29].

Whether or not absolute numbers of lymphocytes within the tumour change may be less important than the phenotype and cytotoxic functionality of those that are present. As described in the previous section, \textit{ex vivo} data indicate that exercise may improve NK cell cytotoxicity. Data on T cells is much scarcer. Some studies suggest that exercise reduces T\textsubscript{reg} cell recruitment to the tumour (inferred from lower levels of the T\textsubscript{reg} cell recruiting cytokine CCL22 or lower mRNA expression of Foxp3, [122,124]), but others have found no change in the proportion of intratumoural T\textsubscript{reg} cells [36,123] or even an increase in intratumoural Foxp3 mRNA (alongside increased expression of inflammatory/cytotoxic cell markers) following exercise training [29]. \textit{Ex vivo} functionality assays investigating the effect of exercise on intratumoural T cells have not yet been conducted.

The tumour microenvironment promotes an immunosuppressive phenotype of infiltrating immune cells, causing them to aid rather than inhibit tumour growth both by the inhibition of cytotoxic immune cells and by secreting factors that aid tumour growth such as VEGF [82]. Myeloid cells seem to be particularly susceptible to this reprogramming and often take on an immunosuppressive phenotype within the TME (e.g. M2 macrophages) [125]. Two studies have found reduced neutrophil infiltration into
tumours with exercise [70,126], and two have found reduced macrophage density [70,127]. Additionally, gene expression of general macrophage markers (F4/80) and M2-specific markers (CD206, arginase) was reduced in mucosal scrapings from exercised compared with non-exercised mice [122]. Together with the above-described ex vivo data, this suggests that exercise reconditions the TME to reduce recruitment of and/or repolarise myeloid cells such as neutrophils and macrophages toward a more anti-tumour phenotype.

Comprehensive analysis of the types and subtypes of immune cells within the tumour microenvironment following exercise is still lacking. Current preliminary evidence suggests that exercise may repolarise immune cells to an anti-tumour phenotype and/or increase numbers of anti-tumour immune cells such as NK cells and CD8+ T cells, but this requires confirmation.

1.6.3.4 Interplay of Immunity with Hypoxia and Angiogenesis

Hypoxia inhibits anti-tumour immunity by inhibiting lytic functions of cytotoxic T cells (CTL) and NK cells and promoting an immunosuppressive phenotype in both lymphoid and myeloid cells (reviewed in [128]). In addition, tumour vasculature is prohibitive to T cell entry in that it downregulates adhesion molecules required for extravasation and upregulates inhibitory and apoptotic ligands [129]. Conversely, Treg cells and M2 macrophages can promote angiogenesis, while Type 1 T helper (Th1) cells can promote intratumoural vessel normalisation [130–132]. Furthermore, hypoxia leads to the stabilisation of HIF-1α and the release of stromal cell-derived factor 1 (SDF-1) into the circulation, which promotes the migration of monocytes from the bone marrow to the tumour, where they initiate vasculogenesis [133]. Thus, the influence of hypoxia on immune cells and their influence on tumour vasculature (and vice versa) are integral to the overall tumour phenotype.

Moreover, hypoxia and the stabilisation of HIF-1α results in upregulation of the HIF-1 target gene Vegf [134]. VEGF promotes tumour angiogenesis and the survival of tumour-associated endothelial cells, which express immune checkpoints such as PD-L1 and the apoptosis-inducing ligand FasL [135]. This results in the inhibition of T cell effector functions and apoptosis of circulating T cells, thus preventing the influx of functional anti-tumour T cells [135]. In addition, VEGF promotes the repolarisation of tumour-
associated macrophages from an anti-tumour M1 to a pro-tumour M2-like phenotype, thus further supporting immune evasion of the tumour [135].

1.6.3.5 Potential Role of Exercise in Combination with Immune Checkpoint Inhibitors

Immune checkpoint inhibitors are a class of cancer therapy aimed at revitalising the host immune system to eradicate the tumour. Although these have shown great promise and long-term response rates in some patients, a large proportion of patients will experience treatment resistance, and checkpoint inhibitors have thus far only proved efficacious in select tumour types such as advanced melanoma [136]. Therefore, there is great interest in the identification of strategies to improve checkpoint inhibitor response rates. Given its possible beneficial effects on anti-tumour immunity, exercise may be one such strategy. The combination of exercise with immunotherapy has only very recently begun to be investigated in preclinical studies [137–139], and shown some promise in that it improves the efficacy of radiotherapy together with anti-PD-1 [137]. However, whether the same is true for exercise and immune checkpoint inhibitors alone remains unclear. This issue is discussed more thoroughly in Section 4.1.

1.6.4 Conclusions on the Immune Microenvironment

In cancer patients, acute exercise-induced lymphocytosis may be partially suppressed [97,98] and chronic exercise may not be able to protect against chemotherapy-induced lymphopenia [104]. However, preclinical and clinical functional data indicate that exercise improves peripheral NK cell cytotoxicity (from both healthy individuals and cancer patients) [103,105,107] and possible repolarisation of macrophages towards an anti-tumour M1 phenotype [140]. It remains unclear whether these improvements in functionality of peripheral immune cells is translated to improved anti-tumour immunity within the TME, but they are a promising indication that exercise could improve immune responses in cancer patients.

1.7 Exercise and Cancer Cachexia

Cancer cachexia is a condition characterised by progressive body weight loss, which is accompanied by a decline in muscle strength, fatigue and anorexia [141]. In addition, recent work suggests that cachexia is preceded by impaired systemic immunity [142]. The syndrome is more common in people with advanced stage cancer, with some cancer
types (such as pancreatic cancer) showing rates of as high as 85% [143]. Cancer cachexia is associated with increased mortality and there is currently no standard of care to improve or prevent the condition.

On a subcellular level, cachectic muscle exhibits mitochondrial dysfunction, increased rates of protein degradation and reduced rates of protein synthesis [144]. These perturbations occur prior to the onset of muscle and body weight loss [145]. Strikingly, exercise induces the exact opposite – enhanced mitochondrial content and function, inhibition of protein degradation and promotion of protein synthesis [146]. This has led to the hypothesis that exercise may be able to prevent or alleviate cachexia.

Preclinical studies have shown almost universally that exercise can alleviate cancer-associated muscle wasting by improving mitochondrial content/function and inhibiting protein degradation [147–155]. However, this is usually achieved by beginning exercise prior to the onset of cachexia (although after tumour initiation), so it is unclear whether exercise can improve the condition once muscle wasting has become noticeable in a clinical setting. Furthermore, few of these studies have investigated how cancer therapy plays into this. Those that have, show that cachexia is worsened by chemotherapy and this attenuates some of the benefits of exercise [147], but next to nothing is known about how more modern therapies (such as immune checkpoint inhibitors) affect the development and progression of cachexia. Moreover, it is unknown how the exercise responsiveness (or adaptability) of the muscle is affected by tumour burden.

1.7.1 Molecular Mechanisms of Cancer Cachexia

At its core, cancer cachexia is a syndrome stemming from energy imbalance and whole-body metabolic perturbations. Much is still unknown about how the tumour induces cachexia, but the current state of knowledge supports a role for the release of inflammatory mediators and other tumour-derived factors into the circulation, which subsequently induce metabolic alterations in many organ systems (including the heart, brain and liver) and lead to the progression of cancer cachexia [144].

Skeletal muscle wasting appears to play a central role in the progression of cancer cachexia [144]. On a subcellular level, cachectic muscle is characterised by upregulation of protein degradation mechanisms such as the ubiquitin proteasome system and autophagy, and downregulation of protein synthesis [156]. Furthermore, mitochondrial
content and function declines, resulting in a loss of muscle oxidative capacity [144]. Importantly, the decline in mitochondrial health appears to be one of the first steps in cancer-associated muscle atrophy and indeed the syndrome of cancer cachexia, occurring prior to any clinical signs of cachexia in animal models [145]. Therefore, strategies to minimise the impact of cancer cachexia could focus on improving mitochondrial health in this early phase, which may halt or slow the progression of cachexia.

1.7.2 Exercise Capacity and Cancer

Exercise capacity is defined as the maximum amount of physical exertion that an individual can sustain and is colloquially referred to as ‘fitness’ [157]. The major variable affecting exercise capacity is, of course, the intensity and duration of exercise performed by an individual on a regular basis. However, other factors can play an important role, including nutrition, age, gender and genetics.

There is a clear heritable component to exercise capacity. Rodents can be selectively bred for high inherent exercise capacity; these animals can run for longer and at a higher speed (in an untrained state) than their low inherent exercise capacity counterparts [158]. In addition, different inbred mouse strains have significantly different exercise capacities in the untrained state [159]. In humans, studies have suggested that there is a large heritable component to exercise capacity (reviewed in [11]).

To my knowledge, there is just one study that has investigated the effect of inherent exercise capacity on cancer risk. Rats were selectively bred for high or low inherent exercise capacity and exposed to the carcinogen 1-methyl-1-nitrosurea (MNU) [160]. Rats with high inherent exercise capacity had lower tumour incidence (fewer rats with any breast malignancy, 14% vs 47.3%), and those that did develop tumours had fewer tumours than rats with low inherent exercise capacity (0.18 vs 0.85 tumours/rat) [160]. This suggests that there may be a large heritable component to the protective effect of exercise on cancer risk.

1.7.3 Exercise Adaptability/Exercise Responsiveness

There also appears to be a heritable component to exercise adaptability (exercise responsiveness), that is, the ability of an individual to effect the physiological changes required to improve exercise capacity in response to exercise. These include muscular adaptations (increased capillary density and mitochondrial expansion) and improved
pulmonary and cardiovascular capacity [12]. In mice, different inbred strains exhibit significantly different changes in exercise capacity following the same training protocol [159]. Similarly, in humans, training-induced increases in maximal oxygen uptake (VO₂ max) vary significantly more between families than within families [161]. It has been suggested that the heritability of exercise adaptability may be as much as 50% (reviewed in [11]).

To my knowledge, there are no published studies specifically investigating the role of exercise adaptability on risk of cancer. However, higher VO₂ max was associated with improved survival in metastatic breast cancer patients and non-small cell lung cancer patients [162,163]. One clinical study compared exercise adaptations in the skeletal muscle of cancer patients with healthy controls [164]. In that study, the authors found that a greater number of healthy individuals had an increase in muscle fibre cross-sectional area with exercise training compared with cancer patients [164]. In addition, healthy individuals had an increase in muscle capillarisation and quadriceps strength while cancer patients did not [164]. Although this study is limited by small numbers (n=12-16 per group), it provides preliminary evidence that cancer patients may not adapt to exercise to the same degree as healthy individuals. Furthermore, comparison of a systematic review on improvements of VO₂ max in cancer patients with data in healthy subjects suggests that the magnitude of improvement in VO₂ max is lower in cancer patients despite following similar exercise programs to the healthy subjects [165].

The role of an individual’s ability to perform the physiological adaptations required for improvements in exercise capacity (exercise adaptability), and indeed inherent exercise capacity itself, have thus far been largely neglected in exercise oncology. Importantly, there is large inter-individual variation in these factors in both rodents and humans, determined both by inherited factors and activity levels [11,158,159]. In simple terms, this means that two individuals undergoing exactly the same exercise program will a) not adapt to exercise to the same degree (chronic response) or b) feel the same level of exertion (acute response). It is unclear what effect (if any) this might have on how exercise affects tumour characteristics, but is an important avenue of investigation if we are to fully understand how exercise effects physiological change in the TME – the key question being whether improvements in exercise capacity/muscular adaptations are required for beneficial effects of exercise on the TME. It is possible that impairments in exercise adaptability due to tumour or treatment burden may limit the effectiveness of
exercise. Parameters such as VO\textsubscript{2} max, muscle protein synthesis/degradation and skeletal muscle mitochondrial content/function can be used to ascertain exercise capacity and exercise adaptability.

1.8 Exercise, Hyperlipidaemia and Cancer

In addition to research in ‘standard’ models (wild-type mice on a chow diet), it is important to understand how exercise affects tumour characteristics in models with additional comorbidities that may be present in a human population, such as hyperlipidaemia. Hyperlipidaemia is the abnormal elevation of plasma lipid levels and is commonly comorbid with obesity. Hyperlipidaemia has been linked to accelerated tumour growth and higher rates of metastasis in mice [166], but it is unknown whether exercise can slow tumour growth rate in a hyperlipidaemic background. This is discussed more thoroughly in Section 6.1.

1.9 Summary of the Interplay of Exercise and Tumour Characteristics

Exercise oncology is a hugely complex field and requires the collaboration of clinical oncologists, preclinical cancer researchers, immunologists and exercise physiologists (to name a few) for a thorough understanding of exercise and tumour physiology. The current state of knowledge supports a beneficial role of exercise in cancer prevention and survival in some cancer types, but comprehensive mechanistic data remain elusive and robust predictors of tumour response to exercise are non-existent. Delineating the effects of exercise on the TME (and of the tumour on the body, Figure 1.5) may be the key to unravelling how and in which situations exercise exerts a tumour growth inhibitory effect.
Chronic exercise induces skeletal muscle remodelling to improve exercise capacity, including an increase in mitochondrial content and function, and improved vascularisation. However, this response may be blunted in cancer patients and varies strongly between individuals. It is unknown whether this variation in exercise adaptability affects the degree to which exercise can alter the TME. Myokines, dopamine, catecholamines and further unknown factors are released into the circulation with exercise and exert effects on the TME. Effects on vascularisation/hypoxia/perfusion and immunity are summarised in figures 1 and 2, respectively. Figure reused from [56] under the Springer Nature author licensing permissions.

1.10 Thesis Overview and Hypotheses

This thesis investigates the effect of exercise on the tumour microenvironment as a single therapy and in combination with anti-PD-1 checkpoint inhibition. In addition, the effect of tumour burden on the adaptation of skeletal muscle mitochondria to exercise is examined, with and without anti-PD-1 treatment. Finally, I investigate whether skeletal muscle mitochondrial content (as a proxy for the response to exercise) is correlated with tumour outcomes in wild-type and hyperlipidaemic ApoE⁻/⁻ mice.

I hypothesise that exercise normalises the tumour microenvironment and improves immune cell infiltration into the tumour. Further, I hypothesise that exercise synergises
with anti-PD-1 to boost the efficacy of immune checkpoint inhibitor treatment. Finally, I hypothesise that tumour burden will impair skeletal muscle mitochondrial adaptations to exercise, but that mice with higher mitochondrial content will show improved tumour outcomes.

Breast cancer and melanoma were selected as cancer types in this thesis due to their relevance to New Zealand health. Breast cancer is the most prevalent cancer in women not only in New Zealand but worldwide, and causes over 600 000 deaths each year [167]. Melanoma is particularly relevant to New Zealand health, as we have the second highest incidence in the world, second only to Australia [167].
2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

Table 2.1: List of chemicals and reagents and their suppliers

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Neutral buffered formalin</td>
<td>Anatomical Pathology, Christchurch Hospital</td>
</tr>
<tr>
<td>10% NP-40 (IGEPAL CA-630)</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
</tr>
<tr>
<td>10% SDS</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
</tr>
<tr>
<td>10% sodium deoxycholate</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
</tr>
<tr>
<td>20x Bolt transfer buffer</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>20x NuPAGE MES SDS running buffer</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>4x LDS sample buffer</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>LabServ, Thermo Fisher Scientific, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>Amersham Hybond P Western blotting membrane, PVDF</td>
<td>GE Healthcare, Chicago, IL, USA</td>
</tr>
<tr>
<td>Bicinchoninic acid solution</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
</tr>
<tr>
<td>Bisbenzimide Hoechst 33342</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
</tr>
<tr>
<td>BSA</td>
<td>Gibco Invitrogen, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Copper II sulfate solution</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
</tr>
<tr>
<td>DMEM with GlutaMax and L-D glucose</td>
<td>Gibco Invitrogen, Carlsbad, CA, USA</td>
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<tr>
<td>DMSO</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
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<tr>
<td>DPX mountant</td>
<td>BDH VWR, Radnor, PA, USA</td>
</tr>
<tr>
<td>DTT</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
</tr>
<tr>
<td>Dual Endogenous Enzyme Block</td>
<td>Dako, Copenhagen, Denmark</td>
</tr>
<tr>
<td>FCS</td>
<td>Gibco Invitrogen, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Gill II Haematoxylin</td>
<td>Leica, Wetzlar, Germany</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>Baxter, Deerfield, IL, USA</td>
</tr>
<tr>
<td>KCl</td>
<td>BDH VWR, Radnor, PA, USA</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>BDH VWR, Radnor, PA, USA</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>BDH VWR, Radnor, PA, USA</td>
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<tr>
<td>Methanol</td>
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</tr>
<tr>
<td>Na2HPO4</td>
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</tr>
<tr>
<td>NaCl</td>
<td>Thermo Fisher Scientific, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>OCT embedding medium</td>
<td>Sakura, Torrance, CA, USA</td>
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<tr>
<td>Name</td>
<td>Supplier</td>
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<tr>
<td>-------------------------------------------------------</td>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>Odyssey Blocking Buffer in TBS</td>
<td>Millennium Science, Auckland, NZ</td>
</tr>
<tr>
<td>PBS tablets</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
</tr>
<tr>
<td>ProLong Diamond antifade mountant</td>
<td>Thermo Fisher Scientific, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>Protease inhibitor cocktail</td>
<td>Roche, Indianapolis, USA</td>
</tr>
<tr>
<td>See-Blue Plus2 Prestained Standard</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>Countdown, NZ</td>
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<tr>
<td>Sodium bicarbonate</td>
<td>BDH VWR, Radnor, PA, USA</td>
</tr>
<tr>
<td>Tris</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
</tr>
<tr>
<td>Trisodium citrate dihydrate</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
</tr>
<tr>
<td>Trypan Blue</td>
<td>Thermo Fisher Scientific, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>TrypLE™ Express</td>
<td>Gibco Invitrogen, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
</tr>
<tr>
<td>Xylene</td>
<td>LabServ, Thermo Fisher Scientific, Sunnyvale, CA, USA</td>
</tr>
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### 2.1.2 Buffers and Media

**Table 2.2: List of buffers and media and their components**

<table>
<thead>
<tr>
<th>Name</th>
<th>Components</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x citrate buffer, pH 6.0</td>
<td>Trisodium citrate dihydrate dH₂O</td>
<td>100 mM</td>
</tr>
<tr>
<td>10x PBS</td>
<td>NaCl</td>
<td>1.37 M</td>
</tr>
<tr>
<td></td>
<td>KCl</td>
<td>27 mM</td>
</tr>
<tr>
<td></td>
<td>Na₂HPO₄</td>
<td>101 mM</td>
</tr>
<tr>
<td></td>
<td>KH₂PO₄</td>
<td>18 mM</td>
</tr>
<tr>
<td>10x TBS, pH 7.6</td>
<td>Tris</td>
<td>200 mM</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>1.37 M</td>
</tr>
<tr>
<td>1x citrate buffer</td>
<td>10x citrate buffer dH₂O</td>
<td>1x (10 mM)</td>
</tr>
<tr>
<td></td>
<td>Tween-20 dH₂O</td>
<td>0.05% (v/v)</td>
</tr>
<tr>
<td>1x PBS</td>
<td>10x PBS dH₂O</td>
<td>1x</td>
</tr>
<tr>
<td>1x PBST</td>
<td>10x PBS dH₂O</td>
<td>1x</td>
</tr>
<tr>
<td></td>
<td>Tween-20 dH₂O</td>
<td>0.05% (v/v)</td>
</tr>
<tr>
<td>Name</td>
<td>Components</td>
<td>Final concentration</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------------</td>
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<tr>
<td>1x running buffer</td>
<td>20x NuPAGE MES SDS running buffer dH$_2$O</td>
<td>1x</td>
</tr>
<tr>
<td>1x TBST</td>
<td>10x TBS, Tween-20, Water</td>
<td>1x 0.1% (v/v)</td>
</tr>
<tr>
<td>1x Transfer buffer</td>
<td>20x Bolt™ transfer buffer Methanol dH$_2$O</td>
<td>1x 10% (v/v)</td>
</tr>
<tr>
<td>Culture media</td>
<td>DMEM with GlutaMax and L-D glucose (Gibco, 10566016) FCS</td>
<td>1x 10% (v/v)</td>
</tr>
<tr>
<td>Freezing media</td>
<td>DMEM with GlutaMax and L-D glucose FCS DMSO</td>
<td>1x 20% (v/v) 10% (v/v)</td>
</tr>
<tr>
<td>RIPA buffer, pH 8.0</td>
<td>NaCl, Tris, NP-40 (IGEPAL CA-630) Sodium deoxycholate SDS dH$_2$O</td>
<td>150 mM 50 mM 1% (v/v) 0.5% (v/v) 0.1% (v/v)</td>
</tr>
<tr>
<td>Scotts’s Tap Water</td>
<td>Sodium bicarbonate, Magnesium sulfate dH$_2$O</td>
<td>23.8 mM 166 mM</td>
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<tr>
<td>Western blot blocking buffer (for initial block)</td>
<td>Skim milk, TBST</td>
<td>5% (w/v) 1x</td>
</tr>
<tr>
<td>Western blot blocking buffer (for secondary antibody dilution)</td>
<td>Odyssey blocking buffer (TBS) TBS</td>
<td>50% (v/v) 1x</td>
</tr>
</tbody>
</table>

### 2.1.3 Kits

**Table 2.3: List of kits and their manufacturers**

<table>
<thead>
<tr>
<th>Name</th>
<th>Catalogue Number</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate Synthase Assay kit</td>
<td>ab239712</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Corticosterone parameter assay kit</td>
<td>KGE009</td>
<td>R&amp;D Systems, Minneapolis, USA</td>
</tr>
<tr>
<td>Creatinine parameter assay kit</td>
<td>KGE005</td>
<td>R&amp;D Systems, Minneapolis, USA</td>
</tr>
</tbody>
</table>
### Name | Catalogue Number | Manufacturer
--- | --- | ---
EnVision™ G/2 System/AP Rabbit/Mouse (Permanent Red) kit | K5355 | Dako, Copenhagen, Denmark
Goat VisUCyte HRP Polymer-DAB Cell and Tissue Staining kit | VCTS004 | R&D Systems, Minneapolis, USA
Hypoxyprobe™ 1-1000 kit | HP1-1000Kit | Hypoxyprobe, Inc., Massachusetts, USA
Mouse L308 Array, Membrane | AAM-BLM-1A-2 | RayBiotech, Georgia, USA
Mouse Pentraxin 3/TSG-14 Quantikine ELISA | MPTX30 | R&D Systems, Minneapolis, USA
Mouse Tumour necrosis factor ligand superfamily member 14(TNFSF14) ELISA kit | CSB-EL023991MO | Cusabio, Texas, USA
REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse | K5007 | Dako, Copenhagen, Denmark

#### 2.1.4 Antibodies

**Table 2.4: List of antibodies (anti-mouse) and their manufacturers**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Catalogue Number</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3</td>
<td>SP7</td>
<td>ab21703</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-CD31</td>
<td>Polyclonal</td>
<td>ab124432</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-CD3e</td>
<td>500A2</td>
<td>553238</td>
<td>BD, California, USA</td>
</tr>
<tr>
<td>Anti-CD8a</td>
<td>EPR21769</td>
<td>ab217344</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-CD8a</td>
<td>4SM15</td>
<td>14-0808-80</td>
<td>eBioscience, San Diego, CA, USA</td>
</tr>
<tr>
<td>Anti-COX-IV</td>
<td>20E8C12</td>
<td>ab14744</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-cytochrome c</td>
<td>7H8.2C12</td>
<td>33-8500</td>
<td>Thermo Fisher Scientific, California, USA</td>
</tr>
<tr>
<td>Anti-FoxP3</td>
<td>FJK-16s</td>
<td>14-5773-80</td>
<td>eBioscience, San Diego, CA, USA</td>
</tr>
<tr>
<td>Anti-GAPDH</td>
<td>EPR16891</td>
<td>ab181602</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Antibody</td>
<td>Clone</td>
<td>Catalogue Number</td>
<td>Manufacturer</td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td>-------------</td>
<td>------------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>Anti-hamster Alexa Fluor 647</td>
<td>Polyclonal</td>
<td>A-21451</td>
<td>Thermo Fisher Scientific, California, USA</td>
</tr>
<tr>
<td>Anti-mouse IRDye 800CW</td>
<td>Polyclonal</td>
<td>ab216772</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-NKp46</td>
<td>Polyclonal</td>
<td>AF2225</td>
<td>R&amp;D Systems, Minneapolis, USA</td>
</tr>
<tr>
<td>Anti-pHH3</td>
<td>Polyclonal</td>
<td>ab5176</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-Pimonidazole (FITC-conjugated)</td>
<td>4.3.11.3</td>
<td>HP1-1000 kit</td>
<td>Hypoxprobe, Inc., Massachusetts, USA</td>
</tr>
<tr>
<td>Anti-rabbit DyLight 755</td>
<td>Polyclonal</td>
<td>SA5-10035</td>
<td>Thermo Fisher Scientific, California, USA</td>
</tr>
<tr>
<td>Anti-rabbit IRDye 680RD</td>
<td>Polyclonal</td>
<td>ab216777</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-rat Alexa Fluor 555</td>
<td>Polyclonal</td>
<td>ab150166</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>InVivoMAb anti-mouse PD-1 (CD279)</td>
<td>RMP1-14</td>
<td>BE0146</td>
<td>Bio-X-Cell, Lebanon, NH, USA</td>
</tr>
<tr>
<td>InVivoMAb rat IgG2a isotype control, anti-</td>
<td>2A3</td>
<td>BE0089</td>
<td>Bio-X-Cell, Lebanon, NH, USA</td>
</tr>
<tr>
<td>trinitrophenol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total OXPHOS Rodent WB Antibody Cocktail</td>
<td>20E9DH10C12</td>
<td>ab110413</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td></td>
<td>21A11A1E7</td>
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<td></td>
</tr>
<tr>
<td></td>
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</tr>
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<td></td>
<td>1D6E1A8</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>15H4C4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 2.2 Methods

All *ex vivo* analysis was done in a blinded manner.

#### 2.2.1 Cell Culture

EO771 cells are a triple-negative medullary breast adenocarcinoma cell line originally derived from a C57BL/6 mouse [168] and kindly gifted by Dr Andreas Moeller (QIMR Berghofer). B16-F10 cells are a skin melanoma cell line originally derived from a C57BL/6 mouse [169] and obtained from ATCC. Cells were brought up from liquid nitrogen and cultured in DMEM culture media (Table 2.2) at 37°C and 5% CO₂ in a 75
cm² flask. Cells were split as required by washing with PBS and then detaching from the flask by adding 1 mL of TrypLE™ Express and incubating at 37°C and 5% CO₂ for approximately 5 minutes. Trypsinisation was stopped with fresh media and diluted cells were replated. Cells used for implant had a maximum passage number of 10 (B16-F10) or 19 (EO771).

Cells were prepared for injection as follows. When they reached approximately 80-90% confluence, cells were detached from the flask as described above and spun down at 600g for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 10 mL PBS per flask to wash cells free of FCS, followed by centrifugation and resuspension in 10 ml PBS. Cells were labelled with Trypan Blue to identify dead cells and counted using a Countess automated cell counter (Invitrogen, Thermo Fisher) and spun down again. Finally, cells were resuspended in PBS at a concentration of 1x10⁷ viable cells/mL (EO771) or 2x10⁷ viable cells/mL (B16-F10).

A portion of cells not needed for injection were frozen down slowly in freezing media (Table 2.2) using a ‘Mr Frosty Freezing Container’ (Thermo Fisher) placed at -80°C before transfer to liquid nitrogen.

2.2.2 In vivo Methods

2.2.2.1 Ethical approval

Ethical approval for the studies in this thesis were obtained from the University of Otago Animal Ethics Committee (C01/16, C04/17, AUP-18-144, AUP-18-179, AUP-18-150). International guidelines on animal welfare in experimental neoplasia were strictly followed [170]. Animal welfare was monitored daily by the use of welfare sheets specifically designed for tumour-bearing mice.

2.2.2.2 Housing, standard care and exercise set-up

C57BL/6 Mice were housed either alone in a standard mouse cage, or in pairs with a cage divider in a standard rat cage (see below and section 2 of chapters 3-5 for details) under a 12:12 hour light-dark cycle. The temperature was maintained around 22°C. Mice were kept on a normal chow diet, provided ad libitum along with water. Female mice aged 6-10 weeks were used for all experiments.

Standard mouse cages were Safesealplus greenline IVC GM500 cages with a floor area of 501 cm² (Tecniplast, Buguggiate, Italy). Rat cages were Safesealplus greenline IVC
GR 900 cages with a floor area of 904 cm² (Tecniplast, Buguggiate, Italy). One section of the divided cage contained a modified Fast-Trac™ saucer wheel (Bio-Serv, Flemington, NJ, USA). Wheels were equipped with a magnetic sensor and digital counter to quantify revolutions. These were designed and purpose-built by Mr Andrew Dachs (Decision Consulting Ltd, NZ). Further details of the electronic design can be found at https://github.com/wirebadger/mouse-wheel. The sensor and counter were confirmed to be working daily by manual spinning of the wheel and visual assessment of counting accuracy. Wheels did not become trapped by the side of the cage, thus preventing them from turning. Mice in exercise groups were allowed constant access to a running wheel. Running distance measures were taken daily.

*ApoE<sup>-/-</sup>* mice were originally generated by Piedrahita *et al* [171] by genetic knockout of apolipoprotein E in C57BL6 mice, and bred in-house from homozygous mice. The *ApoE<sup>-/-</sup>* mice used in Chapter 6 were housed in pairs or groups of three in standard mouse cages (no exercise group) or in pairs in standard rat cages with a running wheel (exercise group). At tumour implant, mice were randomized into either high exercise (HEx, continuous wheel access), low exercise (LEx, wheel access every second day) or sedentary (Sed) groups to investigate whether a dose-effect could be observed (10-14 mice per group). These experiments were performed prior to this PhD, as part of my Honours project. However, muscle samples were analysed for COX-IV expression (by Western blot, as described below) during my PhD and used for additional data analysis in Chapter 6.

### 2.2.2.3 Calculation of Running Distance

Running distance was calculated from measured revolutions by multiplication of the number of revolutions with the wheel circumference (346 mm).

### 2.2.2.4 Injection of tumour cells

Cells were prepared for injection as described in section 2.2.1. Mice were anaesthetised by isoflurane inhalation in an anaesthetic chamber using a precision vaporiser (5% in oxygen for induction of anaesthesia). For EO771 tumour induction, 20 µL of cell suspension (2x10<sup>5</sup> cells) were injected into the 4<sup>th</sup> mammary fat pad of 6-10 week old female C57BL/6 mice using a 29-31 gauge needle. For B16-F10 tumour induction, the right flank of mice was shaved, disinfected with 70% ethanol and 50 µL of cell suspension (1x10<sup>6</sup> cells) injected subcutaneously. Mice were allowed to recover before
being placed into a cage. Mice were monitored daily for tumour growth and assessed for general well-being. Tumour size was measured with callipers at its longest and widest points and the volume estimated using the following formula:

\[ \text{Tumour volume} = \text{width}^2 \times (\text{length}/2) \]

### 2.2.2.5 Euthanasia and organ harvest

When tumour volume reached the ethical limit of 600 mm\(^3\) (EO771) or 1000 mm\(^3\) (B16-F10) or the welfare of the mouse was impacted (by tumour burden, ulceration of the tumour or suspicion of internal tumours) mice were injected intraperitoneally with 60 mg/kg of 30 mg/mL pimonidazole, a hypoxia marker. Mice were anaesthetised by isoflurane inhalation 90 minutes later and 60-80 µL of 5 mg/mL Hoechst 33342 was injected intravenously into the lateral tail vein to allow for analysis of tumour perfusion. One minute after Hoechst 33342 injection, anaesthetised mice were sacrificed by cervical dislocation. Blood was removed from the chest cavity and spun down at 10 600 g for 10 minutes and the supernatant (plasma) transferred to a fresh cryovial. Plasma was stored at -80°C until analysis. In addition, the tumour, liver, kidneys, heart and spleen were removed, as well as internal tumours if present. Internal tumours are highly aggressive tumours established from EO771 tumour cells that have escaped from the primary tumour or from initial implantation of EO771 cells, and approximate the condition of peritoneal carcinomatosis in breast cancer patients. All organs were immediately placed on ice and promptly weighed. The tumour was cut into three portions – one third was frozen at -80°C, one third was embedded in OCT medium for cryosectioning and stored at -80°C, and the final third was formalin fixed for at least 24 hours and paraffin embedded (by Canterbury Health Laboratories) for formalin fixed paraffin embedded (FFPE) sections. In addition, portions of the liver and spleen were embedded in OCT medium or processed into FFPE blocks alongside tumours as positive controls for Hoechst 33342 perfusion and T cell staining, respectively. The left quadriceps femoris muscle was also removed and frozen at -80°C for Western blot analysis and analysis of mitochondrial enzyme activity.

### 2.2.2.6 Urine Collection

Urine for the measurement of corticosterone was collected by placing the mouse in a clean plastic container and waiting for the mouse to urinate. This typically occurred within a few minutes, and urine could then be aspirated with a pipette and transferred to
a microtube. If urination did not occur within a few minutes, the mouse was scruffed and the bladder gently massaged to stimulate urination, as described in [172]. This was likely stressful for the mouse, but given that corticosterone spikes due to acute stress are not visible in the urine until 40 minutes post-stress induction [173], this is unlikely to have affected the measured levels in my study. Urine was collected at the same time each day (9 am) to account for fluctuations in corticosterone due to circadian rhythm.

2.2.3 Muscle Lysate Preparation

2.2.3.1 Muscle lysate preparation for Western blot

In order to analyse the protein content of tissues by Western blotting, the tissues first need to be lysed and the proteins extracted. To this effect frozen muscle samples were split into fragments using a mortar and pestle on dry ice and one fragment transferred to a 2 mL reinforced tube (Bertin, Montigny-le-Bretonneux, France) containing 200 µL ice cold RIPA buffer with freshly added protease inhibitor cocktail (PIC) and 5-6 ceramic beads (2.8 mm, Bertin). Samples were then homogenised by shaking on the Precellys Evolution with Cryolys attachment (Bertin) at 7200 rpm for 2x25 sec, with 10 sec pause at 0°C. Lysates were spun down at 10 600 g at 4°C for 10 minutes and the supernatant transferred to a fresh microtube. Cleared lysates were stored at -80°C.

2.2.3.2 Muscle lysate preparation for citrate synthase activity assay

Frozen muscle samples were split into fragments using a mortar and pestle on dry ice and one fragment transferred to a 1.7 mL tube containing 100 µL ice cold citrate synthase assay buffer (Abcam). Samples were then homogenised using a plastic pestle and sonicated for 10-15 seconds. Tubes were spun down at 10 600 g at 4°C for 10 minutes and the supernatant transferred to a fresh microtube. Lysates were stored at -80°C.

2.2.4 Quantification of Protein Content in Muscle Lysates by BCA Assay

Bicinchoninic acid solution and copper II sulfate solution were mixed together at a ratio of 50:1 and 200 µL/well of the resulting working reagent was pipetted onto a 96 well cell culture plate. 25 µL of each BSA standard (0-2 µg/µL) and diluted sample (1:20) were added to the appropriate wells. The plate was then agitated briefly to ensure even mixing and incubated for 30 minutes at 37°C in the dark before detection with a plate reader at
544 nm. Protein concentrations of the lysates were estimated from the BSA standard curve.

2.2.5 Separation of Proteins by SDS-Page

Muscle samples were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) by combining DTT (final conc. 0.1M), 4x LDS sample buffer (final conc. 1x), RIPA buffer with PIC and the sample (final protein conc. 2 µg/µL) before incubating for 10 minutes at 50 °C. This temperature was selected as OXPHOS complexes I-IV are very sensitive to heating and degrade at higher temperatures. Next, 12.5 µL (25 µg) of the resulting (denatured) mixture was loaded onto a 4-12% BOLT® Bis-Tris gradient SDS gel (Invitrogen, Carlsbad, CA, USA) and separated at 125 V in MES running buffer (Table 2.2) until the protein marker reached the end of the gel (75 minutes).

2.2.6 Western blot

After SDS-Page, the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. To this effect, the gel and membrane were sandwiched between two pieces of filter paper and sponges, and placed under an electric current for 60 minutes at 20 V in transfer buffer (Table 2.2) using a mini blot module (Thermo Fisher). Blots were cut just above the 14 kDa protein marker band for detection of OXPHOS complexes (>14 kDa, top section) and cytochrome c (12 kDa, bottom section) or left whole for detection of COX-IV. All samples were run in duplicate on separate blots. Following this the membrane was blocked in 5% skim milk in TBST at room temperature for 1h. The membrane was then incubated overnight at 4°C with the primary antibody at the appropriate dilution (Table 2.5). All antibody dilutions were determined by titration to determine the optimal dilutions. Next, the membrane was washed three times in TBST for 5 minutes before incubation with the secondary antibody in Odyssey blocking buffer and TBS (1:1) for 1h at room temperature in the dark. After a further three washing steps in TBST the membrane was imaged at tray level 3 with an Alliance Chromapure (Uvitec, Cambridge, UK). IRDye 680 was visualised with excitation using the 690 nm Chromapure module and detection using the F-740 bandpass filter. IRDye 800 was visualised with excitation using the 780 nm Chromapure module and detection using the F-850 bandpass filter. Automatic exposure times were used in order to prevent saturation of the signal.
Table 2.5: Western blot antibody dilution scheme

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution (primary antibody)</th>
<th>Secondary antibody conjugation</th>
<th>Dilution (secondary antibody)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-COX-IV</td>
<td>1:2 000</td>
<td>IRDye 800 CW</td>
<td>1:5 000</td>
</tr>
<tr>
<td>Anti-cytochrome c</td>
<td>1:1 000</td>
<td>IRDye 800 CW</td>
<td>1:5 000</td>
</tr>
<tr>
<td>OXPHOS rodent antibody cocktail</td>
<td>1:250</td>
<td>IRDye 800 CW</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-GAPDH</td>
<td>1:10 000</td>
<td>IRDye 680 RD</td>
<td>1:10 000</td>
</tr>
</tbody>
</table>

**2.2.6.1 Quantification of Protein Bands**

Densitometric quantification of protein bands was performed using Image J software. The signal was normalised between blots by the use of the same positive control (25 µg muscle lysate) and between samples within the same blot by the use of GAPDH as an internal loading control. Specifically, the sample band intensity was first divided by the control band intensity. This value was then divided by the GAPDH band intensity for that sample to obtain the relative protein expression.

**2.2.7 Citrate synthase activity assay**

Citrate synthase activity was determined in muscle homogenates using the Citrate synthase assay kit (Table 2.3) according to the manufacturer’s instructions. Specifically, 10 µL of each sample and 100x diluted positive control were loaded into a 96-well plate and the volume adjusted to 50 µL with citrate synthase assay buffer. Standards were loaded at a range of 0-40 nmol GSH. Then, 50 µL reaction mix was added to each well. The first reading was taken immediately using a plate reader set to 412 nm and 25°C incubation temperature. Absorbance was then measured at 2 minute intervals for 30 minutes. Sample values were obtained by interpolation from the standard curve. Citrate synthase activity was calculated by first subtracting the 0 standard reading from all other readings, then calculating activity by the following formula:

\[
\text{CS activity} = \frac{\text{interpolated sample value} \times \text{sample dilution factor}}{\Delta \text{time} \times \text{sample volume}}
\]
The L308 mouse antibody array (Ray Biotech) was used to identify candidate exercise-regulated circulating proteins according to the manufacturer’s instructions. Four plasma samples each from exercising and sedentary mice were pooled for the experiment.

Specifically, samples were dialysed overnight at 4°C with constant stirring. Dialysed samples were transferred to a clean Eppendorf tube and centrifuged for 5 min at 10 600 g. The supernatant was transferred to a fresh tube. A BCA assay was performed to determine protein content.

Samples were incubated with 7.2 µL Labelling Reagent per 1 mg protein for 30 min at room temperature with gentle shaking. Next, 5 µL Stop Solution was added. Spin columns were spun at 1000 g for 3 minutes to remove storage solution. Then, the columns were washed 3x with PBS. Samples were loaded into the columns and spun to remove unbound biotin.

Membranes were blocked using Blocking Buffer for 1 hour at room temperature with gentle agitation. Blocking buffer was then aspirated and replaced with samples. Sample incubation occurred for 2 hours at room temperature with gentle agitation. Membranes were washed 3x5 min with Wash buffer before incubation with HRP-conjugated streptavidin at 4°C overnight with gentle agitation. Membranes were then washed 3x5 min with Wash Buffer.

Detection was done by incubating membranes for 2 minutes at room temperature with 1:1 Detection Buffers C and D. Membranes were imaged with a UVItec Alliance 4.7. Relative protein abundances were obtained by quantifying dot intensities using Image J software and normalising to the strongest positive control dot (HRP) in the top left corner of the membrane.

### 2.2.8 Enzyme-Linked Immunosorbent Assay (ELISA)

#### 2.2.8.1 Detection of urinary corticosterone

The amount of corticosterone in urine samples was determined using the Corticosterone parameter assay kit (Table 2.3) according to the manufacturer’s instructions. Specifically, 50 µL of corticosterone primary antibody solution was added to each well (except for the non-specific binding control wells) and incubated for 1 h at room temperature with gentle mixing on a horizontal orbital microplate shaker. Then, wells were aspirated and washed four times with wash buffer (inverting the plate and blotting against paper towels after
each wash step; this was done for all ELISAs) before addition of 100 µL Pretreatment F. The standard, control and samples (50 µL each, samples were diluted 100-fold) were added to the appropriate wells, and 50 µL Calibrator Diluent RD5-43 to the zero standard and non-specific binding control wells. Next, 50 µL of the corticosterone conjugate was added to all wells and the mixture incubated for 2 h at room temperature on the shaker. All wells were washed four times before addition of 200 µL substrate solution to each well and incubation for 30 min at room temperature on the benchtop in the dark. Finally, 100 µL of stop solution was added to each well and mixed by gentle tapping before measurement in a microplate reader set to 450 nm with wavelength correction at 540 nm. Sample concentrations were determined by interpolation from the standard curve using a four parameter logistic curve fit (using Graphpad Prism software).

2.2.8.2 Detection of plasma LIGHT

The amount of LIGHT in plasma samples was determined using the Mouse TNFSF14 ELISA kit (Table 2.3) according to the manufacturer’s instructions. Specifically, 100 µL of standard and sample were added to the appropriate wells of the provided 96-well plate and incubated for 2 h at 37°C. Next, the liquid was removed from each well and 100 µL of 1x biotin antibody added to each well. The plate was incubated for 1 h at 37°C. Then, the wells were aspirated and washed 3x with wash buffer. HRP-avidin (100 µL) was then added to each well and incubated for 1 h at 37°C. The wells were then washed 5x and 90 µL of TMB substrate added to each well. The plate was protected from light and incubated for 15 minutes at 37°C. Finally, 50 µL of stop solution was added to each well and the optical density determined using a microplate reader set to 450 nm with wavelength correction at 540 nm. Sample concentrations were determined by interpolation from the standard curve using a four parameter logistic curve fit (using Graphpad Prism software).

2.2.8.3 Detection of plasma pentraxin 3

Pentraxin 3 is involved in the regulation of innate immune responses [174], is oncosuppressive [175] and is increased in the plasma by exercise [176]. The amount of pentraxin 3 in plasma samples was determined using the Mouse pentraxin 3 Quantikine ELISA kit (Table 2.3) according to the manufacturer’s instructions. Specifically, 50 µL of assay diluent was added to each well, followed by 50 µL of standard, control or diluted (1:4) sample and incubation for 2 h at room temperature with gentle agitation. Wells were
then washed 5x with wash buffer and 100 µL of mouse pentraxin 3 conjugate added to each well. The plate was incubated for 2 h at room temperature on the shaker. Next, wells were washed 5x and 100 µL substrate solution added. The plate was incubated for 30 minutes at room temperature in the dark before addition of 100 µL stop solution. The optical density was determined using a plate reader set to 450 nm with wavelength correction at 540 nm. Sample concentrations were determined by interpolation from the standard curve using a four parameter logistic curve fit.

### 2.2.9 Creatinine Assay

Urinary creatinine was measured by Jaffe reaction using the Creatinine parameter assay kit (Table 2.3) according to the manufacturer’s instructions. Specifically, 50 µL of standards, control and samples (diluted 10-fold) were added to each well. Then, 100 µL of alkaline picrate solution was added to each well and the plate incubated for 30 minutes at room temperature. The optical density of each well was determined using a microplate reader set to 490 nm. Sample concentrations were determined by interpolation from the standard curve.

### 2.2.10 Cryosectioning

OCT embedded samples were cut to a thickness of 8 µm on a Leica CM1860 UV cryostat and adhered to Superfrost Plus adhesion slides (Thermo Fisher Scientific, Sunnyvale, CA, USA). Adhered sections were stored at -20 to -80°C in the dark (to protect Hoechst 33342 from photobleaching) prior to staining.

### 2.2.11 Immunofluorescence

#### 2.2.11.1 Analysis of intratumoural T cells, hypoxia and perfusion in Chapters 3 and 4

5-colour staining and imaging experiments (as described below) were performed in the Dunbar lab at the University of Auckland. B16-F10 melanomas in Chapter 3 were stained with two separate 4 colour stains on different sections to stain for CD8 and Foxp3, as 5 colour optimisation succeeded only at a later time-point.

Sections were kept in the dark throughout to prevent photobleaching of Hoechst 33342. Frozen sections were removed from the freezer and allowed to warm up for 30 minutes before commencing with the staining protocol. Following this, sections were fixed for 10
minutes in 10% neutral buffered formalin. All further incubation steps were done in a dark humid chamber. Sections were outlined with a Dako pen and slides were blocked in 0.25% casein for 1 hour at room temperature. Slides were then incubated with the primary antibodies (Table 2.7) overnight at 4°C. Next, slides were washed 3 times for 5 minutes in PBST before incubation with the secondary antibodies (Table 2.7) in 0.125% casein for 1 h at room temperature. Slides were then washed 3 times for 5 minutes in PBST before draining excess liquid and manual coverslipping using ProLong Diamond as a mounting medium. Sections were imaged using a Nikon Ni-U epifluorescent microscope with bandpass filter cubes fitted as outlined in Table 2.6.

Table 2.6: Filter cubes for Nikon Ni-U microscope.

<table>
<thead>
<tr>
<th>Cube name</th>
<th>Excitation (nm)</th>
<th>Split (nm)</th>
<th>Emission (nm)</th>
<th>Supplier/ part no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>325-375</td>
<td>400</td>
<td>435-485</td>
<td>Chroma 49000</td>
</tr>
<tr>
<td>GFP</td>
<td>450-490</td>
<td>495</td>
<td>500-550</td>
<td>Chroma 49002</td>
</tr>
<tr>
<td>TRITC</td>
<td>530-560</td>
<td>570</td>
<td>590-650</td>
<td>Chroma 49005</td>
</tr>
<tr>
<td>Cy5</td>
<td>590-650</td>
<td>660</td>
<td>662-738</td>
<td>Chroma 49006</td>
</tr>
<tr>
<td>Cy7</td>
<td>672-748</td>
<td>760</td>
<td>765-855</td>
<td>Chroma 49007</td>
</tr>
</tbody>
</table>

Five hotspot clusters of T cells and 10 random tumour fields were imaged using the 20x objective to obtain two measures of intratumoural T cells. Images were analysed using my own custom-written macros (Section 9.1) to count the total number of CD3\(^+\), CD8\(^+\) and Foxp3\(^+\) cells per field. It was observed that a vanishingly small number (<1 per tumour) of CD8\(^+\) or Foxp3\(^+\) cells were not also CD3\(^+\); therefore, macros did not analyse for double-positivity of CD3 and CD8/Foxp3. Macros also quantified the hypoxic and perfused areas (separate macro for each) and counted the number of CD3\(^+\), CD8\(^+\) and Foxp3\(^+\) cells in these areas to obtain the T cell density in these areas.

Perfusion was quantified by manually counting the number of Hoechst 33342 perfused vessels in 10 random fields at using the 20x objective.

Hypoxia was quantified by automated analysis of pimonidazole\(^+\) area in 10 random fields using the 10x objective. The percent hypoxic area was then calculated (necrotic areas and empty space were excluded to make a percentage of viable tumour area).
Table 2.7: Antibody dilution scheme for immunofluorescence (Chapters 3 and 4)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3 [500A2]</td>
<td>1:200</td>
<td>Anti-hamster Alexa Fluor 647</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-CD8a [EPR21769]</td>
<td>1:100</td>
<td>Anti-rabbit Alexa Fluor 755</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-FoxP3</td>
<td>1:300</td>
<td>Anti-rat Alexa Fluor 555</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-pimonidazole (FITC)</td>
<td>1:500</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

2.2.11.2 Analysis of intratumoral T cells in Chapter 6

Frozen sections were removed from the freezer and allowed to warm up for 30-60 minutes before commencing with the staining protocol. Following this, sections were fixed for 10 minutes in acetone and allowed to air dry for 15 minutes. All further incubation steps were done in a humid chamber. Sections were outlined with the Dako pen and slides were blocked in 10% BSA in PBS for at least 1 hour at room temperature. FoxP3 and CD8a antibodies were diluted in the pre-diluted CD3 antibody for doublestaining. Slides were then incubated with the primary antibodies (Table 2.8) overnight at 4°C. Next, slides were washed 3 times for 5 minutes in PBST before incubation with the secondary antibodies (Table 2.8) in 5% BSA in PBS for 40 minutes at room temperature. Slides were then washed 3 times for 5 minutes in PBST before draining excess liquid and manual coverslipping using ProLong Gold with DAPI as a mounting medium. Sections were imaged using a Zeiss AxioObserver Z1 microscope (equipped with an ApoTome.2 to enable optical sectioning).

Table 2.8: Antibody dilution scheme for immunofluorescence (Chapter 6)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3 [SP7]</td>
<td>Prediluted</td>
<td>Donkey anti-rabbit Alexa Fluor 594</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-CD8a [4SM15]</td>
<td>1:1000</td>
<td>Donkey anti-rat Alexa Fluor 488</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-FoxP3</td>
<td>1:300</td>
<td>Donkey anti-rat Alexa Fluor 488</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
Sections were quantified by counting the total number of CD3+ T cells and number of either CD3+CD8+ CTLs or CD3+FoxP3+ T\textsubscript{reg} cells per field using the 40x objective. An average number of cells per field was obtained for 5 random fields. The relative proportion of CTLs and T\textsubscript{reg} cells of total T cells was obtained by calculating the percentage of CTLs or T\textsubscript{reg} cells of total T cells.

2.2.11.3 Analysis of hypoxia and perfusion in Chapter 3

In Chapter 3, analysis of hypoxia and perfusion was done on sections stained for pimonidazole only, as this was done before the collaboration in Auckland was established and thus occurred in Christchurch. Staining was performed as above, except blocking and antibody incubation was done in 10% and 5% BSA, respectively. Casein was used in Auckland as BSA was unavailable. Sections were imaged using a Zeiss AxioObserver Z1 microscope (equipped with an ApoTome.2 to enable optical sectioning).

Hypoxic area was quantified as above, as was perfusion (with the exception that images taken using the 10x objective rather than the 20x objective were used).

2.2.12 Microtome Sectioning

FFPE embedded samples were cut to a thickness of 3 \( \mu \text{m} \) on a Leica RM2125RT microtome and adhered to Superfrost Plus adhesion slides (Thermo Fisher Scientific, Sunnyvale, CA, USA). Adhered sections were stored at room temperature prior to staining.

2.2.13 Immunohistochemistry

2.2.13.1 Method

Tumour sections were stained using primary antibodies (Table 2.9) and the EnVision\textsuperscript{TM} G/2 System/AP Rabbit/Mouse (Permanent Red) kit (Dako, Chapter 6), the goat VisUCyte kit (for NK cells, R&D Systems) or the REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Chapters 3 and 4).

Specifically, slides were first baked for at least 1 hour at 60°C. Sections were then deparaffinised and rehydrated as follows: first, slides were incubated in xylene for 2x5 minutes, followed by 95% ethanol for 2x3 minutes and distilled water for 2x1 minute. Slides were then rinsed briefly in PBS before antigen retrieval.
Antigen retrieval was performed in citrate buffer (Table 2.2) by incubating for 3 minutes in a fully pressurised pressure cooker. The pressure cooker was then depressurised and left to cool for 40 minutes.

Slides were washed twice for 2 minutes in PBST before commencing with the staining procedure. All incubation steps were done in a humid chamber. Sections were outlined with the Dako pen and blocked for 5 minutes with Dual Endogenous Enzyme Block (Dako kits) or Peroxidase Blocking Reagent (R&D kit). Slides were then washed for 5 minutes in PBST. Serum Blocking Reagent D was then added for 15 minutes (R&D kit only) before incubation with the primary antibody (Table 2.9) overnight at 4°C. Next, slides were washed 3x for 5 minutes in PBST before incubation with the secondary antibody for 30 minutes (Dako kits) or one hour (R&D kit) at room temperature.

### Table 2.9: Antibody dilution scheme and developing times for IHC

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Developing Time (min)</th>
<th>Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-pHH3</td>
<td>1:10 000 (DAB)</td>
<td>13</td>
<td>REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (Chapters 3 and 4) EnVision™ G/2 System/AP Rabbit/Mouse (Permanent Red) kit (Chapter 6)</td>
</tr>
<tr>
<td></td>
<td>1:100 (Permanent Red)</td>
<td></td>
<td>EnVision™ G/2 System/AP Rabbit/Mouse (Permanent Red) kit (Chapter 6)</td>
</tr>
<tr>
<td>Anti-CD31</td>
<td>1:5000 (DAB)</td>
<td>10</td>
<td>REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse EnVision™ G/2 System/AP Rabbit/Mouse (Permanent Red) kit (Chapter 6)</td>
</tr>
<tr>
<td></td>
<td>1:200 (Permanent Red)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-pimonidazole</td>
<td>1:1000</td>
<td>14</td>
<td>EnVision™ G/2 System/AP Rabbit/Mouse (Permanent Red) kit (Chapter 6)</td>
</tr>
<tr>
<td>Anti-NKp46*</td>
<td>1:400</td>
<td>20</td>
<td>Goat VisUCyte kit</td>
</tr>
</tbody>
</table>

*This antibody is extremely sensitive to freeze-thawing and cannot be refrozen after thawing.
Following this, slides were washed twice for 5 minutes in PBST before incubation with AP Enzyme (enhancer) for 10 minutes at room temperature (Permanent Red kit only). This was followed by a further three washes in PBST for 5 minutes each and incubation with the chromogenic working reagent (prepared according to the manufacturer’s instructions) for a primary antibody dependent time period (Table 2.9). Slides were then placed in distilled water and counterstained by incubation in haematoxylin for 45 seconds, followed by a rinse in tap water and incubation in Scott’s tap water (a blueing reagent) for 20 seconds. Slides were then dehydrated by graded alcohol washes (95%, 95%, 100% and 100%) and cleared in xylene. Coverslipping was performed using DPX mountant.

2.2.13.2 Quantification (for experiments in Chapters 3 and 4)

Sections were imaged using a Zeiss AxioObserver Z1 microscope. All analysis was done in Image J. All bright field images were taken with automatic exposure and white balance settings.

**pHH3**

The total number of cells per field was counted using a custom-written macro to count nuclei on images (Section 9.1).

The number of pHH3 positive cells per field was manually counted from 10 random fields and the average used to calculate a percentage of pHH3 positive cells. Counting was done on images taken using the 20x objective.

**CD31 and NKp46**

CD31+ vessels and NKp46+ cells were quantified by counting their respective numbers in 10 random fields using the 20x objective.

2.2.13.3 Quantification (for experiments in Chapter 6)

All scoring was done (manually) in a blinded manner by one (pHH3, CD31) or two (pimonidazole) observers.

**pHH3**

The total number of cells per field was estimated by counting all cells in the first row of a graticule using the 40x objective and multiplying by the number of rows (ten). The same was done using the last column. This was done for the first and last fields counted, and the average of all four values calculated to give the mean total cell number per field.
The number of pHH3 positive cells per field was counted from 10 random fields and the average used to calculate a percentage of pHH3 positive cells. Counting was done using the 40x objective.

**CD31**

Angiogenic potential was estimated by counting the number of CD31 positive vessels in 5 hotspot areas using the 40x objective and calculating the average. Hotspot areas were identified by scanning of the whole section at low power.

**Pimonidazole**

Pimonidazole staining was quantified using a modified H score as follows. Specifically, the percent area of the tumour (estimated by eye) which was positive for pimonidazole was multiplied by the intensity of the staining (scored from 1-3), giving a score between 0 and 300. Examples of staining intensities can be viewed in Figure 9.1.

### 2.2.14 Statistical Analysis

All data were analysed using GraphPad Prism 7 or 8. The D’Agostino-Pearson normality test was used to determine if data was from a Gaussian distribution to inform whether data should be analysed by parametric or non-parametric test.

Correlations were determined using Pearson (normally distributed) or Spearman (non-normally distributed) correlation according to the result of the normality test.

Comparison between two groups was done using an unpaired, two-tailed student’s t test (normally distributed data) or Mann-Whitney test (non-normally distributed data).

Comparison of the effect of one variable between more than two groups was performed using one-way analysis of variance (ANOVA) with Tukey’s multiple comparison post-hoc test to determine differences between individual groups. If the data was not normally distributed, the Kruskal-Wallis test was used.

Comparison of the effect of two variables and their interaction was performed using two-way ANOVA with Tukey’s or Holm-Sidak’s multiple comparison post-hoc tests to determine differences between individual groups.

P values less than 0.05 were considered significant.
3 The Effect of Exercise on the Tumour Microenvironment

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3.1 Introduction

3.1.1 Effect of Exercise on Breast Cancer and Melanoma

It is now well-established that exercise is efficacious for patients with cancer, in a number of different ways and at different time-points along the cancer continuum. Pre-diagnosis physical activity reduces the risk of developing a range of different cancers, including breast cancer, esophageal adenocarcinoma, liver cancer, lung cancer, kidney cancer, gastric cancer, endometrial cancer, myeloid leukaemia, myeloma, colorectal cancer, head and neck cancer, and bladder cancer [177]. One apparent exception is that the risk of developing malignant melanoma is increased with leisure-time physical activity, but this loses significance when adjusted for UV radiation exposure [177]. A large body of data indicates that during treatment, exercise can help to reduce treatment-related side-effects, improve symptoms of anxiety and depression, and improve health-related quality of life (reviewed in [3]). In terms of survival outcomes, post-diagnosis exercise reduces breast cancer mortality by approximately 40% (systematically reviewed in [178]). On the other hand, clinical data on melanoma are sparse, there being (to my knowledge) only one study investigating the effect of exercise on survival in melanoma patients. In that study, the authors found that pre-diagnosis exercise did not affect survival outcomes in patients with high-risk primary melanoma [179]. However, survival rates for primary melanoma are very high regardless (>90%, [180]), which means there is little room for improvement. It is unknown whether post-diagnosis exercise affects survival outcomes in either primary or metastatic melanoma patients.
3.1.2 Exercise and the TME

In preclinical studies, varying and conflicting data have been reported with regards to the effect of exercise on tumour growth and the TME (discussed in Section 1.3). There have been numerous reports of the effects of exercise on tumour progression in rodents (reviewed in 2,6,8), but it remains unclear how post-implantation exercise affects the TME in different tumour types. In particular, it has been suggested that exercise can increase tumour perfusion [27,30,31], vascularity [27,65] and/or reduce hypoxia [27,32], thus ‘normalising’ the tumour microenvironment. Although this has been shown in breast cancer models [27], it has not yet been demonstrated in a melanoma model.

The anti-cancer effect of exercise in melanoma has largely been attributed to improved immune function, with pre-implantation exercise increasing T cells, natural killer (NK) cells and dendritic cell numbers within the tumour [29]. It is unclear whether this is also the case with post-implantation exercise. Similarly, pre-implantation exercise increases NK cell infiltration and the proportion of CD8+ T cells in breast tumours [181,182].

Although some preclinical studies have begun to elucidate the mechanisms of the potential anti-tumour effect of exercise in terms of the effect on the tumour microenvironment, very little is known about which factors actually mediate these effects. Contracting muscle tissue releases a variety of proteins into the bloodstream – myokines. It is likely that these act as messengers between muscle and tumour tissue, thus altering tumour characteristics. Post-exercise serum has been shown to directly inhibit tumour cell growth in vitro, and a few individual myokines have been identified that exert anti-tumour effects (such as SPARC, irisin and oncostatin M. [46,48,49]). However, these myokines are likely to be just a drop in the ocean of exercise-regulated factors. The identification of additional myokines is required to further our understanding not only of how exercise affects the tumour microenvironment, but also how it affects other tissues.

3.1.3 The Role of Stress in Exercise Oncology

It has been suggested that stress due to excessive tumour burden, forced exercise modalities or isolated housing may confound exercise effects in preclinical studies [21]. In order to minimise these stressors, my study utilised a maximum tumour size of 1000 mm³ for the subcutaneous B16-F10 tumours (as per institutional ethical guidelines) and
a more conservative maximum size of 600 mm³ for the orthotopic EO771 tumours as their localisation on the abdomen of the mouse may impede movement or normal functioning at an earlier stage than tumours on the flank. In addition, I selected voluntary wheel running as my exercise modality, which has been shown to be less stressful than treadmill running or swimming [183,184]. Resolving the issue of stress due to isolated housing proved to be more of a challenge. During a previous study in which mice were housed with one running wheel per pair, I discovered that inter-individual running distance is highly variable, which meant that taking the daily revolutions run per cage was not able to provide me with an accurate indication of how far each individual mouse was running [36]. Thus, I decided to use cage dividers with holes to allow mice to see, smell and hear each other, while preventing physical contact, in an attempt to minimise isolation stress while still allowing for individual measurement of running distance. It is unknown how semi-isolated housing, such as with a cage divider (as used in my study), affects mouse stress levels.

The hypothalamic-pituitary-adrenal (HPA) axis is one of the major neuroendocrine axes regulating homeostasis in mammals [185]. In response to stress, the HPA axis causes the glucocorticoids cortisol and corticosterone to be released from the adrenal gland [185]. In humans, cortisol is the major glucocorticoid, but in mice corticosterone is primarily released [185]. Thus, plasma, urinary or faecal corticosterone are commonly used measures of stress in rodents.

### 3.2 Chapter Aim and Experimental Approach

**Hypothesis:** That tumours would exhibit increased perfusion and reduced hypoxia, as well as improved immune cell infiltration with post-implantation exercise.

The primary aim for this chapter was to determine how post-implantation exercise affects the tumour microenvironment in two different cancer types: melanoma and breast cancer. In addition, I aimed to determine whether housing with a cage divider reduced isolation stress. In order to assess this, the following specific objectives were addressed:

1. Measure urinary corticosterone over a period of time in mice housed either alone, in pairs or in pairs with a cage divider to assess stress levels.

2. Measure B16-F10 melanoma and EO771 breast tumour growth in exercising vs non-exercising mice.
3. Use immunohistochemical techniques to investigate levels of overall tumour hypoxia, perfusion, blood vessel density, immune cell infiltration and tumour cell proliferation in tumours from exercising compared with non-exercising mice. The immune cell populations chosen were T cells, as they are the traditional targets of immune checkpoint blockade (the combination of which with exercise was investigated in Chapter 4), and NK cells because they are the most responsive to acute exercise. In addition, two specific T cell subtypes were studied: cytotoxic CD3⁺CD8⁺ T cells and regulatory CD3⁺Foxp3⁺ T cells.

4. Use immunohistochemical techniques to investigate the intratumoural localisation of T cell types and subsets with respect to hypoxic areas and perfused blood vessels.

5. Identify candidate exercise-regulated proteins in plasma that may be exerting anti-tumour effects.

### 3.2.1 Study Design

To investigate stress levels in mice in different housing conditions, urine was collected from 6-10 week old female mice housed either alone, in pairs or in pairs with a cage divider (Figure 3.1). Urine was collected at baseline (before housing change), daily for the first week and weekly until day 28. Urinary corticosterone was investigated instead of plasma as it requires less invasive sampling and responds less quickly to acute stress. Urine was collected at the same time each day to control for fluctuations in corticosterone due to circadian rhythm and the vaginal opening was visually inspected to determine oestrous cycle stage.

To study the effect of exercise on the TME, mice were subcutaneously (B16-F10) or orthotopically (EO771) inoculated with tumour cells before randomisation to either exercise (using a voluntary running wheel placed in the cage) or non-exercising control (Figure 3.2). Tumours were grown to maximal ethical size (B16-F10: 1000 mm³, EO771: 600 mm³) and mice were euthanised following IP injection with pimonidazole (a hypoxia marker) 90 minutes prior, and IV injection with Hoechst 33342 (to label perfused blood vessels) 1 minute prior. The tumour, heart, spleen, kidneys, liver, quadriceps femoris muscle and plasma were removed for analysis.
Figure 3.1: Experimental design to examine stress levels of mice in different housing conditions.
Female, 6-10 week old mice were housed either in pairs in a regular mouse cage, in pairs with a cage divider in a rat cage, or individually in a regular mouse cage. Urine was collected daily for a week, then weekly until day 28.

T cell subtypes, hypoxia and perfusion were analysed by 5-colour immunofluorescent staining on frozen sections (Section 2.2.11). NK cells, vascularity and tumour cell proliferation were investigated by immunohistochemical single-stains on FFPE sections (Section 2.2.13).

Images were analysed by custom-written macros (Section 9.1). For quality control, analysis using these macros was compared with manual analysis for a subset of images as described in Section 9.2.2.2.
3.3 Acknowledgements

I would like to acknowledge Dr Abel Ang for his assistance with in vivo experiments in this chapter (mouse euthanasia and organ harvest if I was sick or injured).

3.4 Results

3.4.1 Model Development and Cohort Characterisation

3.4.1.1 Mouse stress levels according to housing condition

In order to attempt to minimise isolation stress while still allowing quantification of individual mouse running distance, I had custom cage dividers designed (Figure 9.2) that allowed mice in either half of the cage to see, smell and hear each other, but prevented physical contact. To determine whether this set-up did indeed reduce isolation stress, I measured urinary corticosterone from mice housed either singly, in pairs or in pairs with a cage divider.

There was no difference in urinary corticosterone between groups at any time-point (Figure 3.3). Housing change also did not appear to cause much stress in any of the three groups, as values obtained from post-baseline samples (day 1-28) were similar to those obtained at baseline (day 0) (Figure 3.3). Large spikes in corticosterone were seen in some mice (Figure 3.4); it is unclear what the reason for these were as they did not appear to be associated with housing condition or oestrus stage.
Figure 3.3: Stress levels are similar in mice housed individually, in pairs or with a cage divider.

Average corticosterone levels in the urine of mice housed alone, in pairs (group) or in pairs with a cage divider. Corticosterone levels were normalised to creatinine to control for filtration rate. Data are presented as mean with 95% CI. n=6-8 per data point (some urine samples could not be obtained for every time point). Mice were tumour-free.
Figure 3.4: Urinary corticosterone levels in individual mice across the study period.
Red dots indicate that mice were in pro-oestrus or oestrus at this time point. Mice were tumour-free.
3.4.1.2 Exercise levels in tumour-bearing mice

Mice in the exercise group ran an average of 8 km/day across the duration of the study, although this was subject to large inter and intra-individual variation (range: <1 km/day – 23 km/day; Figure 3.5). Average daily running distance was steady throughout, indicating that tumour burden did not affect activity towards the end of the study. Daily running distance was similar in mice bearing subcutaneous melanoma tumours (flank) and mice bearing orthotopic breast tumours (mammary fat pad), suggesting that tumour type/location did not affect ability to use the wheel. The distance run by mice in this study is similar to previously published reports of running distance in young, female C57BL/6 mice without tumours [186], indicating that tumour burden did not reduce running activity.

![Graphs of running distance](image)

Figure 3.5: Running distance of tumour-bearing mice is subject to large inter- and intra-individual variability.

Individual daily running distance for mice bearing B16-F10 (a) or EO771 (b) tumours. Average daily running distance for mice bearing B16-F10 (c) or EO771 (d) tumours. Data are presented as mean ± SD. n=3-12 per data point (numbers reduce as mice are progressively euthanised).
3.4.1.3 Body and organ weights of tumour-bearing, exercising mice

Most mice lost weight in the first few days after tumour implant (Figure 3.6). Weight returned to baseline or increased by the end of the study in 50% of mice, but remained below starting weight in the other 50% of mice (Table 3.1), regardless of tumour type or exercise group. Using a cut-off of 5% body weight loss, five mice with B16-F10 melanoma can be described as having developed cachexia (No Ex: n=2, Ex: n=3) and two mice with EO771 breast cancer (No Ex: n=1, Ex: n=1). A cut-off of 10% weight loss has also been described for cancer cachexia in mice [187], but given that other symptoms of cachexia (such as anorexia) begin prior to a noticeable weight loss in mice [188], I chose to use a more conservative cut-off.

![Figure 3.6: Individual weight change curves for mice bearing B16-F10 or EO771 tumours.](image)

<table>
<thead>
<tr>
<th>a</th>
<th>B16-F10</th>
<th>No Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Change from initial weight</td>
<td>Days after implant</td>
<td></td>
</tr>
<tr>
<td>-15</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b</th>
<th>EO771</th>
<th>No Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Change from initial weight</td>
<td>Days after implant</td>
<td></td>
</tr>
<tr>
<td>-15</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>c</th>
<th>Ex</th>
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<tbody>
<tr>
<td>% Change from initial weight</td>
<td>Days after implant</td>
</tr>
<tr>
<td>-15</td>
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<table>
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<tr>
<th>d</th>
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<tbody>
<tr>
<td>% Change from initial weight</td>
<td>Days after implant</td>
</tr>
<tr>
<td>-15</td>
<td>0</td>
</tr>
</tbody>
</table>

The spleen, liver, both kidneys and heart were removed and weighed after euthanasia. Organ weights were normalised to final body weight (minus tumour weight) for analysis. Hearts from exercising mice with EO771 tumours were significantly heavier than those
from non-exercising mice (p=0.0008, Table 3.1) and a similar trend was seen for mice with B16-F10 tumours (p=0.095, Table 3.1). No other organ weights were significantly different between exercising or non-exercising mice, for either tumour type (Table 3.1).

Table 3.1: Body and organ weights of non-exercising vs exercising mice with B16-F10 or EO771 tumours.

<table>
<thead>
<tr>
<th></th>
<th>B16 No Ex</th>
<th>B16 Ex</th>
<th>p-value</th>
<th>EO771 No Ex</th>
<th>EO771 Ex</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>20.0±1.85</td>
<td>19.2±1.68</td>
<td>0.293</td>
<td>18.8±1.27</td>
<td>18.3±1.38</td>
<td>0.344</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>20.3±1.04</td>
<td>19.3±1.56</td>
<td>0.0615</td>
<td>18.8±0.89</td>
<td>18.4±1.10</td>
<td>0.271</td>
</tr>
<tr>
<td>Change in body weight (%)</td>
<td>1.45±5.22</td>
<td>1.39±6.66</td>
<td>0.980</td>
<td>0.32±3.94</td>
<td>0.68±4.07</td>
<td>0.832</td>
</tr>
<tr>
<td>Heart/body weight (mg/g)</td>
<td>5.95±0.46</td>
<td>6.45±0.88</td>
<td>0.095</td>
<td>6.13±0.52</td>
<td>6.98±0.52</td>
<td>0.0008***</td>
</tr>
<tr>
<td>Spleen/body weight (mg/g)</td>
<td>6.98±6.27</td>
<td>4.73±0.88</td>
<td>0.232</td>
<td>5.22±1.28</td>
<td>4.67±0.78</td>
<td>0.260</td>
</tr>
<tr>
<td>Liver/body weight (mg/g)</td>
<td>45.1±5.96</td>
<td>46.8±6.07</td>
<td>0.494</td>
<td>46.8±6.08</td>
<td>50.4±3.11</td>
<td>0.099</td>
</tr>
<tr>
<td>Kidney/body weight (mg/g)</td>
<td>14.5±1.40</td>
<td>14.7±1.05</td>
<td>0.615</td>
<td>15.7±0.74</td>
<td>15.9±1.02</td>
<td>0.654</td>
</tr>
</tbody>
</table>

1 Values are means±SD. p-values are for exercising (Ex) vs non-exercising (No Ex) control for the respective tumour types. Data were analysed using a two-tailed student’s t test; n=10-12.

3.4.2 Effect of Exercise on Tumour Growth

Some mice were euthanised early due to the following reasons and so excluded from the survival analysis: ulceration of the tumour (B16-F10 n=4, EO771 n=2), presence of intraperitoneal tumours (EO771 only, n=3) or pyometra (EO771 n=1).

Tumour volume was estimated daily using calliper measurement. The median time to endpoint was 17 days for mice bearing melanomas and 21 days for mice bearing breast tumours. I saw no difference in tumour growth rate between mice with or without access
to a running wheel for both B16-F10 and EO771 tumours (Figure 3.7, Figure 3.8c and d), and there was no difference in survival (Figure 3.8a and b). In addition, exercise did not affect the lag (defined as the time to reach 175 mm$^3$ in B16-F10 melanoma or 100 mm$^3$ in EO771 tumours) or exponential phase (defined as the time for the tumour to quadruple in volume) of tumour growth in either tumour type (Figure 3.8e-h). Similarly, average daily running distance was not correlated with tumour growth rate (Figure 3.9a and b). One exercising mouse with B16-F10 melanoma survived much longer than all the others, but this did not appear to be due to exercise or mouse characteristics.

Figure 3.7: Individual tumour growth curves for non-exercising and exercising mice bearing B16-F10 melanoma or EO771 breast cancer.
B16-F10: n=12 per group; EO771: n=10 per group.
Figure 3.8: Tumour growth rate and mouse survival are unaffected by exercise in B16-F10 and EO771 tumours.

Survival curves for mice bearing B16-F10 (a) or EO771 tumours (b) (endpoint due to tumour size only). Lag phase of tumour growth for mice bearing B16-F10 (c) or EO771 tumours (d): B16-F10: n=12 per group; EO771: n=9-10 per group. Exponential phase of tumour growth for mice bearing B16-F10 (e) or EO771 tumours (f): B16-F10: n=8-11 per group; EO771: n=7-10 per group. Data are shown as individual data points and mean with 95% CI. Survival analysis was performed using the Log rank (Mantel-Cox) test.
Figure 3.9: Average daily running distance is not correlated with time to euthanasia in B16-F10 or EO771 tumours.

Correlation of the time to euthanasia (due to maximum tumour size) with average daily running distance in mice with B16-F10 (a) or EO771 (b) tumours. Data analysed by Pearson (b) or Spearman correlation (a). Data shown as scatter plot with best fit line with 95% CI bands. B16-F10: n=12, EO771: n=10.

3.4.2.1 Tumour growth according to weight change

In mice that had lost weight over the course of the study (including cachectic mice), EO771 tumours grew more rapidly to palpable size (100 mm$^3$, defined as lag phase of tumour growth), than those whose weight remained stable or who gained weight (Figure 3.10b), regardless of whether mice exercised or not. There was no difference in lag phase growth (time to reach 175 mm$^3$) in mice with B16-F10 tumours (Figure 3.10a). Exponential tumour growth rate (time for the tumour to quadruple in volume) was unchanged by weight loss in both tumour types (Figure 3.10c and d). It is noteworthy that mice that lost weight while bearing EO771 tumours had poorer overall survival (time to euthanasia due to tumour burden) than those that did not lose weight (p=0.0008, Figure 3.10f), while those bearing B16-F10 tumours had similar survival regardless of weight change (Figure 3.10e). It is unclear whether accelerated tumour growth is causing the weight loss or whether weight loss supports more rapid tumour growth.
Figure 3.10 Weight loss after implant is associated with a shorter tumour lag phase and shorter survival in mice with EO771 breast cancer.

Tumour establishment time (lag phase, time to 175 or 100 mm$^3$) in mice with B16-F10 (a) or EO771 (b) tumours according to mouse weight change. Exponential tumour growth rate (time for the tumour to quadruple in volume) in mice that did or did not lose weight with B16-F10 (c) or EO771 (d) tumours. Data are shown as individual data points and mean ± 95% CI. Data analysed using a two-tailed students t test. p<0.05*. B16-F10 lag phase weight loss: n=9, no weight loss: n=15; EO771 lag phase weight loss: n=8, no weight loss: n=11; B16-F10 exponential phase weight loss: n=7, no weight loss: n=12; EO771 exponential phase weight loss: n=6, no weight loss: n=11. Survival curves for mice with or without weight loss while bearing B16-F10 (e) or EO771 (f) tumours. Animals were included in survival analysis only if euthanasia was due to tumours reaching maximum ethical size. Data analysed using Log-rank test.

3.4.3 Effect of Exercise on Tumour Hypoxia, Perfusion, Blood Vessel Density and Proliferation

Previous reports in 4T1 and EO771 murine breast tumours have indicated that post-implantation exercise can increase tumour perfusion and vascularity, and reduce hypoxia,
compared with non-exercising mice [27]. I aimed to confirm this in EO771 breast tumours and determine whether it holds true for melanoma.

Intra-peritoneal tumours were excluded from the histological analyses (n=3), and one mouse was euthanised before tumour development due to pyometra. In addition, one tumour exhibited low-level, diffuse perfusion which could not be quantified accurately by vessel count. This reduced the number of tumours used for perfusion analysis to n=9 in the EO771 No Ex group and n=10 in the EO771 Ex group. All B16-F10 tumours were suitable for analysis.

Figure 3.11: Representative B16-F10 and EO771 tumour sections stained for CD31, pimonidazole and with Hoechst 33342. Red: CD31, green: pimonidazole, blue: Hoechst 33342. Closed arrows indicate examples of perfused CD31+ vessels and open arrows indicate examples of unperfused CD31+ vessels.
Figure 3.12: Exercise reduces variance in perfusion in EO771 tumours.

(a) Representative immunofluorescent images of sections containing Hoechst 33342 (perfused blood vessels, blue) and stained for pimonidazole (hypoxia, green) in B16-F10 and EO771 tumours from non-exercising vs exercising mice. Quantification of hypoxic area in B16-F10 (b) and EO771 (d) tumours from non-exercising vs exercising mice. Quantification of perfused blood vessels in B16-F10 (c) and EO771 (e) tumours from non-exercising vs exercising mice. B16-F10 No Ex and Ex: n=12, EO771 No Ex and Ex: n=10. Difference in variance analysed using the F test. Data are presented as individual data points and mean ± 95% CI.

Oxygen delivery must have occurred in perfused vessels (according to Hoechst 33342 staining), because hypoxia was not observed immediately adjacent to those vessels.
In addition, Hoechst 33342 staining co-localised with CD31+ blood vessels, but not all CD31+ vessels were perfused (Figure 3.11). I found that hypoxic area and perfused vessel number were unchanged in tumours from exercising compared with non-exercising mice, for both B16-F10 and EO771 tumours (Figure 3.12a-e). However, it was noteworthy that there was significantly less variation between EO771 tumours in the number of perfused vessels from exercising compared with non-exercising mice (F test, p=0.013, Figure 3.12e). A similar trend was seen in B16-F10 tumours, but the effect was much less pronounced (Figure 3.12c).

The number of animals in each group are lower for the CD31 stain for the B16-F10 tumours as I encountered technical difficulty in measuring staining in some of the tumours. In a small number of melanomas, dark pigmentation was observed, making true DAB staining difficult to identify and quantification unreliable. For this reason, these tumours were excluded from the CD31 analysis. Four tumours were removed from analysis in the B16-F10 No Ex group, and two from the B16-F10 Ex group for this reason, bringing the numbers to n=8 and n=10, respectively. As for the perfusion analysis, intra-peritoneal EO771 tumours were excluded.

There was no difference in CD31+ vessel density in tumours from exercising compared with non-exercising mice, for both B16-F10 and EO771 tumours (Figure 3.13). Similarly, average daily running distance was not correlated with either the number of perfused vessels, tumour hypoxia or the number of CD31+ vessels (Figure 3.14).
Figure 3.13: Exercise does not change CD31+ vessel density in melanoma or breast cancer.

(a) Representative immunohistochemical images of B16-F10 and EO771 tumours from exercising vs non-exercising mice. Quantification of the number of CD31+ vessels in B16-F10 (b) and EO771 tumours (c) from non-exercising vs exercising mice. B16-F10 No Ex: n=8, B16-F10 Ex: n=10, EO771 No Ex: n=11, EO771 Ex: n=10. Data are presented as individual data points and mean ± 95% CI.
Figure 3.14: Average daily running distance is not correlated with tumour perfusion, hypoxia or vascularity in B16-F10 or EO771 tumours.

Correlation of perfused vessel number with average daily running distance in mice with B16-F10 (a) or EO771 (b) tumours. Correlation of hypoxic area with average daily running distance in mice with B16-F10 (c) or EO771 (d) tumours. Correlation of CD31+ vessel number with average daily running distance in mice with B16-F10 (e) or EO771 (f) tumours. Data analysed by Pearson (a, d, e, f) or Spearman correlation (b, c). Data shown as scatter plot with best fit line with 95% CI bands. B16-F10: n=10-12, EO771: n=9-10.

The area of tumour hypoxia did not correlate with either the total number of vessels or the number of perfused vessels in either tumour type (Figure 3.15). Likewise, the total number of vessels did not correlate with the number of perfused vessels in either tumour type (Figure 3.15).
Figure 3.15: Correlations between hypoxia, perfusion and CD31+ vessel density in B16-F10 and EO771 tumours.

Correlation of perfused vessel number with CD31+ vessel number in B16-F10 (a) or EO771 tumours (b). Correlation of hypoxia with CD31+ vessel number in B16-F10 (c) or EO771 tumours (d). Correlation of hypoxic area with perfused vessel number in B16-F10 (e) or EO771 (f) tumours. Data analysed by Pearson (a, b, d, f) or Spearman correlation (c, e). Data shown as scatter plot with best fit line with 95% CI bands. B16-F10: perfused vs CD31 vessels n=16; hypoxia vs CD31 vessels n=18; hypoxia vs perfusion n=22; EO771: perfused vs CD31 vessels n=25; hypoxia vs CD31 vessels n=18; hypoxia vs perfusion n=19.

Tumour cell proliferation was measured by immunohistochemical staining for pHH3, a mitotic marker, and by calculating the percentage of pHH3 positive nuclei. No difference
was observed in the proliferation of tumours from exercising versus non-exercising mice for both B16-F10 and EO771 tumours (Figure 3.16).

Figure 3.16: Exercise does not change tumour cell proliferation in melanoma or breast cancer.
(a) Representative immunohistochemical staining for pHH3, a mitotic marker, in tumour sections from exercising vs non-exercising mice with B16-F10 melanoma or EO771 breast cancer. Quantification of the percentage of pHH3+ cells in B16-F10 (b) and EO771 (c) tumours from exercising vs non-exercising mice. B16-F10 No Ex and Ex: n=12, EO771 No Ex and Ex: n=10. Data are shown as individual data points and mean ± 95% CI.

Taken together, my data show that exercise beginning after tumour implantation does not alter the mean level of tumour hypoxia, perfusion, CD31+ vessel density or cancer cell
proliferation. However, inter-tumour perfusion heterogeneity was reduced with exercise in EO771 tumours.

3.4.4 Effect of Exercise on Intratumoural Immune Cells

Previous studies have shown that pre-implantation exercise increases numbers of intratumoural T and NK cells in B16-F10 melanoma and 4T1 breast cancer [29,137,181]. I investigated whether post-implant exercise affected numbers of infiltrating immune cells in B16-F10 and EO771 tumours.

3.4.4.1 Effect of exercise on intratumoural natural killer cells

NK cells were analysed by imaging 10 random fields per tumour and taking the average. Numbers of infiltrating NK cells were unchanged with exercise in both tumour types, although much higher numbers were present in EO771 compared with B16-F10 tumours (B16-F10 No Ex=2.2±2.2 cells per field, EO771 No Ex=17.2±13.9 cells per field; Figure 3.17).
Figure 3.17: Exercise does not alter NK cell number in B16-F10 or EO771 tumours.

(a) Representative immunohistochemical images of B16-F10 and EO771 tumours stained for NKp46, from exercising vs non-exercising mice. Arrows indicate cells positive for NKp46. Quantification of the number of NKp46+ cells in B16-F10 (b) and EO771 (c) tumours from non-exercising vs exercising mice. B16-F10 No Ex: n=8, B16-F10 Ex: n=10, EO771 No Ex: n=11, EO771 Ex: n=10. Data are presented as individual data points and mean ± 95% CI.

3.4.4.2 T cell infiltrate and exercise

I also investigated whether T cell infiltrate varied with exercise. CD3 was used as a marker to identify all T cells, Foxp3 was used in addition to identify T_{reg} cells and CD8 was used in conjunction with CD3 to identify cytotoxic T cells. In B16-F10 melanomas, T cells tended to cluster in high-density hotspots, whereas in EO771 tumours, T cells were more evenly distributed, although higher density clusters were still present (Figure
The quantity of T cells per tumour was estimated using two imaging strategies – hotspots and random fields. For hotspots analysis, 5 images of high-density T cell clusters per tumour were analysed for T cell localisation and abundance. For random fields analysis, 10 random tumour fields were analysed for T cell localisation and abundance (details in Section 2.2.11.1).

![Representative immunofluorescent images of T cell clusters within a B16-F10 and EO771 tumour.](image)

Green: pimonidazole (hypoxia), blue: Hoechst 33342 (perfusion), red: CD3 (pan T cell marker), yellow: CD8 (cytotoxic T cell marker), white: Foxp3 (regulatory T cell marker). Arrows indicate examples of CD3⁺ cells (red), CD3⁺CD8⁺ cells (yellow) and CD3⁺Foxp3⁺ cells (white).

I found that exercise did not alter the number of infiltrating T cells in hotspot areas in B16-F10 melanomas (Figure 3.19a-c). However, in EO771 tumours, the number of CD3⁺ cells and CD8⁺ cells was significantly reduced by exercise (p=0.023 and p=0.0011, respectively; Figure 3.19d and f). The number of Foxp3⁺ cells was unchanged by exercise in both tumour types. Similar results were found when using random fields for analysis (Figure 3.20).
Figure 3.19: Exercise reduces CD3⁺ and CD8⁺ T cell number in EO771 breast tumours but not B16-F10 melanomas (hotspots).

Average number of CD3⁺ cells per field in B16-F10 (a) or EO771 (d) tumours from non-exercising compared with exercising mice. Average number of Foxp3⁺ cells in B16-F10 (b) or EO771 (e) tumours from non-exercising compared with exercising mice. Average number of CD8⁺ cells in B16-F10 (c) or EO771 (f) tumours from non-exercising compared with exercising mice. Cell densities were quantified by imaging 5 hotspot areas of T cell clusters and counting the total number of CD3⁺, Foxp3⁺ and CD8⁺ cells per field. No Ex: no exercise, Ex: exercise. p<0.05*, p<0.01**. Data are shown as individual data points and mean with 95% CI. B16-F10: n=12; EO771: n=10. Data analysed by unpaired, two-tailed student’s t test.

Figure 3.20: Exercise reduces CD3⁺ and CD8⁺ T cell number in EO771 breast tumours but not B16-F10 melanomas (random fields).

Average number of CD3⁺ cells per field in B16-F10 (a) or EO771 (d) tumours from non-exercising compared with exercising mice. Average number of Foxp3⁺ cells in B16-F10 (b) or EO771 (e) tumours from non-exercising compared with exercising mice. Average number of CD8⁺ cells in B16-F10 (c) or EO771 (f) tumours from non-exercising compared with exercising mice. Cell densities were quantified by imaging 10 random tumour fields and counting the total number of CD3⁺, Foxp3⁺ and CD8⁺ cells per field. No Ex: no exercise, Ex: exercise. p<0.01**, p<0.001***. Data are shown as individual data points and mean with 95% CI. B16-F10: n=12; EO771: n=10. Data analysed by unpaired, two-tailed student’s t test.
When analysing the relative proportion of Foxp3+ and CD8+ T cells in hotspot areas, I saw that the percentage Foxp3+ cells within the CD3+ population was significantly increased by exercise in B16-F10 tumours (p=0.040) and tended to be increased in EO771 tumours (Figure 3.21a and c; p=0.12). Moreover, the percentage CD8+ cells within the CD3+ population was decreased by exercise in both tumour types (Figure 3.21b and d; B16-F10: p=0.039, EO771: p=0.0015).

![Figure 3.21: Exercise alters proportions of intratumoural CD8+ and Foxp3+ T cells (hotspots).](image)

Percentage of Foxp3+ T cells in B16-F10 (a) or EO771 (c) tumours from non-exercising compared with exercising mice. Percentage of CD8+ T cells in B16-F10 (b) or EO771 (d) tumours from non-exercising compared with exercising mice. No Ex: no exercise. Ex: exercise. p<0.05*, p<0.01**. Data are shown as individual data points and mean with 95% CI. B16-F10: n=12; EO771: n=10. Data analysed by two-tailed Mann-Whitney test (B16-F10 Foxp3+ cells, EO771 CD8+ cells) or unpaired, two-tailed student’s t test (B16-F10 CD8+ cells, EO771 Foxp3+ cells).

When analysing random fields, only the percentage CD8+ of CD3+ T cells in EO771 tumours was significantly changed by exercise (decreased, Figure 3.22d; p=0.029). In addition, the percentage of Foxp3+ cells tended to be increased by exercise in EO771 tumours (p=0.087, Figure 3.22c), but the proportions of either subtype were unchanged by exercise in B16-F10 tumours (Figure 3.22a and b).
3.4.4.3 T cell infiltrate in different tumour regions

In order to gain a more complete picture of the TME, I analysed T cell density in three discrete microenvironmental zones – perfused areas (high Hoechst 33342 fluorescence, indicating a perfused blood vessel), hypoxia (positive for pimonidazole) and non-hypoxic intervascular space (negative/low Hoechst 33342 and negative for pimonidazole, Figure 3.23).

Figure 3.22: Exercise reduces proportion of intratumoural CD8+ T cells in EO771 breast tumours (random fields).

Percentage of Foxp3+ T cells in B16-F10 (a) or EO771 (c) tumours from non-exercising compared with exercising mice. Percentage of CD8+ T cells in B16-F10 (b) or EO771 (d) tumours from non-exercising compared with exercising mice. No Ex: no exercise, Ex: exercise. p<0.05*. Data are shown as individual data points and mean with 95% CI. B16-F10: n=12; EO771: n=10. Data analysed by two-tailed Mann-Whitney test (EO771 CD8+ cells) or two-tailed, unpaired student’s t test (EO771 Foxp3+ cells, B16-F10 Foxp3+ and CD8+ cells).
Using the hotspots imaging strategy, it appeared that T cells clustered primarily around perfused vessels, in both tumour types, with significantly lower densities in the intervascular space and hypoxic areas (Figure 3.24a and d; p<0.0001 for localisation effect in both tumour types).

Interestingly, the number of Foxp3+ cells was similar in perfused and hypoxic areas, with lower densities in the intervascular space (Figure 3.24b and e; B16-F10: p=0.0071; EO771: p=0.049 for perfusion vs intervascular space, No Ex). This was true for both tumour types, although the density of Foxp3+ cells in hypoxic areas in B16-F10 tumours from exercising mice was still significantly lower than in perfused areas (p=0.037). These results align with published data indicating that hypoxia induces an immunosuppressive phenotype and/or promotes the recruitment of immunosuppressive cells [189,190].

CD8+ T cell localisation followed a similar trend to total T cells, with the highest densities being found close to perfused blood vessels, and lower densities in intervascular space and hypoxia, in both B16-F10 and EO771 tumours (Figure 3.24c and f; B16-F10: p=0.0047, EO771: p=0.0033 for localisation effect). Exercise did not affect the localisation pattern of CD3+, Foxp3+ or CD8+ cells in either tumour type (Figure 3.24; p>0.05 for interaction effect between localisation and exercise). Results followed a similar trend using the random fields imaging strategy (Figure 3.25).
Figure 3.24: T cells preferentially localise close to perfused blood vessels in both B16-F10 melanomas and EO771 breast tumours (hotspots).

Average density of CD3⁺ T cells localised close to perfused blood vessels (perfusion), in intervascular space or in hypoxic areas, in B16-F10 (a) or EO771 (d) tumours from non-exercising compared with exercising mice. Average density of Foxp3⁺ cells localised close to perfused blood vessels (perfusion), in intervascular space or in hypoxic areas, in B16-F10 (b) or EO771 (e) tumours from non-exercising compared with exercising mice. Average density of CD8⁺ cells localised close to perfused blood vessels (perfusion), in intervascular space or in hypoxic areas, in B16-F10 (c) or EO771 (f) tumours from non-exercising compared with exercising mice. Cell densities were quantified by imaging 5 hotspot areas of T cell clusters, counting the numbers of CD3⁺, Foxp3⁺ and CD8⁺ cells in each tumour environment and forming a ratio of the number of cells to the area. No Ex: no exercise, Ex: exercise. * indicates significantly different from Perfusion, same exercise group. p<0.05*; p<0.01**; p<0.001***; p<0.0001****. No significant differences were found between cell density in hypoxia and intervascular space. Data are shown as individual data points and mean with 95% CI. B16-F10: n=9-12 per group; EO771: n=9-10 per group. Data analysed by two-way ANOVA with Tukey’s post-hoc test.
Figure 3.25: T cells preferentially localise close to perfused blood vessels in both B16-F10 melanomas and EO771 breast tumours (random fields).

Average density of CD3+ T cells localised close to perfused blood vessels (perfusion), in intervascular space or in hypoxic areas, in B16-F10 (a) or EO771 (d) tumours from non-exercising compared with exercising mice. Average density of Foxp3+ cells localised close to perfused blood vessels (perfusion), in intervascular space or in hypoxic areas, in B16-F10 (b) or EO771 (e) tumours from non-exercising compared with exercising mice. Average density of CD8+ cells localised close to perfused blood vessels (perfusion), in intervascular space or in hypoxic areas, in B16-F10 (c) or EO771 (f) tumours from non-exercising compared with exercising mice. Cell densities were quantified by imaging 10 random fields, counting the numbers of CD3+, Foxp3+ and CD8+ cells in each tumour environment and forming a ratio of the number of cells to the area. No Ex: no exercise, Ex: exercise. * indicates significantly different from Perfusion, same exercise group. p<0.05*,#; p<0.01*; p<0.001***. Data are shown as individual data points and mean with 95% CI. B16-F10: n=11-12 per group; EO771 n=9-10 per group. Data analysed by two-way ANOVA with Tukey’s post-hoc test.

Taken together, my results show that while exercise does not alter numbers of intratumoural NK cells or the localisation of intratumoural T cells, it does reduce CD3+ and CD8+ T cell number in EO771 breast tumours. In addition, the percentage CD3+/CD8+ T cells in hotspot clusters is reduced by exercise in both tumour types, and the percentage CD3+/Foxp3+ T cells is increased by exercise in both tumour types.

3.4.5 Comparison of EO771 and B16-F10 Tumours

I compared microenvironmental features of B16-F10 and EO771 tumours to determine differences between the two tumour models. Where no differences were found with exercise in the investigated features, I pooled results from non-exercising and exercising mice for each tumour type. Where differences were found with exercise, I used only the non-exercising condition for analysis.
EO771 tumours were significantly more proliferative than B16-F10 tumours (p<0.0001, Figure 3.26a). EO771 tumours were also significantly more hypoxic (p=0.0063) and had a higher CD31+ vessel density (p=0.0005) than B16-F10 tumours (Figure 3.26b and c), while the number of perfused vessels per field was similar in EO771 compared with B16-F10 tumours (Figure 3.26d).

![Figure 3.26: EO771 tumours are more proliferative, more vascular and more hypoxic than B16-F10 tumours.](image)

Exercising and non-exercising mice were pooled for this analysis as no difference was seen with exercise. (a) Quantification of the percentage of pHH3+ cells in B16-F10 vs EO771 tumours. B16-F10: n=24, EO771: n=20. (b) Comparison of the number of CD31+ vessels in B16-F10 and EO771 tumours. B16-F10: n=18, EO771: n=21. Quantification of hypoxia (c) and perfusion (d) in B16-F10 vs EO771 tumours. B16-F10: n=24, EO771: n=20. Data analysed using two-tailed Mann-Whitney test (hypoxia) or two-tailed student’s t test (proliferation and vascularity). Data are presented as individual data points and mean ± 95% CI.

EO771 tumours contained significantly more NK cells than B16-F10 tumours (Figure 3.27).
Figure 3.27: EO771 tumours contain higher numbers of NK cells than B16-F10 tumours.
Exercising and non-exercising mice were used for this analysis as no differences were found between exercise groups. Comparison of the number of NK cells in B16-F10 and EO771 tumours. Data analysed using two-tailed Mann-Whitney test. p<0.0001****. Data are presented as individual data points and mean ± 95% CI. B16-F10: n=20, EO771: n=21.

When comparing numbers of CD3+, Foxp3+ and CD8+ cells in B16-F10 and EO771 tumours, I found that EO771 tumours contained significantly more of each subtype than B16-F10 tumours when sections were analysed by random fields (Figure 3.28d-f; CD3+ cells: p=0.0003, Foxp3+ cells: p=0.009, CD8+ cells: p=0.0006). Numbers of T cells were comparable in hotspot clusters between tumour types (Figure 3.28a-c).

Figure 3.28: EO771 tumours contain higher numbers of CD3+, Foxp3+ and CD8+ cells than B16-F10 tumours.
Non-exercising mice were used for this analysis. Comparison of the number of CD3+ cells in B16-F10 and EO771 tumours using hotspots (a) or random fields (d). Comparison of the number of Foxp3+ cells in B16-F10 and EO771 tumours using hotspots (b) or random fields (e). Comparison of the number of CD8+ cells in B16-F10 and EO771 tumours using hotspots (c) or random fields (f). Data analysed using two-tailed Mann-Whitney test (CD8+ cells random fields). Data analysed using unpaired, two-tailed student’s t test (CD3+ and Foxp3+ cells random fields). p<0.01**, p<0.001***. Data are presented as individual data points and mean ± 95% CI. B16-F10: n=12, EO771: n=10.
These results suggest that EO771 tumours have a more aggressive phenotype than B16-F10 tumours, characterised by higher levels of tumour hypoxia, more CD31+ vessels (but no increase in perfusion) and more proliferative tumour cells. Conversely, they also appear to be more permissive to immune cell infiltration, as evidenced by higher numbers of T cells and NK cells.

### 3.4.6 Effect of Exercise on Levels of Circulating Factors

Studies suggest that the effects of exercise on the tumour microenvironment may be mediated by factors secreted by contracting muscle tissue, termed ‘myokines’ (described in Section 1.4). I aimed to identify exercise-regulated proteins in plasma that may be acting either directly on the tumour cells, or indirectly via immune cells. In order to achieve this, I used a commercially available antibody array containing probes for more than 300 proteins, mostly cytokines and chemokines.

Signals were quantified by taking the average of the duplicates for each protein and normalising to the positive control (HRP). Duplicate signals were very similar and no background signal was observed (Figure 3.29).

![Figure 3.29: Antibody array membranes probed with plasma from non-exercising or exercising mice with EO771 tumours.](image)

Plasma from 4 exercising and 4 non-exercising mice was pooled and used to probe the blot. All antibodies were present in duplicate. Antibody array membranes were analysed by densitometry and normalised to the expression of the positive control antibody (HRP, lowest dilution used for normalisation, i.e. that on the far left).
Figure 3.30: Relative levels of circulating proteins in exercising vs non-exercising mice.

Plasma from 4 exercising and 4 non-exercising mice was pooled to obtain data. Antibody array membranes were analysed by densitometry and normalised to the positive control protein. Red boxes indicate a 2-fold or greater increase in plasma from exercising compared to non-exercising mice. Blue boxes indicate a 2-fold or greater decrease in plasma from exercising compared to non-exercising mice.
The assay detected 91/308 possible proteins in the plasma of exercising vs non-exercising mice with EO771 tumours. Of these, 24 were increased by at least 2-fold in the plasma of exercising compared with non-exercising mice (Figure 3.30). Only MCP-5 and ubiquitin were decreased by more than 2-fold in the exercising mice.

Tumour necrosis factor superfamily member 14 (TNFSF14, aka LIGHT) showed the highest fold change (13-fold) increase in the plasma of exercising compared with non-exercising mice (Figure 3.30). LIGHT is a costimulator for T cell activation [191], can induce vessel normalisation [192], augments effector cell priming at tumour sites to induce anti-tumour immunity [193] and is expressed in skeletal muscle [194]. Based on this, I selected LIGHT as a candidate exercise-regulated myokine with potential anti-tumour function to investigate further.

I used a commercially available ELISA kit to validate the results found in the antibody array in a larger cohort of exercising compared with non-exercising mice bearing B16-F10 or EO771 tumours. There was no difference in plasma LIGHT in exercising compared with non-exercising mice with either tumour type, although there was a large spread of data in mice with B16-F10 tumours (Figure 3.31).

![Figure 3.31: Exercise does not alter plasma levels of LIGHT in mice bearing B16-F10 or EO771 tumours.](image)

Levels of LIGHT in plasma from exercising vs non-exercising mice with B16-F10 melanoma (a) or EO771 breast cancer (b). Data are shown as individual data points and mean ± 95% CI. EO771 No Ex: n=8, EO771 Ex: n=10, B16-F10: n=12 per group. Plasma samples from mice with EO771 tumours were obtained from a previous cohort of mice in 2016 (details in Section 6.2); in this cohort, mice were housed in pairs with a running wheel rather than with a cage divider.

As LIGHT failed to validate as an exercise-regulated plasma protein, I selected a second candidate. Pentraxin 3 showed a fold-change increase of 1.75 in exercising compared with non-exercising mice (Figure 3.30) and is involved in the regulation of innate
immune responses [174]. Its role in cancer is unclear, but it is expressed in the skeletal muscle of mice [195] and plasma levels have been reported to rise with exercise in humans [176].

However, using ELISA, I found no difference in plasma levels of pentraxin 3 in exercising compared with non-exercising mice with B16-F10 or EO771 tumours (Figure 3.32).

![Figure 3.32: Exercise does not alter plasma levels of pentraxin 3 in mice bearing B16-F10 or EO771 tumours.](image)

Plasma levels of pentraxin 3 in non-exercising and exercising mice with B16-F10 (a) or EO771 (b) tumours. n=12 per group, except EO771 Ex: n=11. Data are shown as individual data points and mean ± 95% CI.

### 3.5 Discussion

Experiments in this chapter have shown that short-term, post-implantation exercise did not alter tumour growth rate, hypoxia, perfusion, blood vessel density or cell proliferation in either tumour type in a murine model. However, the number of intratumoural CD3$^+$ and CD8$^+$ T cells was reduced by exercise in EO771 tumours, but not B16-F10 tumours. In addition, the relative proportion of CD8$^+$ T cells in hotspot areas was reduced by exercise in both tumour types, and the proportion of Foxp3$^+$ T cells was increased. Mice bearing EO771 tumours who lost weight had a shorter tumour lag growth phase and poorer survival than those who did not lose weight. Additionally, I observed that EO771 tumours had increased levels of tumour cell proliferation, hypoxia, CD31$^+$ vessel density, intratumoural NK cells and intratumoural T cells when compared with B16-F10 tumours.

*Use of mouse models for exercise research:* There is much discussion as to which mouse exercise modality is best-suited to generate translational data. Forced modalities such as
treadmill running and swimming allow better control of exercise dosage, but are inherently stressful [183,184]. Given that there may be a link between chronic stress and cancer progression [196], it is possible that stress due to forced exercise could impact results. A recent review article argues that because mice naturally run far more than most humans are physically capable of, voluntary wheel running experiments cannot lead to human relevant data [38]. I reason that although exercise doses that elicit a physiological response will naturally be different between the two species, mouse exercise studies can and do provide useful mechanistic data in the exercise oncology setting. Indeed, I observed a significant increase in heart to body weight ratio in exercising mice with EO771 tumours, and a similar trend in mice with B16-F10 tumours. Exercise-induced cardiac hypertrophy is a well-established phenomenon in humans [197]. In addition, healthy female mice exposed to 21 days of voluntary wheel running had a significantly higher heart to body weight ratio than their non-exercising counterparts [198]. This also holds true in tumour-bearing mice. Sturgeon et al. found that mice bearing B16-F10 melanoma and exposed to 16 days of treadmill running had significantly higher heart to body weight ratios than their non-exercising counterparts [199]. Together, this indicates that in mice, heart weight increases within a week or two of exercise, and this remains true in tumour-bearing mice. Thus, my cohort showed the expected increase in cardiac size, demonstrating that my exercise protocol was having a physiological effect.

Effect of exercise on tumour growth: I observed no change in tumour growth rate in exercising vs non-exercising mice, regardless of tumour type (Figure 3.8). For B16-F10 melanoma, this is in agreement with Pedersen et al., who found that wheel running beginning at tumour implant did not alter tumour growth rate, although exercise beginning 4 weeks prior to tumour implant significantly slowed tumour growth [29]. In two previous studies in mice, exercise beginning at tumour implant significantly slowed orthotopic EO771 tumour growth [4,27]. However, this difference was small (tumour volume at endpoint approx. 1300-1500 mm$^3$ for non-exercising mice and 900 mm$^3$ for exercising mice). Importantly, this only became apparent once tumours exceeded 600 mm$^3$ (i.e. there was no difference in growth rate up to a tumour size of 600 mm$^3$ [4,27]), which was the maximum ethical size used in my study. This limit was used to prevent hindrance of mouse movement due to tumour size. Thus, my study results are consistent with previous data for EO771 tumours up to 600 mm$^3$. 


Most preclinical studies that reported a statistical reduction in tumour growth rate with post-implantation exercise show only a marginal slowing of tumour growth, with tumours from exercising mice having 80-90% the weight or volume of those from non-exercising mice at endpoint [24,25], or a difference of 150-400 mm³ at endpoint [26,27]. To provide context, transplantable tumour models (such as those used in my study and those used in the above-mentioned studies) typically grow extremely rapidly and can increase in size by 200 mm³ or more each day once they reach the exponential growth phase. A recent systematic review and meta-analysis found a “small to moderate” effect size for exercise to reduce final tumour size [19]. However, of the 8 studies included that showed a statistically significant difference in tumour size, one of these showed an increase with exercise, one had a small effect size and four had a ‘probably high’ risk of bias [19]. As there is little consistency between studies in terms of the effect of post-implantation exercise on tumour growth, it seems unlikely that exercise as a sole intervention (monotherapy) has a meaningful effect.

Comparing the effect of pre- vs post-diagnosis exercise on survival outcomes in clinical populations is much more difficult than in preclinical studies. Few such studies exist, and those that do are observational studies which cannot entirely rule out the possibility of reverse causation [200–202]. An intervention study would be required to determine whether beginning exercise after diagnosis can improve survival outcomes, or whether the survival improvement seen in cancer patients who exercise is due to either a pre-conditioning effect of pre-diagnosis exercise, or to reverse causation.

Effect of exercise on tumour blood flow and hypoxia: Previous studies in orthotopic breast (4T1 and EO771) and prostate tumours have reported a reduction in tumour hypoxia [27,32], increase in tumour perfusion [27,30,31] and increase in CD31⁺ vessel density with post-implantation exercise [27]. In contrast, I found no change in the mean value of any of these parameters with exercise, in either B16-F10 or EO771 tumours (Figure 3.12, Figure 3.13). For B16-F10 melanoma, this could be due to differences in tumour type and location (subcutaneous melanoma vs orthotopic breast and prostate cancer). Garcia et al. found that, during exercise, blood flow was increased to orthotopic prostate tumours in rats, but decreased to subcutaneous prostate tumours using the same cell line and rat strain [50]. This was paralleled by decreased blood flow to subcutaneous adipose tissue and skin (i.e. the tissues adjacent and attached to the subcutaneous tumour). Therefore, although tumour vessels themselves are less able to respond to
haemodynamic cues than normal vessels [50], the response of the surrounding tissue to exercise seems to play an important role in regulating blood flow.

In the case of EO771 breast tumours, those grown in our study were smaller than those in the earlier study by Betof et al. [27], which may explain the lower level of hypoxia observed in our study and may influence the physiological response of the tumour to exercise.

There was significantly less variation in perfusion between EO771 tumours from exercising compared with non-exercising mice, and this trend was also seen in B16-F10 tumours. This suggests that exercise may improve the regulation of vascular maturation (although this was not tested for in my study), particularly in EO771 tumours. Indeed, Betof et al. have seen higher pericyte coverage in 4T1 breast tumours from exercising mice, suggesting improved vascular maturation [27]. My results support previous work which reported more homogenous perfusion across orthotopic breast [27] and prostate [31] tumours with exercise.

In normal tissue, blood flow is locally regulated by contraction and dilation of arterioles. However, tumour vessels exhibit not only lower contractility upon noradrenergic stimulation compared with normal vessels [50,61], but also reduced responsiveness to vasodilators [60]. Together, this reflects the impaired ability of tumour vessels to respond to haemodynamic cues. In addition, oxygen delivery is determined not only by blood flow, but also by additional properties such as the oxygen consumption rate of tumour cells and the red blood cell flux [64]. This may explain the lack of association between tumour perfusion and hypoxia seen by me and others [32], and the differing effects of exercise on tumour perfusion across different studies. More research is required to gain a full picture of tumour haemodynamics relating to tissue perfusion both during acute exercise and following a training period (chronic exercise).

Effect of exercise on immune infiltrate: I observed no difference in the numbers of intratumoural NK cells in either B16-F10 or EO771 tumours from non-exercising compared with exercising mice (Figure 3.17). Conversely, two studies in B16-F10 melanoma [29] and 4T1 breast cancer [181] found that exercise increased numbers of intratumoural NK cells. However, both of these studies used pre-implantation exercise, whereas my study used post-implantation exercise. This suggests that a pre-conditioning effect prior to tumour initiation may be required in order to enhance NK cell infiltration.
In humans, it has been shown that cancer patients may have a blunted lymphocytosis in response to acute exercise [97,98]. To my knowledge, it has never been assessed whether tumour-bearing mice also exhibit this altered lymphocytosis, but if so, it could be that post-implant exercise alone is unable to increase tumour infiltration by NK cells simply because they are not being mobilised into the bloodstream in sufficient numbers.

Contrary to my hypothesis, numbers of intratumoural CD3+ T cells and CD8+ T cells were significantly reduced by exercise in EO771 tumours (Figure 3.19), and the relative proportion of CD8+ cells was reduced with exercise in both tumour types, while the proportion of Foxp3+ cells was increased (Figure 3.21). Previous studies have shown increased numbers of intratumoural CD8+ T cells and/or reduced numbers of T\text{reg} cells with exercise, but these all utilised pre-implantation exercise, which may have a preconditioning effect on the body [24,29,182].

It is important to note that I did not stain for CD4 and so was unable to detect T helper subsets other than T\text{reg} cells. For example, the fact that the proportion of CD8+ T cells was reduced by exercise in EO771 tumours, but the proportion of Foxp3+ T cells was unchanged, indicates that the proportion of a different, unstained, subset must have increased. The role of T helper cell subsets in cancer is highly complex. In general, more inflammatory subtypes such as T\text{H}1 cells are thought to have anti-cancer functions, while suppressive subtypes such as T\text{reg} cells are thought to have primarily pro-tumour actions. However, in melanoma T\text{reg} cells have been associated with a favourable prognosis [203] and it has been suggested that T\text{H}1 cells may in some situations promote tumour growth [204]. It is likely that the pro- or anti-tumour action of each subset is interdependent on other subtypes present. Therefore, in future, a more comprehensive staining panel including markers for additional T cell subsets would help clarify the effect of exercise on the immune tumour microenvironment.

Previous reports have indicated that hypoxia induces Foxp3 expression, creating an immunosuppressive phenotype [205]. In agreement with this, my data show that Foxp3+ T cells are present in similar densities in hypoxic and perfused areas, whereas CD8+ T cells are found in lower densities in hypoxic areas than in perfused areas (Figure 3.24). In addition, my work expands on previous work showing that T cells are found at lower frequencies in hypoxic areas [206], demonstrating for the first time that T cells are present at low densities in both hypoxic and normoxic intervascular space, but cluster
close to perfused blood vessels. This suggests that T cells may be hindered from migrating further into tumour tissue after extravasation due to cues (or the lack thereof) in the tumour microenvironment. For example, T cells move using the extracellular matrix as a scaffold [207]. B16-F10 tumours are extremely soft, almost liquid, whereas EO771 tumours are firmer. This could provide an explanation as to why T cells are primarily present in high density clusters close to perfused blood vessels in B16-F10 tumours but found very sparsely in other areas, whereas in EO771 tumours they are found to be more widely distributed.

*Effect of mouse housing on stress markers*: I found no significant differences in urinary corticosterone levels in mice housed alone, in pairs or in pairs with a cage divider (Figure 3.3). Meijer et al. showed that baseline levels of urinary corticosterone in female C57BL/6 mice were approximately 175 nmol/mmol creatinine (equivalent to approximately 550 ng/mg creatinine) [172]. The mice in my study had comparable levels of corticosterone (300-700 ng/mg creatinine), aside from the spikes in corticosterone seen in some mice on some days. This suggests that overall, my mice were not overtly stressed and, given that I saw no differences between groups, nor did housing condition have a significant effect.

*Plasma markers of exercise*: Using an antibody array, I was able to detect numerous plasma proteins that were increased by exercise training. However, this result was not validated for either of the two candidate proteins (LIGHT and pentraxin 3) selected (Figure 3.31, Figure 3.32). This may be due to the small numbers of mice used for the antibody array (pooled plasma from 4 mice per group) or the nature of the array itself, being a semi-quantitative assay. Future attempts to identify novel myokines could use a more sensitive technique such as mass spectrometry.

*Strengths and limitations of study*: I have used transplantable tumour models in this work. These models have the advantage of a clearly defined tumour initiation and rapid growth, thus shortening experimental time. However, these models do not capture the full developmental process of cancer and show a relatively uniform histology. In addition, the rapid growth is a double-edged sword, as it provides a very narrow window in which interventions can be applied with any success. In preclinical exercise studies outside the field of oncology, training interventions aimed at achieving physiological adaptation are
typically a minimum of 4 weeks [159,208], whereas in my study some mice had as little as two weeks. This likely limited training efficacy.

This brings me to a second point – in the tumour-bearing mice, the training period was not a consistent length due to variable tumour growth rates. Time to euthanasia typically fell somewhere between two and four weeks, with some as long as five weeks. Therefore, some mice had more than double the training time as others, which likely impacted the degree to which exercise could affect the tumour.

I was unable to measure dynamic changes in perfusion and hypoxia in the whole tumour. Immunofluorescence, as used in my study, provides a snapshot of blood flow and hypoxia within the tumour at the time the mouse was euthanised. Furthermore, vessels with low red blood cell flux (and thus low oxygen delivery capacity) would have been labelled as well as those with high red blood cell flux. Therefore, Hoechst 33342 labelled vessels do not necessarily provide an accurate indication of oxygen-carrying capacity. Techniques such as magnetic resonance imaging (MRI) or the infusion of labelled microspheres can be used to measure whole tumour perfusion and tissue blood flow, respectively [27,50], and would allow measurement of changes both during exercise (acute changes) and at rest in trained animals (chronic changes). However, the advantage of Hoechst fluorescence is the relative ease with which the experiment can be conducted. Furthermore, it has been able to detect differences in perfusion in the past [62], and has been validated for this purpose [209].

Similarly, staining for basic immune cell markers provides an indication of the abundance of specific cell types within the tumour, but provides little indication of functionality. Multiplex immunohistochemistry (for example using the Opal system) enables staining for additional markers and so could be utilised to detect markers of exhaustion or activation in addition to general subtype markers. However, this system is more labour-intensive and requires more complex data analysis. Mass or flow cytometry enables more accurate quantification of cell numbers and the detection of additional markers, but loses information on tissue architecture. Alternatively, functional assays could be used on isolated tumour-infiltrating lymphocytes ex vivo.

Strengths of my study include the use of two different tumour types, allowing comparison of exercise effects on different cancers in different anatomical locations. In addition, the use of post-implantation exercise eliminates the possibility of an exercise effect due to
pre-conditioning of the body. It is possible that the strong tumour growth-inhibitory effect of pre-implantation exercise seen in some preclinical studies is due to host tissues being less receptive to tumour cell seeding. This would be seen as a tumour growth curve with a delayed tumour establishment time but relatively unaltered exponential growth rate. In support of this idea, Pedersen et al. reported slower growth of B16-F10 tumours with pre- but not post-implant exercise [29]. Inspection of the tumour growth curves revealed that the tumour volume of mice in the pre-implantation exercise group was close to zero (<50 mm$^3$) for longer (approx. 1 week) than those in the post-implant group, while exponential growth was similarly rapid (quadrupling of tumour size in 2-3 days).

**Conclusion:** Exercise as a monotherapy post-implant may have limited effects on tumour growth, although numbers of CD8$^+$ T cells were reduced by exercise in EO771 tumours. A small number of studies have used exercise in combination with conventional cancer therapies and demonstrated potentiation of the effect of the accompanying therapy, even in the absence of an exercise-only effect on tumour growth [27,30,199,210]. As this also reflects a more clinically relevant scenario, future preclinical studies should focus on the combination of exercise with other treatments such as chemotherapy, radiotherapy or immunotherapy. In line with this, the combination of exercise with immune checkpoint inhibition is investigated in the next chapter.
4 Exercise in Combination Therapy with Anti-PD-1

4.1 Introduction

In recent years, immunotherapy for cancer has increasingly gained traction as a promising treatment approach. Although many different approaches (including T cells with specifically engineered receptors, adoptive cell therapy and interferon therapy) have been tested and are under development, the approach which has garnered the most attention is the inhibition of immune checkpoint proteins. Immune checkpoint inhibitors have shaken the field of oncology due to the long-term response rates seen in some patients, particularly in high grade melanoma, leading to a plateau in the survival curve [211]. This phenomenon had very rarely been seen before in advanced melanoma, not even with targeted therapies such as BRAF inhibitors.

TCR signalling is essential for T cell activation and is initiated by the binding of the TCR to the MHC-antigen complex on an antigen-presenting cell (APC), engagement of co-stimulatory receptors and the secretion of stimulatory cytokines from the APC [73]. However, if a co-inhibitory receptor (such as PD-1 or CTLA-4) binds to its ligand on the surface of the APC instead, the TCR signalling cascade is inhibited [73]. This ultimately results in T cell anergy and the downregulation of effector functions. In addition, T cell exhaustion can occur from chronic antigen exposure, which leads to the persistent upregulation of inhibitory receptors such as PD-1 [85].

Ligation of PD-1 to the APC through concomitant binding of the TCR to the MHC-antigen complex and PD-1 to its ligand, PD-L1 or PD-L2, leads to auto-phosphorylation of the cytoplasmic tail of PD-1 [73]. Phosphorylation occurs on the structural immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) (Figure 4.1). This allows binding of the Src-homology domain proteins (SHP) 1 and 2, which then dephosphorylate phosphatidylinositol-3-kinase (PI3K), leading to the inhibition of the Akt pathway [73]. The PI3K/Akt pathway plays an important role in cell proliferation and survival, and its inhibition results in T cell anergy [73].
PD-1 signalling is also an important inhibitory mechanism in NK cells [212]. PD-L1 blockade revitalises NK cell-mediated anti-tumour immunity in a pre-clinical setting [212], and high expression of PD-1 on patient-derived NK cells is associated with poorer prognosis in digestive cancers [213].

Immune checkpoint inhibitors are monoclonal antibodies targeted against a particular checkpoint protein. Those most commonly used (and with FDA approval) are targeted against CTLA-4 (ipilimumab), PD-1 (pembrolizumab, nivolumab) or PD-L1.
(atezolizumab, avelumab, durvalumab) [214]. Binding of the antibody to the target prevents binding to the ligand, allowing T cell signalling to continue uninhibited. However, only 35-60% of patients with metastatic melanoma respond initially to treatment with anti-PD-1 based therapy, and of these patients approximately 43% will go on to experience relapse and acquired resistance to treatment [136]. For anti-CTLA-4 based therapy, less than 30% of patients respond initially [136]. Therefore, new treatment approaches are needed for use in combination with immune checkpoint inhibition, in the hope of overcoming resistance to this treatment.

In an attempt to improve response rates, checkpoint inhibitors have been combined with various agents including other checkpoint inhibitor classes, chemotherapy, radiotherapy and anti-angiogenic agents [215–217]. While effective, these combinations are inevitably associated with higher rates of severe treatment-related adverse events, which can lead to discontinuation of treatment or even death [218].

As discussed in Chapter 1, exercise has been shown to have immune-stimulatory effects. In addition, it is not associated with significant adverse events and is safe for people with cancer to practice. In fact, it even helps to alleviate treatment-related side effects and improve quality of life (reviewed in [3]). I postulate that exercise may boost the efficacy of immune checkpoint inhibitor treatment by overcoming resistance mechanisms such as impaired T cell recruitment. These mechanisms and the potential role of exercise are summarised in Figure 4.2.

In support of the notion that exercise may boost the efficacy of existing therapies, some preclinical studies have found that exercise potentiates the effect of the accompanying therapy, even in the absence of an exercise-only effect on tumour growth [27,30,199,210]. Two studies have investigated the combination of exercise with anti-PD-1 therapy in mice [137,139]. The first study used nivolumab to treat non-obese diabetic, severe combined immunodeficient (NOD/SCID) gamma mice with non-small cell lung cancer patient-derived xenografts (PDX) [139]. The authors found that while exercise alone reduced tumour growth rate, the addition of nivolumab had no effect. However, the obvious limitation to this study is the use of the severely immunodeficient NOD/SCID gamma mice, which lack lymphocytes – the primary target cell for immune checkpoint inhibitors. The second study investigated the combination of exercise with anti-PD-1 treatment and radiation therapy in mice with subcutaneous 4T1 breast cancer
In this study, the authors found that exercise increased the tumour growth inhibitory effect of the combination of anti-PD-1 and radiation therapy by a small amount (approx. 100 mm$^3$ difference in final tumour volume).

Figure 4.2: Proposed mechanisms by which exercise might overcome resistance to immune checkpoint inhibitors.

Tumours can develop resistance to immune checkpoint inhibition by a number of mechanisms, such as the upregulation of alternative inhibitory ligands and immunosuppressive molecules, dysregulation of tumour cell signalling pathways and the presence of immunosuppressive cell types such as myeloid derived suppressor cells (MDSCs). I hypothesise that exercise can overcome aspects of resistance to immune checkpoint blockade by improving T cell activation and recruitment, improving dendritic cell (DC) recruitment and reducing recruitment/activity of immunosuppressive cell types. Green arrows denote stimulation and red arrow denotes inhibition.

In addition to promoting T and NK cell effector functions, immune checkpoint inhibitor treatment can also increase tumour perfusion and reduce hypoxia in EO771 (anti-CTLA-4 or combination anti-CTLA-4 and anti-PD-1) and MCA38 tumours (anti-PD-1) in mice [132,219,220]. This was shown to be T cell dependent, highlighting the important role of T cells in mediating checkpoint-inhibitor-induced vascular normalisation. The mechanism is thought to be via a shift towards a $T_{H1}$ cell phenotype through IFN$\gamma$
signalling [132], but the exact mechanisms by which T_H1 cells mediate vessel normalisation is unknown.

My study aimed to determine whether the combination of post-implant exercise with anti-PD-1 treatment could boost the effect of anti-PD-1 alone to alter the tumour microenvironment and slow tumour growth. I hypothesised that exercise would improve the efficacy of anti-PD-1 treatment in B16-F10 melanoma and EO771 breast cancer.

*Note: This study was conducted and the hypothesis formed before I had obtained the results in Chapter 3 suggesting that exercise might actually reduce T cell recruitment in EO771 tumours, and so does not reflect those results. Rather, it represents the original hypothesis made at the beginning of the study, based on published literature.

4.2 Chapter Aim and Experimental Approach

**Hypothesis:** That exercise would boost the anti-tumour efficacy of anti-PD-1 treatment.

The primary aim for this chapter was to determine whether post-implantation exercise could boost the efficacy of immune checkpoint inhibitor treatment, specifically anti-PD-1 treatment, in mouse models of melanoma and breast cancer. To achieve this, the following specific objectives were addressed:

1. Determine tumour growth-delay following treatment with anti-PD-1 or IgG2a isotype control in exercising vs non-exercising mice with B16-F10 melanoma or EO771 breast cancer.

2. Use immunohistochemical techniques to investigate levels of overall tumour hypoxia, perfusion, blood vessel density, CD3+ T cells, CD3+Foxp3+ T cells, CD3+CD8+ T cells, NK cells and tumour cell proliferation in tumours from exercising compared with non-exercising mice treated with either anti-PD-1 or an isotype control antibody.

3. Use immunohistochemical techniques to investigate the intratumoural localisation of T cell types and subsets with respect to hypoxic areas and perfused blood vessels in tumours from exercising compared with non-exercising mice treated with either anti-PD-1 or an isotype control antibody.
4. Use immunohistochemical techniques to investigate levels of tumour hypoxia and perfusion in tumours from exercising compared with non-exercising mice treated with either anti-PD-1 or an isotype control antibody.

The anti-PD-1 antibody used in this study is raised in rat and targeted against mouse PD-1 (clone: RMP1-14, catalogue no. BE0146, BioXCell). This antibody has been extensively used for preclinical research as a checkpoint inhibitor in vivo [221–227]. The treatment regime (dose and scheduling) used in this study was determined by literature review to identify commonly used treatment regimens [221–227]. The IgG2a isotype control is recommended by the manufacturer for in vivo use alongside the anti-PD-1 antibody.

4.2.1 Study Design

Mice were subcutaneously (B16-F10, melanoma) or orthotopically (EO771, breast cancer) inoculated with tumour cells before randomisation to either no exercise + IgG2a, exercise (using a running wheel placed in the cage) + IgG2a, no exercise + anti-PD-1 or exercise + anti-PD-1 (Figure 4.3). Twice-weekly treatment with 200 µg of anti-PD-1 or IgG2a began when tumours reached 50-100 mm³. Tumours were grown to maximal ethical size (B16-F10: 1000 mm³, EO771: 600 mm³) and mice were euthanised following IP injection with pimonidazole (a hypoxia marker) 90 minutes prior, and IV injection with Hoechst 33342 (to label perfused blood vessels) 1 minute prior. The tumour, heart, spleen, kidneys, liver, quadriceps femoris muscle and serum were removed for analysis (details in Sections 2.2.3 to 2.2.13).
Female mice aged 6-10 weeks were subcutaneously inoculated with either B16-F10 melanoma cells or orthotopically with EO771 breast cancer cells. Mice were then randomised to non-exercising control, no exercise + immune checkpoint inhibition, voluntary exercise (with access to a modified running wheel with revolution counter) or voluntary exercise + immune checkpoint inhibition. Mice were housed at two per cage with a cage divider to enable accurate quantification of running distance. Mice were euthanised when tumours reached 1000 mm$^3$ (melanoma) or 600 mm$^3$ (breast cancer), after which point samples were collected for analysis of perfusion, hypoxia and immune infiltrate.

4.3 Acknowledgements

I would like to acknowledge Thomas Williams (summer student) for performing the staining and imaging of pHH3, CD31 and NKp46 in this chapter, under my supervision.

4.4 Results

Due to Covid-19 restrictions, staining and imaging for hypoxia, perfusion and T cells were unable to be completed for all samples, leading to lower numbers for analysis of these parameters.

Some mice were euthanised early due to the following reasons: ulceration of the tumour (B16-F10, No Ex, IgG2a: n=2; B16-F10, No Ex, aPD-1: n=1; B16-F10, Ex, aPD-1: n=3; EO771, No Ex, aPD-1: n=1) and presence of peritoneal metastases (EO771, No Ex, aPD-1: n=1). In addition, one mouse intended for the EO771, No Ex, aPD-1 group did not develop a tumour. This brought numbers down in the EO771, No Ex, aPD-1 group from n=10 to n=8. Peritoneal tumours were excluded from the analyses. Mice euthanised early
due to ulceration of the tumour were excluded from the survival analysis but the tumours were included in the microenvironmental analyses.

4.4.1 Effect of Exercise and Anti-PD-1 Treatment on Running Distance, Body and Organ Weights

I first wanted to determine whether there were any overt toxicities due to anti-PD-1 treatment. While conducting the study, I observed no sign of immune-related adverse events such as skin irritation or colitis. In order to further delve for the presence of toxicities, I investigated mouse running behaviour, body weight and organ weights.

Mice bearing B16-F10 tumours ran an average of 7 km/day over the course of the experiment, and this was unaltered by anti-PD-1 treatment (Figure 4.4a, c and e). Similarly, mice with EO771 tumours ran an average of 7.5 km/day and this was not significantly changed by anti-PD-1 treatment (Figure 4.4b, d and f). As in Chapter 3, running distance was highly variable between mice, and for individual mice on any given day.
Figure 4.4: Running behaviour is unaltered by anti-PD-1 treatment.

Individual daily running distance for mice bearing B16-F10 (a, c) or EO771 (b, d) tumours and treated with either an isotype control antibody (IgG2a, a, b) or anti-PD-1 (aPD-1, c, d). Average daily running distance for mice bearing B16-F10 (e) or EO771 (f) tumours and treated with either IgG2a or aPD-1. Data are presented as mean ± SD. n=3-12 per data point (numbers reduce as mice are progressively euthanised).

In mice with B16-F10 tumours, there was a significant effect of anti-PD-1 treatment on the change in body weight over the course of the study, with mice receiving anti-PD-1 treatment gaining less weight than mice receiving IgG2a (Table 4.1). This was not seen in mice with EO771 tumours (Table 4.2).

Interestingly, exercise significantly increased liver weight relative to body weight in mice with B16-F10 tumours (Table 4.1) but not EO771 tumours (Table 4.2).
In mice with EO771 tumours, exercise significantly increased heart weight relative to body weight (Table 4.2), while this was not the case in mice with B16-F10 tumours (Table 4.1). This aligns with results from Chapter 3.

I found no other significant effects of either exercise or anti-PD-1 treatment on body and organ weights, indicating that exercise and/or anti-PD-1 treatment does not affect relative spleen or kidney weight (Table 4.1 and Table 4.2).

**Table 4.1: Body and organ weights of B16-F10 melanoma-bearing non-exercising vs exercising mice receiving anti-PD-1 or IgG2a treatment.**

<table>
<thead>
<tr>
<th></th>
<th>B16 No Ex IgG2a</th>
<th>B16 No Ex aPD-1</th>
<th>B16 Ex IgG2a</th>
<th>B16 Ex aPD-1</th>
<th>p-value treatment effect</th>
<th>p-value exercise effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>18.1±1.00</td>
<td>19.0±1.52</td>
<td>18.3±0.96</td>
<td>19.1±1.36</td>
<td>0.105^2</td>
<td></td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>19.9±1.13</td>
<td>19.8±2.10</td>
<td>19.3±0.89</td>
<td>19.4±1.56</td>
<td>0.949</td>
<td>0.326</td>
</tr>
<tr>
<td>Change in body weight (%)</td>
<td>9.78±9.62</td>
<td>4.00±5.91</td>
<td>5.73±5.59</td>
<td>1.84±5.40</td>
<td>0.032*</td>
<td>0.161</td>
</tr>
<tr>
<td>Heart/body weight (mg/g)</td>
<td>6.26±0.80</td>
<td>6.63±0.55</td>
<td>6.51±0.31</td>
<td>6.63±0.55</td>
<td>0.187</td>
<td>0.486</td>
</tr>
<tr>
<td>Spleen/body weight (mg/g)</td>
<td>6.26±3.92</td>
<td>4.75±1.73</td>
<td>3.95±0.69</td>
<td>4.40±1.03</td>
<td>0.457</td>
<td>0.067</td>
</tr>
<tr>
<td>Liver/body weight (mg/g)</td>
<td>46.8±2.55</td>
<td>43.6±4.75</td>
<td>48.7±1.61</td>
<td>48.2±3.30</td>
<td>0.088</td>
<td>0.0033*</td>
</tr>
<tr>
<td>Kidney/body weight (mg/g)</td>
<td>13.6±1.22</td>
<td>13.7±1.00</td>
<td>13.8±0.88</td>
<td>14.1±0.66</td>
<td>0.524</td>
<td>0.424</td>
</tr>
</tbody>
</table>

^1 Values are means±SD. Data were analysed using two-way ANOVA. n=10.

^2 Test for pre-study differences between groups using Kruskal-Wallis test.
Table 4.2: Body and organ weights of EO771 breast tumour-bearing non-exercising vs exercising mice receiving anti-PD-1 or IgG2a treatment.

<table>
<thead>
<tr>
<th></th>
<th>EO771 No Ex IgG2a</th>
<th>EO771 No Ex aPD-1</th>
<th>EO771 Ex IgG2a</th>
<th>EO771 Ex aPD-1</th>
<th>p-value treatment effect</th>
<th>p-value exercise effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>17.2±0.65</td>
<td>17.7±1.61</td>
<td>16.9±0.82</td>
<td>17.0±0.78</td>
<td>0.323²</td>
<td></td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>18.0±0.38</td>
<td>17.9±0.78</td>
<td>17.6±0.71</td>
<td>18.1±0.25</td>
<td>0.277</td>
<td>0.588</td>
</tr>
<tr>
<td>Change in body weight (%)</td>
<td>4.73±3.79</td>
<td>1.75±7.06</td>
<td>4.58±5.86</td>
<td>6.77±4.76</td>
<td>0.821</td>
<td>0.175</td>
</tr>
<tr>
<td>Heart/body weight (mg/g)</td>
<td>6.09±0.55</td>
<td>6.17±0.34</td>
<td>6.64±0.31</td>
<td>6.44±0.40</td>
<td>0.670</td>
<td>0.0044**</td>
</tr>
<tr>
<td>Spleen/body weight (mg/g)</td>
<td>3.95±0.50</td>
<td>4.19±0.55</td>
<td>3.78±0.72</td>
<td>3.76±0.92</td>
<td>0.635</td>
<td>0.195</td>
</tr>
<tr>
<td>Liver/body weight (mg/g)</td>
<td>45.5±3.10</td>
<td>46.2±2.60</td>
<td>47.1±3.68</td>
<td>47.7±5.16</td>
<td>0.614</td>
<td>0.228</td>
</tr>
<tr>
<td>Kidney/body weight (mg/g)</td>
<td>14.2±0.55</td>
<td>14.3±0.47</td>
<td>13.9±1.10</td>
<td>13.7±1.02</td>
<td>0.790</td>
<td>0.157</td>
</tr>
</tbody>
</table>

¹ Values are means±SD. Data were analysed using two-way ANOVA. n=8-10.
² Test for pre-study differences between groups using one-way ANOVA.

4.4.2 Effect of Exercise and Anti-PD-1 Treatment on Tumour Growth and Tumour Cell Proliferation

There is a large proportion of patients who do not respond to checkpoint inhibitor treatment or who will develop resistance [136]. I aimed to determine whether post-implant exercise can boost responses (i.e. reduce the incidence of resistance, as outlined in Figure 4.2) to anti-PD-1 treatment in mice with B16-F10 melanoma or EO771 breast cancer.

Tumour growth and survival data were analysed as the time from treatment start to euthanasia to remove confounding variation in the initial phase of tumour growth (before
treatment begin). Tumour growth and survival data from implant until euthanasia can be viewed in Figure 9.8.

I found no differences in mouse survival (defined as time until maximum ethical tumour size and euthanasia), tumour growth rate, or the time from treatment begin to euthanasia in exercising compared with non-exercising mice treated with IgG2a or anti-PD-1, for either tumour type (Figure 4.5, Figure 4.6). Low numbers precluded analysis of exercise subsets (high vs low exercise groups).
Figure 4.5: Individual tumour growth curves for exercising or non-exercising mice receiving IgG2a or aPD-1 treatment.

B16-F10: n=10 per group; EO771: n=8-10 per group.
Figure 4.6: Exercise and anti-PD-1 treatment do not alter growth rate of B16-F10 or EO771 tumours. Survival curves for mice bearing B16-F10 (a) or EO771 (b) tumours (endpoint due to tumour size only). Time from treatment begin to euthanasia for mice bearing B16-F10 (c) or EO771 (d) tumours. B16-F10: n=8-10 per group; EO771: n=8-10 per group. Data are shown as individual data points and mean with 95% CI. Data analysed by two-way ANOVA. Survival analysis was performed using the Log rank (Mantel-Cox) test.

Similarly, exercise and anti-PD-1 treatment did not alter tumour cell proliferation in either tumour type, as measured by the percent pHH3+ cells in immunohistochemically stained sections (Figure 4.7).
Figure 4.7: Exercise and anti-PD-1 treatment do not alter the percentage proliferating cells in B16-F10 or EO771 tumours.

Quantification of the percentage of pHH3⁺ cells in B16-F10 (a) or EO771 (b) tumours from exercising vs non-exercising mice treated with IgG2a or anti-PD-1. B16-F10: n=10 per group, EO771: n=8-10 per group. Data are shown as individual data points and mean ± 95% CI. Data analysed by two-way ANOVA. Refer to Section 3.4.3 for examples of staining.

These data show that anti-PD-1 treatment was not effective in reducing tumour growth in either of these two mouse models compared to an IgG2a control treatment. It also showed that short-term, post-implant exercise did not improve treatment efficacy of anti-PD-1 in either B16-F10 melanoma or EO771 breast cancer.

### 4.4.3 Effect of Exercise and Anti-PD-1 on Tumour Hypoxia, Perfusion and Vascularity

Previous reports in orthotopic breast and subcutaneous colon tumours have shown that checkpoint inhibition can modify the tumour microenvironment in such a way as to improve perfusion and reduce hypoxia [132,219,220]. I aimed to determine whether exercise could act synergistically with anti-PD-1 treatment to further improve tumour blood flow, thereby reducing hypoxia.

There was no change in tumour hypoxia or perfusion in either B16-F10 or EO771 tumours from mice receiving anti-PD-1 treatment compared with those receiving the IgG2a isotype control, and this was not affected by exercise (Figure 4.8).
Figure 4.8: Exercise and anti-PD-1 treatment do not alter tumour hypoxia or perfusion in B16-F10 and EO771 tumours.

Quantification of hypoxic area in B16-F10 (a) and EO771 (c) tumours from non-exercising vs exercising mice receiving anti-PD-1 treatment or isotype control. Quantification of perfused blood vessels in B16-F10 (b) and EO771 (d) tumours from non-exercising vs exercising mice receiving anti-PD-1 treatment or isotype control. B16-F10 hypoxia: n=5-8 per group, B16-F10 perfusion: n=8-10 per group, EO771 hypoxia: n=5-9 per group, EO771 perfusion: n=5-9 per group. Due to Covid-19 restrictions, staining and imaging for hypoxia and perfusion were unable to be completed for all samples, leading to lower numbers for analysis. Data are presented as individual data points and mean ± 95% CI. Data analysed by two-way ANOVA. Refer to Section 3.4.3 for examples of staining.

Similarly, anti-PD-1 treatment and exercise did not change the number of CD31⁺ blood vessels in B16-F10 or EO771 tumours (Figure 4.9).
Figure 4.9: Exercise and anti-PD-1 treatment do not alter CD31⁺ vessel density in B16-F10 and EO771 tumours.

Quantification of CD31⁺ blood vessels in B16-F10 (a) and EO771 (b) tumours from non-exercising vs exercising mice receiving anti-PD-1 treatment or isotype control. B16-F10: n=10 per group, EO771: n=8-10 per group. Data are presented as individual data points and mean ± 95% CI. Data analysed by two-way ANOVA. Refer to Section 3.4.3 for examples of staining.

These results show that anti-PD-1 treatment with or without exercise did not improve tumour perfusion, hypoxia or vascularity in these models.

4.4.4 Effect of Exercise and Anti-PD-1 Treatment on Intratumoural Immune Cells

Disinhibition of TCR signalling through anti-PD-1 treatment may lead to increased recruitment of immune cells to the tumour, both T cells and other immune cell types such as NK cells. I hypothesised that T and NK cell recruitment may be further boosted by exercise.

4.4.4.1 Effect of exercise and anti-PD-1 treatment on intratumoural NK cells

I found no change in the number of infiltrating NK cells with exercise and/or anti-PD-1 treatment, in either tumour type (Figure 4.10).
Figure 4.10: Exercise and anti-PD-1 do not alter numbers of infiltrating NK cells in B16-F10 melanomas or EO771 breast tumours.

Quantification of the number of NKp46+ cells in B16-F10 (a) and EO771 (b) tumours from non-exercising vs exercising mice treated with IgG2a or anti-PD-1. B16-F10: n=10 per group; EO771: n=8-10 per group. Data are presented as individual data points and mean ± 95% CI. Data analysed by two-way ANOVA. Refer to Section 3.4.4.1 for examples of staining.

4.4.4.2 Effect of exercise and anti-PD-1 treatment on intratumoural T cell abundance

Given that image analysis using hotspots and random fields returned similar results in Chapter 3 (Section 3.4.4.2) I elected to use only images of hotspot areas for analysis of intratumoural T cells in this chapter.

I found that anti-PD-1 treatment significantly increased the number of CD8+ T cells in B16-F10 tumours (p=0.017, Figure 4.11c). Interestingly, exercise prevented this increase (interaction effect of exercise and anti-PD-1: p=0.047). These results were mirrored by the number of infiltrating CD3+ cells, but did not reach statistical significance (effect of anti-PD-1 treatment: p=0.17, interaction effect: p=0.13; Figure 4.11a). The number of Foxp3+ T cells was unchanged by exercise and anti-PD-1 treatment in B16-F10 tumours (Figure 4.11b).

In EO771 tumours, anti-PD-1 treatment did not affect numbers of infiltrating T cells, regardless of subtype (Figure 4.11d-f). There was a significant main effect of exercise to reduce numbers of CD3+ cells and Foxp3+ T cells (p=0.027, p=0.026; Figure 4.11d and e), but post-hoc analysis did not reveal significant differences between groups. This is likely due to low numbers. Numbers of CD8+ T cells were unchanged by exercise in EO771 tumours (Figure 4.11f).
Figure 4.11: Anti-PD-1 treatment increases numbers of tumour-infiltrating CD8+ cells in B16-F10 melanoma, and this is reduced by exercise.

Average number of CD3+ cells per field in B16-F10 (a) or EO771 (d) tumours from non-exercising (No Ex) compared with exercising (Ex) mice treated with anti-PD-1 (aPD-1) or isotype control (IgG2a). Average number of Foxp3+ cells in B16-F10 (b) or EO771 (e) tumours from non-exercising compared with exercising mice treated with anti-PD-1 or isotype control. Average number of CD8+ cells in B16-F10 (c) or EO771 (f) tumours from non-exercising compared with exercising mice treated with anti-PD-1 or isotype control. Cell densities were quantified by imaging 5 hotspot areas of T cell clusters and counting the total number of CD3+, Foxp3+ and CD8+ cells per field. *indicates statistically significantly different from No Ex, same treatment group. #indicates statistically significantly different from IgG2a, same exercise group. p<0.05*,#. Data are shown as individual data points and mean with 95% CI. Due to Covid-19 restrictions, staining and imaging for T cells was unable to be completed for all samples, leading to lower numbers for analysis. B16-F10: n=5-8 per group; EO771: n=5-9 per group. Data analysed by two-way ANOVA followed by Sidak’s multiple comparisons test. Refer to Section 3.4.4 for examples of staining.

I observed that there was a significant main effect of exercise to reduce the percentage of CD8+ T cells in B16-F10 tumours (p=0.031), but post-hoc analysis did not reveal any significant differences between groups (Figure 4.12b). As mentioned above, this is likely due to low numbers. In EO771 tumours, the combination of anti-PD-1 treatment and exercise significantly increased the percentage of intratumoural CD8+ T cells (p=0.039), while exercise in combination with IgG2a did not (Figure 4.12d). Neither exercise nor anti-PD-1 treatment altered the percentage of Foxp3+ T cells in either tumour type (Figure 4.12a and c).
The combination of exercise and anti-PD-1 treatment increases the percentage of CD8+ cells in EO771 but not B16-F10 tumours.

Percentage Foxp3+ T cells in B16-F10 (a) or EO771 (c) tumours from non-exercising (No Ex) compared with exercising (Ex) mice treated with anti-PD-1 (aPD-1) or isotype control (IgG2a). Percentage CD8+ cells in B16-F10 (b) or EO771 (d) tumours from non-exercising compared with exercising mice treated with anti-PD-1 or isotype control. p<0.05* (compared with non-exercise control in same treatment group). Data are shown as individual data points and mean with 95% CI. Due to Covid-19 restrictions, staining and imaging for T cells was unable to be completed for all samples, leading to lower numbers for analysis. B16-F10: n=5-8 per group; EO771: n=5-9 per group. Data analysed by two-way ANOVA followed by Sidak’s multiple comparisons test. Refer to Section 3.4.4 for examples of staining.

4.4.4.3 Effect of exercise and anti-PD-1 treatment on intratumoural T cell localisation

I investigated the effect of anti-PD-1 treatment and exercise on T cell densities in three discrete microenvironmental areas – in immediate proximity to perfused blood vessels, in normoxic intervascular space and in hypoxic regions. In accordance with the results obtained in Chapter 3 (Section 3.4.4.3), CD3+, CD3+Foxp3+ and CD3+CD8+ T cells preferentially clustered close to perfused blood vessels in both tumour types (Figure 4.13, Figure 4.14).

Neither anti-PD-1 treatment nor exercise significantly altered the localisation pattern of CD3+, CD3+Foxp3+ or CD3+CD8+ T cells in B16-F10 or EO771 tumours, although there was a trend for anti-PD-1 treatment to reduce Foxp3+ T cell density in hypoxia in EO771 tumours from non-exercising mice (p=0.087; Figure 4.13, Figure 4.14).
Figure 4.13: Anti-PD-1 treatment and exercise do not alter the tendency of T cells to cluster close to perfused blood vessels in B16-F10 tumours.

Density of CD3+ (a, d), CD3+Foxp3+ (b, e) and CD3+CD8+ (c, f) cells in different microenvironmental areas (in immediate proximity to perfused blood vessels, intervascular space or hypoxia) in B16-F10 tumours from non-exercising (No Ex) compared with exercising (Ex) mice treated with anti-PD-1 (aPD-1) or isotype control (IgG2a). Data are shown as individual data points and mean with 95% CI. Due to Covid-19 restrictions, staining and imaging for hypoxia, perfusion and T cells were unable to be completed for all samples, leading to lower numbers for analysis. n=5-8 per group. Data analysed by two-way ANOVA. Refer to Section 3.4.4 for examples of staining.
Figure 4.14: Anti-PD-1 treatment and exercise do not alter the tendency of T cells to cluster close to perfused blood vessels in EO771 tumours.

Density of CD3⁺ (a, d), CD3⁺Foxp3⁺ (b, e) and CD3⁺CD8⁺ (c, f) cells in different microenvironmental areas (in immediate proximity to perfused blood vessels, intervascular space or hypoxia) in EO771 tumours from non-exercising (No Ex) compared with exercising (Ex) mice treated with anti-PD-1 (aPD-1) or isotype control (IgG2a). Data are shown as individual data points and mean with 95% CI. Due to Covid-19 restrictions, staining and imaging for hypoxia, perfusion and T cells were unable to be completed for all samples, leading to lower numbers for analysis. n=5-9 per group. Data analysed by two-way ANOVA.

4.4.5 Effect of IgG2a and Anti-PD-1 on Intratumoural Immune Cells and Tumour Growth Compared with Untreated Mice

Previous work has shown that mice receiving control IgG (IgG2a) have slower tumour growth than NK-cell depleted mice receiving either anti-PD-1 or control IgG [212]. Although not acknowledged in the article itself, these data suggest that an anti-drug immune reaction may be occurring which could affect tumour growth. Because of this, I investigated whether mice receiving anti-PD-1 or IgG2a had altered levels of intratumoural immune cells and tumour growth rate compared with the untreated mice from Chapter 3.

The results in this section come from a retrospective analysis of two separate experiments and as such should be interpreted with caution.
4.4.5.1 Effect of IgG2a and anti-PD-1 on intratumoural NK cells

I found that B16-F10 tumours from mice receiving either IgG2a or anti-PD-1 contained significantly higher numbers of NK cells than those from untreated mice (p=0.0025 vs IgG2a, p=0.038 vs aPD-1; Figure 4.15a). In EO771 tumours, anti-PD-1 but not IgG2a significantly elevated the number of NK cells (p=0.0088; Figure 4.15b).

![Figure 4.15: Anti-PD-1 and IgG2a increase numbers of intratumoural NK cells.](image)

Quantification of the number of NKp46+ cells in B16-F10 (a) and EO771 (b) tumours from untreated non-exercising vs exercising mice, or mice treated with IgG2a or anti-PD-1. B16-F10: n=9-11 per group; EO771: n=8-10 per group. Data are presented as individual data points and mean ± 95% CI. Data analysed by Two-way ANOVA with Tukey’s post hoc test. No exercise effect was found, so data from exercising and non-exercising mice were pooled for post-hoc comparisons. p<0.05*, p<0.01**. Refer to Section 3.4.4.1 for examples of staining.

4.4.5.2 Effect of IgG2a and anti-PD-1 on intratumoural T cells

Neither IgG2a nor anti-PD-1 affected overall CD3+ T cell numbers in either B16-F10 or EO771 tumours (Figure 4.16a and d). However, there was a significant main effect of treatment to reduce Foxp3+ T cell numbers in B16-F10 but not EO771 tumours (p=0.035; Figure 4.16b and e). Post-hoc analysis to assess differences in Foxp3+ T cell numbers between individual groups was borderline significant for B16-F10 tumours (p=0.060 vs IgG2a, p=0.11 vs aPD-1).

Interestingly, IgG2a administration reduced CD8+ T cell numbers in both B16-F10 and EO771 tumours (B16-F10: p=0.023, EO771: p=0.0005; Figure 4.16c and f). In addition, CD8+ cell numbers were lower in anti-PD-1 treated EO771 tumours compared with untreated (p=0.0003; Figure 4.16f).
Figure 4.16: Anti-PD-1 and IgG2a reduce CD8+ T cell numbers in B16-F10 and EO771 tumours.

Average number of CD3+ cells per field in B16-F10 (a) or EO771 (d) tumours from untreated, non-exercising (No Ex) compared with exercising (Ex) mice, or mice treated with anti-PD-1 (aPD-1) or isotype control (IgG2a). Average number of Foxp3+ cells in B16-F10 (b) or EO771 (e) tumours from untreated non-exercising compared with exercising mice, or mice treated with anti-PD-1 or isotype control. Average number of CD8+ cells in B16-F10 (c) or EO771 (f) tumours from untreated, non-exercising compared with exercising mice, or mice treated with anti-PD-1 or isotype control. Cell densities were quantified by imaging 5 hotspot clusters and counting the total number of CD3+, Foxp3+ and CD8+ cells per field. p<0.01**, p<0.0001****. Data are shown as individual data points and mean with 95% CI. Due to Covid-19 restrictions, staining and imaging for T cells from IgG2a and anti-PD-1 treated tumours was unable to be completed for all samples, leading to lower numbers for analysis in these groups. B16-F10: n=5-12 per group; EO771: n=5-10 per group. Data analysed by two-way ANOVA followed by Tukey’s multiple comparisons test. Non-exercising mice were compared for post-hoc analysis. Refer to Section 3.4.4 for examples of staining.

Furthermore, anti-PD-1 and IgG2a altered the relative proportion of Foxp3+ and/or CD8+ T cells in B16-F10 and EO771 tumours. Specifically, B16-F10 tumours from mice receiving IgG2a or anti-PD-1 had a significantly lower percentage of Foxp3+ T cells compared with tumours from untreated mice (p=0.0039 vs IgG2a, p=0.0011 vs aPD-1; Figure 4.17a). This was not observed in EO771 tumours (Figure 4.17c).

I found that there was a significant main effect of treatment to reduce the percentage of CD8+ T cells in B16-F10 tumours (p=0.0065), for which the post-hoc analysis was borderline significant for non-exercising mice (p=0.069 vs IgG2a, p=0.077 for aPD-1; Figure 4.17b). In addition, IgG2a and anti-PD-1 decreased the percentage of CD8+ T cells in EO771 tumours compared with untreated mice (p<0.0001; Figure 4.17d).
Figure 4.17: IgG2a and anti-PD-1 reduce the percentage of CD8+ T cells in B16-F10 and EO771 tumours.

Percentage Foxp3+ T cells in B16-F10 (a) or EO771 (c) tumours from untreated, non-exercising (No Ex) compared with exercising (Ex) mice, or mice treated with anti-PD-1 (aPD-1) or isotype control (IgG2a). Percentage CD8+ cells in B16-F10 (b) or EO771 (d) tumours from untreated, non-exercising compared with exercising mice, or mice treated with anti-PD-1 or isotype control. p<0.01**, p<0.0001****. Data are shown as individual data points and mean with 95% CI. Due to Covid-19 restrictions, staining and imaging for T cells was unable to be completed for all samples treated with IgG2a or aPD-1, leading to lower numbers for analysis in these groups. B16-F10: n=5-12 per group; EO771: n=5-10 per group. Data analysed by two-way ANOVA followed by Tukey’s multiple comparisons test. If no exercise effect was found, data from exercising and non-exercising mice were pooled for post-hoc analysis (a). If an exercise effect was found, non-exercising mice were compared for post-hoc analysis (d). Refer to Section 3.4.4 for examples of staining.

4.4.5.3 Effect of IgG2a and anti-PD-1 on tumour growth rate

Given that IgG2a and anti-PD-1 administration both altered numbers of NK cells and T cell subsets, I next investigated whether either antibody altered tumour growth rate.

I found that mouse survival was significantly increased by anti-PD-1 and IgG2a administration for mice bearing B16-F10 but not EO771 tumours (p=0.002; Figure 4.18a and b). Similarly, IgG2a and anti-PD-1 increased the time to euthanasia from treatment begin (or tumour size=50-100 mm3 for untreated mice), for mice with B16-F10 but not EO771 tumours (Figure 4.18c and d).
Figure 4.18: IgG2a and anti-PD-1 slow growth rate of B16-F10 but not EO771 tumours.
Survival curves for untreated, non-exercising compared with exercising mice, or mice treated with IgG2a or anti-PD-1, bearing B16-F10 (a) or EO771 (b) tumours (endpoint due to tumour size only). Data analysed by log-rank (Mantel-Cox) test. Time from treatment begin (or 50-100 mm³ for untreated groups) for mice bearing B16-F10 (c) or EO771 (d) tumours. B16-F10: n=8-11 per group; EO771: n=7-10 per group. Data are shown as individual data points and mean with 95% CI. Data analysed by two-way ANOVA with Tukey’s multiple comparisons test. As no exercise effect was seen, data from exercising and non-exercising mice were pooled for post-hoc analysis.

4.5 Discussion

Experiments in this chapter have shown that while the combination of exercise and anti-PD-1 treatment did not affect the growth rate of B16-F10 or EO771 tumours, it did significantly increase the proportion of intratumoural CD8⁺ T cells in EO771 tumours. However, in B16-F10 tumours, exercise prevented the anti-PD-1-induced influx of CD8⁺ T cells. The localisation pattern of T cells in relation to perfused areas, intervascular space and hypoxia was unchanged by exercise and anti-PD-1 treatment. In addition, exercise and anti-PD-1 treatment did not alter intratumoural NK cell numbers, tumour hypoxia, perfusion or the number of CD31⁺ blood vessels. However, both IgG2a and anti-PD-1 administration induced significant alterations in the composition of the
immune tumour microenvironment, in both tumour types, resulting in a tumour growth delay in B16-F10 melanomas compared with untreated tumours.

**Effect of anti-PD-1 on tumour growth:** Consistent with previous reports, neither B16-F10 nor EO771 tumours responded to anti-PD-1 monotherapy in this study (Figure 4.6) [223,228]. A range of resistance mechanisms have been proposed for non-response to immunotherapy. These include, but are not limited to, low tumour mutational burden, high expression of alternative inhibitory ligands and dysregulated IFNγ signalling [229]. In B16-F1 tumours (a less metastatic variant of B16-F10), exposure of the tumour cells to a mutagen prior to implant significantly slowed tumour growth in immunocompetent but not immune-deficient mice, suggesting that this effect was immune-mediated [230]. Therefore, it is possible that low tumour mutational burden may be one factor contributing to the lack of response to anti-PD-1 seen in this study.

**Effect of exercise and anti-PD-1 on tumour growth:** This was the first study to investigate the effect of post-implant exercise on anti-PD-1 treatment efficacy in immune-competent mice. However, two other studies have investigated the combination of exercise with anti-PD-1 in alternative settings. Asuncion-Ruiz et al. showed that exercise did not significantly alter the efficacy of nivolumab treatment in NOD-SCIDγ mice with non-small cell lung cancer PDXs [139]. However, the obvious limitation of that study was the lack of lymphoid cells in NOD-SCIDγ mice, thus removing the primary target of nivolumab treatment and making this a suboptimal model to investigate the combined effect of exercise and anti-PD-1 treatment.

Interestingly, Wennerberg et al. found that post-implant exercise improved the efficacy of anti-PD-1 and radiation combination therapy in mice with 4T1 breast cancer to a small but statistically significant degree [137]. This was accompanied by a significant increase in the proportion of splenic NK cells and a decrease in the proportion of intratumoural MDSCs, suggesting that exercise was able to enhance therapeutic efficacy by further boosting the anti-tumour immune response. Radiation has a known bystander effect in which tumour cell death induces an immune response (known as immunogenic cell death), which can result in tumour cell killing even in areas that have not been irradiated [231]. Therefore, the addition of radiation to the treatment regimen in the study by Wennerberg et al. likely induced immunogenic cell death leading to enhanced anti-tumour responses. In support of this, Dufresne et al. showed that exercise enhanced the
Effect of radiation therapy in athymic nude mice with human prostate cancer [232]. Although the tumour growth rate with exercise and radiation was only marginally slower than that with radiation alone, intratumoural gene expression of NK cell markers was significantly higher with the combination than with either treatment alone [232]. Given that tumours were only grown to a relatively small size (approx. 400 mm³), a larger effect of exercise and radiation may have been seen if the study had continued for longer. These results suggest that immunogenic cell death may be required as an initial stimulus upon which exercise can act to further potentiate immune responses.

Effect of immune checkpoint inhibition on tumour hypoxia and perfusion: Previous work has shown that immune checkpoint blockade can increase perfusion in orthotopic EO771 breast tumours [132,219,220] and reduce hypoxia [132,219]. Similarly, anti-PD-1 treatment reduced hypoxia and increased perfusion in MCA38 colon tumours [219]. I found no difference in tumour perfusion or hypoxia in B16-F10 or EO771 tumours from mice treated with anti-PD-1 compared with IgG2a (Figure 4.8), but this is not surprising given that I also found no difference in tumour growth rate with anti-PD-1 treatment. Zheng et al. showed that increased perfusion following treatment predicted response of EO771 tumours to anti-CTLA-4, showing that vascular normalisation is indicative of an effective response to checkpoint inhibitor therapy [219].

Zheng et al. showed differences in tumour hypoxia and perfusion with anti-PD-1 or anti-CTLA-4 treatment using the same method as I have (immunofluorescent detection of pimonidazole and Hoechst 33342, respectively), indicating that this method is robust enough to detect differences in these parameters [219].

Effect of exercise and anti-PD-1 on immune cell infiltration: Anti-PD-1 therapy significantly increased CD8⁺ T cell numbers in B16-F10 tumours. However, rather than further increasing this infiltration of CD8⁺ T cells as I had hypothesised, exercise completely prevented it (Figure 4.11). It is unclear why exercise prevented the anti-PD-1-induced influx (or expansion) of CD8⁺ T cells in this model. It would be of interest to investigate the effector function and/or activation status of these cells in tumours from exercising mice, as it is possible that they exhibit increased activation markers and/or decreased exhaustion markers despite showing no increase in numbers.

In EO771 tumours, however, exercise significantly increased the proportion of intratumoural CD8⁺ T cells in conjunction with anti-PD-1 treatment (although absolute
numbers remained unchanged, Figure 4.12, Figure 4.11). Similarly, Wennerberg et al. showed that exercise tended to increase the proportion of CD8+ T cells in the spleens of mice with 4T1 breast tumours that had been treated with anti-PD-1 and radiation [137]. This suggests that exercise can modulate both the local and the systemic immune landscape in mice with breast cancer receiving immunotherapy.

Given that exercise has been shown to potentiate the effect of anti-cancer therapy even in the absence of an exercise-only or therapy-only effect [30], I hypothesised that exercise would be sufficient to boost the effect of anti-PD-1 and generate anti-tumour immunity sufficient to slow the growth of the tumour. This was not the case. However, some promising alterations in tumour-infiltrating T cells were observed (as described above) suggesting that although a significant growth inhibitory effect did not occur, exercise was able to modify the effect of anti-PD-1 on the tumour microenvironment. Future studies could investigate whether exercise can enhance the response rate to immune checkpoint inhibitors by the use of a model which typically exhibits some responders and some non-responders, such as the use of anti-CTLA-4 in EO771 tumours [219,220].

Effect of IgG2a on anti-tumour immunity: It has been reported that tumours grown in mice receiving the same isotype control used in this study grow more slowly than NK-cell depleted tumours treated with either anti-PD-1 or IgG2a [212]. This suggests that there may be an anti-drug immune response occurring independently of the targeted action of anti-PD-1. In support of this, I observed striking differences in NK cell and T cell subtype frequencies in both B16-F10 and EO771 tumours treated with IgG2a compared with untreated tumours (Figure 4.15, Figure 4.16), and tumour growth was slowed compared with untreated mice, in mice with B16-F10 (but not EO771) tumours, receiving IgG2a or anti-PD-1 (Figure 4.18).

Inoculation with antibodies from foreign species can induce an immune response against these antibodies. In order to minimise this response, approved immune checkpoint inhibitors have been developed as a fully humanised monoclonal antibodies [233]. However, the generation of antibodies against these monoclonals does still occur in humans, and these can induce immune complex formation and subsequent activation of myeloid cells via Fc receptor engagement [234]. Even small amounts of immune complexes can trigger a range of immune responses [235], which may provide a modulatory effect on anti-tumour immune responses. Because the antibodies used in my
study are derived from rat (and not ‘murinised’) and being administered repeatedly to mice, it is highly likely that anti-rat antibodies and therefore immune complexes are being induced even in the mice treated with the isotype control. This may result in altered immune responses and may explain why IgG2a administration altered numbers of tumour-infiltrating NK and T cells compared with untreated mice. This is integral to the interpretation of results, as it emphasises that the isotype control antibody is not inert and can have unintended effects.

Anti-drug immunity would likely be mediated by a T<sub>H</sub>2 response in order to support antibody production by plasma cells, thereby shifting away from a T<sub>H</sub>1 response which supports CD8<sup>+</sup> T cell function [73]. This could explain the drop in CD8<sup>+</sup> T cell numbers and proportion seen with IgG2a administration in both B16-F10 and EO771 tumours. The altered proportion of CD8<sup>+</sup> T cells suggests that numbers of one or more T cell subtypes not investigated in this study were increasing. If the hypothesis that the anti-IgG2a immune response is T<sub>H</sub>2-mediated is correct, it is likely that these were T<sub>H</sub>2 cells. Future work could investigate this by additionally staining for CD4 and GATA-3 (the signature transcription factor of T<sub>H</sub>2 cells).

The role of T<sub>H</sub>2 cells in anti-tumour immunity is not well-defined. They have been reported to have both pro- and anti-tumour functions and as such, it is unclear what effect a T<sub>H</sub>2-mediated systemic anti-IgG2a response would have on tumour growth [236].

Body and organ weights: Unexpectedly, I found an increase in the liver to body weight ratio in mice with B16-F10 tumours in this study (Table 4.1). Although this increase was statistically significant, the values were within the normal range of 3-5% of mouse body weight [237]. This suggests that the observed increase in liver weight was unlikely to be pathological.

Similarly, although anti-PD-1 treatment significantly attenuated the weight gain in these young mice with B16-F10 tumours, this is unlikely to have been detrimental as a net weight gain was still observed in the treated groups. Overall, this suggests that anti-PD-1 treatment was well-tolerated at the doses and frequencies used in this study.

Limitations of this study: A major limitation of this study was the low numbers available for analysis of immunofluorescent staining for hypoxia, perfusion, CD3, CD8 and Foxp3. Due to the global Covid-19 outbreak, I was unable to complete the staining and imaging on all sections as this would have required travel to Auckland. This resulted in numbers
as low as 5 in some groups – half the intended number. Therefore, it is possible that some changes in hypoxia, perfusion or T cell parameters were missed due to insufficient statistical power. I aim to finish staining and imaging the remaining samples for publication.

As mentioned above, neither EO771 nor B16-F10 tumours typically exhibit a strong response to anti-PD-1 treatment [223,228]. It may be that for exercise to boost the effect of immunotherapy, an initial, successful, immune stimulus is required. This could occur through the use of an agent to induce immunogenic cell death, such as radiation, or an alternate immunotherapeutic to which the tumour responds better, such as anti-CTLA-4 for B16-F10 or EO771 tumours.

**Conclusion:** Although the combination of exercise and anti-PD-1 did not result in a significant reduction in tumour growth rate, modulation of the immune microenvironment within the tumour occurred which differed from that seen with exercise or anti-PD-1 alone. Together with previous work indicating that exercise can enhance tumour growth inhibition and modulate peripheral and intratumoural immune phenotypes in mice treated with a combination of anti-PD-1 and radiation [137], this suggests that exercise may be able to modify anti-tumour immune responses when provided with an initial, immunogenic stimulus.
5 The Effect of Cancer and Immunotherapy on Skeletal Muscle Mitochondrial Adaptations to Exercise

5.1 Introduction

Cancer cachexia is a syndrome characterised by involuntary weight loss, fatigue, anorexia and muscle wasting. It is associated with a loss of physical functioning, poorer quality of life and a worse prognosis, contributing to approximately 20% of cancer deaths [238]. It occurs in 40-80% of cancer patients, depending on cancer type [144]. Impairments in muscle function begin before any significant weight loss, at least in animal models [239], highlighting the importance of treating the condition at an early stage (before noticeable weight loss).

Approaches to managing cancer cachexia include nutritional control and pharmacological interventions (such as appetite stimulants or anti-inflammatory medication), although there is no current standard of care [240]. However, these approaches are not sufficient to reverse the condition. Given that exercise can improve muscle mass and function, it is unsurprising that an increasing number of studies are investigating whether exercise can alleviate cachexia, including as part of a multimodal (exercise, nutrition and anti-inflammatory medication) intervention in a phase 3 trial [241]. To date, studies have been almost exclusively in animals, and show that, while exercise can indeed alleviate symptoms of cachexia on a systemic and molecular level, it is usually insufficient to return the muscle to a normal state [147–149,152,242]. Some preclinical studies have shown that exercise beginning prior to the onset of cachexia can completely prevent weight loss (or a lack of weight gain in young animals) [153,154]. However, impairments in some functional measures (grip strength) and mitochondrial markers were still seen, and these would likely eventually result in progression of cachexia [153]. In one model (C26 colon carcinoma), exercise beginning prior to the onset of cachexia attenuated but did not prevent the loss of body weight [151]. These results suggest that exercise may delay rather than prevent the onset of cachexia, at least in models where the cancer is not treated and the tumour can progress unchecked.
These studies are primarily focussed on whether exercise can return muscle function to control levels, rather than on differences in exercise adaptations compared with healthy groups. Given the profound systemic effect of cancer, it would be unwise to assume that exercise induces muscular adaptations with the same efficiency in people with cancer as in healthy individuals. Understanding how tumour burden changes exercise adaptations is key to optimising exercise interventions to treat cachexia and pre-cachectic muscle alterations.

Exercise adaptations in healthy skeletal muscle are well-characterised and very similar between mice and humans. Resistance and endurance exercise induce distinct alterations; I will here focus on those induced by endurance exercise, as voluntary wheel running induces endurance-like adaptations [243]. Endurance exercise increases the level of intracellular free calcium and the AMP:ATP ratio, which stimulate the activity of calcium-sensitive signalling molecules and AMP kinase (AMPK), respectively [146]. In turn, this leads to the transcription and increased expression of Pgc-1α [146]. PGC-1α is a transcription factor and the master regulator of endurance training adaptations – it induces the expression of genes related to fatty acid oxidation, mitochondrial respiration and mitochondrial biogenesis [146]. As a result, mitochondrial content within the cell increases, which is reflected by increased protein levels and activity of the oxidative phosphorylation complexes, and increased activity of metabolic enzymes such as citrate synthase.

Importantly, muscle regeneration following damage (including ultrastructural damage caused by exercise [244]) is highly dependent on a tightly controlled sequence of events orchestrated by the immune system [245,246]. Initially, localised inflammation occurs, characterised by enhanced infiltration of neutrophils and M1-type macrophages, which phagocytose cell debris from damaged muscle cells, and release proteolytic enzymes to break down damaged muscle tissue [244,245]. In addition, CD8+ T cells are early-responders to muscle injury and release CCL2, which attracts neutrophils and monocytes [245,246]. Following this initial inflammatory phase, a shift to a more regulatory, wound-healing immune response occurs [246]. This is characterised by high numbers of M2-type macrophages and T_{reg} cells, which peak at 4-7 days post-injury [245,246]. Deregulation of this process can affect the health of the muscle – for example, chronic inflammation can lead to muscle damage [245].
Given this important role of the immune system in muscle recovery, it is likely that immune cells also play a key role in muscle adaptation to exercise, likely driven by exercise-induced damage driving similar processes as described in the previous paragraph [244]. Current knowledge is largely extrapolated from more severe models of muscle damage such as cardiotoxin injection, and few studies have examined the relationship between the local muscle immune response and exercise adaptation directly [244]. However, histological evidence of leukocyte accumulation in human muscle following exercise has been reported [247]. In addition, exercise induced the gene expression of macrophage-associated chemokines and cytokines in muscle following exercise [248], and anti-inflammatory medication may modulate muscle adaptation to exercise [249]. This suggests that exercise effects immune alterations in skeletal muscle which may be important for muscle adaptation to exercise. Importantly, wheel running has been shown to induce a degree of muscle damage [250]; thus, an immune response likely occurs post-wheel running exercise. It is unclear whether the immune system is also important for muscle adaptation to non-damaging exercise.

Due to the role of the immune system in muscle regeneration and adaptation to exercise, it is likely that immunotherapies such as anti-PD-1 will affect muscle tissue. Myopathies are a possible side effect of checkpoint inhibitors [251], but it has also been reported that checkpoint inhibition may prevent the onset of cachexia in some patients, as evidenced by a higher proportion of patients than expected showing no change or an increase in skeletal muscle index (limb skeletal muscle mass divided by the square of the height) following anti-PD-1 treatment [252]. Therefore, the effect of anti-PD-1 on muscle tissue and muscle response to exercise is unclear.

As indicated by their nickname, the powerhouse of the cell, the key function of mitochondria is ATP production. This occurs through the process of oxidative phosphorylation (oxphos) at the inner mitochondrial membrane (Figure 5.1). Complex I and II oxidise NADH and FADH₂, respectively, thereby passing electrons to ubiquinone, which shuttles these on to complex III [253]. Complex III passes electrons to cytochrome c, which is oxidised by complex IV, utilising molecular oxygen and producing water. Complexes I, III and IV are proton pumps, and this process of electron transport generates a proton gradient across the inner mitochondrial membrane. This drives the activity of ATP synthase (complex V), which phosphorylates ADP to generate ATP [253]. The citric acid cycle feeds into this process by generating NADH and FADH₂ [253]. Therefore,
expression and activity of the OXPHOS complexes (and citrate synthase, which is involved in the citric acid cycle) can be used as markers of mitochondrial content and function.

**Figure 5.1: Oxidative phosphorylation in mitochondria.**
The electron transport chain (complexes I-IV) generates a proton gradient to drive the activity of ATP synthase (complex V). Ubiquinone (Q) receives electrons from the oxidation of NADH and FADH$_2$ (provided by the citric acid cycle) by complex I and II, respectively, which it passes on to complex III. Complex III transfers these to cytochrome c, which is then oxidised by complex IV by the use of molecular oxygen. COX-IV is subunit 4 of complex IV. The citric acid cycle feeds NADH and FADH$_2$ into the electron transport chain. Citrate synthase is the initial and rate-limiting step of the citric acid cycle. Figure modified from “Oxidative phosphorylation: Figure 3,” by Openstax College, Biology (reused under the creative commons licence CC BY 4.0).

In this chapter, I investigated changes in muscular expression of the five OXPHOS complexes, cytochrome c, cytochrome c oxidase (aka complex IV) subunit IV (COX-IV), and citrate synthase activity following exercise training of tumour-free and tumour-bearing (B16-F10 and EO771) mice. Subsequently, I investigated changes in the expression of the above markers in the muscle of tumour-bearing mice receiving anti-PD-1 or isotype control (IgG2a) treatment. I hypothesised that tumour burden would impair exercise adaptation of skeletal muscle mitochondria.
5.2 Chapter Aim and Experimental Approach

**Hypothesis:** That tumour burden would impair skeletal muscle mitochondrial adaptations to exercise.

The aim for this chapter was to investigate how cancer and anti-PD-1 immunotherapy affect physiological adaptations of skeletal muscle mitochondria to exercise. I hypothesised that mitochondrial adaptations to exercise would be dysregulated and/or impaired in tumour-bearing mice. It is important to emphasise that the tumour models used in this study do not induce high rates of ‘clinical’ cachexia (>5% body weight loss) at the tumour sizes used here; thus, I have investigated the effects of exercise on *precachectic* perturbations in muscle mitochondria. This was addressed using the following specific objectives:

1. Investigate the effect of tumour burden on skeletal muscle mitochondrial adaptation to exercise by:

   a. Using Western blotting, measure protein levels of cytochrome c oxidase subunit 4 (COX-IV), cytochrome c and the five oxidative phosphorylation (oxphos) complexes of the electron transport chain in the quadriceps femoris muscles of non-exercising compared with exercising, tumour-bearing vs tumour-free mice.

   b. Using a commercially available kit, measure activity of citrate synthase in the quadriceps femoris muscles of non-exercising compared with exercising, tumour-bearing vs tumour-free mice.

   c. Comparing mitochondrial protein levels and enzyme activities between two different tumour models (B16-F10 melanoma and EO771 orthotopic breast cancer).

2. Investigate the effect of immunotherapy on skeletal muscle mitochondrial adaptation to exercise by:

   a. Measuring mitochondrial markers, as for Specific Objective 1a, in exercising/non-exercising tumour-bearing mice receiving anti-PD-1 treatment or isotype control.
b. Measuring mitochondrial marker activity, as for Specific Objective 1b, in exercising/non-exercising tumour-bearing mice receiving anti-PD-1 treatment or isotype control.

5.2.1 Study Design

Muscle from tumour-bearing mice was obtained from mice used in the experiments in Chapters 3 and 4. In addition, muscle was obtained from a cohort of tumour-free mice of the same age, similarly housed in pairs in cages with cage dividers, with or without access to a running wheel (Figure 5.2), as for the experiments with tumour-bearing mice (Section 3.2 and 4.2). Tumour-free mice were euthanised after 19 days, which was the median time taken for tumours to reach maximum size.

Western blots were carefully optimised to enable semi-quantitative assessments of relative protein levels. The optimal protein loading amount to avoid saturation of the signal and to maintain a constant ratio to the loading control was determined by titrating protein amounts and densitometry (see Appendix, Figure 9.10).

Figure 5.2: Experimental design to investigate the effect of tumour burden on exercise adaptability in mice.

Female mice aged 6-10 weeks were subcutaneously inoculated with B16-F10 melanoma cells or orthotopically inoculated with EO771 breast cancer cells, or served as tumour-free controls. Mice were then randomised to either no exercise control or voluntary exercise (with access to a modified running wheel with revolution counter). Mice were housed at two per cage with a cage divider to enable accurate quantification of running distance. Mice were euthanised when tumours reached 1000 mm³ (melanoma) or 600 mm³ (breast cancer), or 19 days post-experiment begin (median time for tumour-bearing mice to reach endpoint), after which point muscle samples were taken from the left quadriceps femoris muscles and analysed for expression and activity of mitochondrial proteins/enzymes. In addition, hearts were removed and weighed.
5.3 Acknowledgements

I would like to acknowledge Dr Troy Merry (Faculty of Medical and Health Sciences, University of Auckland) for his technical input (clarification of questions regarding lysis buffers and which proteins to analyse) and assistance with the data analysis in this chapter (discussion of data in section 5.4.2).

5.4 Results

Cancer can have profound systemic effects on the body, including muscle wasting and dysfunction of skeletal muscle mitochondria [6]. While it has been shown that exercise can attenuate cancer-associated loss of mitochondrial content [153], it is unclear whether tumour burden affects exercise responses of skeletal muscle mitochondria.

5.4.1 Effect of Tumour Burden on Running Distance, Heart Weight and Body Weight

The running distance, heart and body weight data from tumour-bearing mice in this section has already been presented in Chapter 3, but is compared to data from tumour-free mice in this chapter.

Running distance was highly variable in both tumour-free and tumour-bearing mice (Figure 5.3). Despite this, average daily running distance was similar across all groups (tumour-free = 7.91 ± 2.85 km/day, B16-F10 = 8.43 ± 3.17 km/day, EO771 = 7.81 ± 1.41 km/day; Figure 5.3).

Figure 5.3: Tumour burden does not alter mouse running behaviour.
Average daily running distance for tumour-free mice (a) or mice bearing B16-F10 (b) or EO771 (c) tumours. Data are presented as mean ± SD. Tumour-Free: n=3-6 per data point (total number of mice=9, but running distance was not able to be calculated each day for every mouse due to the availability of wheels fitted with a revolution counter). B16-F10 and EO771 n=3-12 per data point (numbers reduce as mice are progressively euthanised).
I found that the change in body weight at endpoint was significantly reduced in tumour-bearing mice compared with tumour-free mice (B16-F10: p=0.012, EO771: p=0.0025; Figure 5.4a). This was due to most mice remaining weight stable or losing weight, whereas non-exercising, tumour-free mice all gained weight. Exercise attenuated this weight gain, although this was not significant. It should be noted that only 1-3 mice in each tumour-bearing group met the cut-off for cachexia (>5% body weight loss; B16-F10 No Ex: n=2, B16-F10 Ex: n=3, EO771 No Ex: n=1, EO771 Ex: n=2), indicating that the majority of mice were pre-cachectic.

Exercise significantly increased the heart/body weight ratio in mice with EO771 tumours, but not tumour-free mice or mice with B16-F10 tumours (p=0.0030, Figure 5.4b). This led to a significantly higher heart/body weight ratio in exercising mice with EO771 tumours compared with exercising tumour-free mice (p=0.0090, Figure 5.4b).

Figure 5.4: Tumour burden results in weight loss or attenuation of weight gain.
(a) Percent body weight change at endpoint in non-exercising or exercising tumour-free mice, mice with B16-F10 melanoma or mice with EO771 breast cancer, corrected for tumour weight. (b) Heart to body weight ratio in non-exercising or exercising tumour-free mice, mice with B16-F10 melanoma or mice with EO771 breast cancer. Data shown as individual data points and mean with 95% CI. Data analysed by two-way ANOVA with Sidak’s or Tukey’s multiple comparisons post-test. Exercise effect: * indicates significantly different from No Ex, same tumour group. Tumour effect: # indicates significantly different from Tumour-Free, same exercise group. p<0.05#; p<0.01**,##. Tumour-Free: n=9-10; B16-F10: n=12; EO771: n=11-12.

5.4.2 Effect of Tumour Burden on Exercise Adaptations of Skeletal Muscle Mitochondria

Western blotting was used to investigate protein levels of components of the electron transport chain in muscle tissue from tumour-free compared with tumour-bearing mice.
Figure 5.5: Tumour burden alters adaptation of skeletal muscle mitochondria to exercise.
Representative Western blots of quadriceps femoris muscle homogenates from exercising or non-exercising tumour-free mice, mice with B16-F10 melanoma or mice with EO771 breast cancer. Blots were probed for either OXPHOS complexes I-V (CI-V), cytochrome c and GAPDH, or for COX-IV and GAPDH. All samples were run on duplicate gels and the points shown are means of the duplicates. Samples were normalised to a positive control (run on all blots, see Section 2.2.6.1 for details) and GAPDH. Densitometric quantification of blots in (a). Exercise effect: * indicates statistically significantly different from No Ex (same tumour group). Tumour effect: # indicates statistically significantly different from tumour-free (same exercise group). Data analysed by two-way ANOVA with Sidak’s or Tukey’s multiple comparison’s post-test. Data shown as individual data points and mean with 95% CI. Tumour-Free: n= 9 per group, B16-F10 and EO771: n=11-12 per group.

I found that mice with B16-F10 melanoma, but not EO771 breast cancer, had elevated levels of cytochrome c (p=0.0052) and complex I (p=0.005) compared with tumour-free mice (Figure 5.5a and b). Conversely, the expression of complex IV was significantly reduced by tumour burden in mice with either tumour type (B16-F10: p=0.0078, EO771: p=0.048, Figure 5.5a and b). No other significant differences in expression were found between tumour groups (Figure 5.5a and b). Similarly, tumour burden did not significantly alter citrate synthase enzyme activity (Figure 5.5c).

In order to better visualise the effect of exercise on OXPHOS protein expression and enzyme activity, I normalised values to the respective no exercise control for each tumour group (Figure 5.6). However, statistical analysis was only performed on the raw data.

Cytochrome c expression was non-significantly increased by exercise in muscle from tumour-free mice (p=0.26) and in muscle from mice with B16-F10 melanoma (p=0.0027), but not mice with EO771 tumours (Figure 5.5a and b, Figure 5.6a). Similarly, expression of complex III was non-significantly increased by exercise in tumour-free mice (p=0.090) and significantly increased in mice with B16-F10 tumours (p=0.0068), but not mice with EO771 tumours (Figure 5.5a and b, Figure 5.6a).

The expression of complexes II (p=0.039) and V (p=0.023) was increased by exercise in mice with B16-F10 tumours, but not in tumour-free mice or mice with EO771 tumours.

I observed that while exercise induced a significant increase in complex IV expression in all groups (Tumour-Free: p<0.0001; B16-F10: p=0.046; EO771: p=0.015), the magnitude of this increase in the tumour-bearing groups was only half that seen in tumour free mice (tumour-free: 0.53, B16-F10: 0.25, EO771: 0.24; Figure 5.5a and b, Figure 5.6a).
Finally, I found that citrate synthase activity was increased by exercise in muscle from mice with EO771 tumours, but not in tumour-free mice or mice with B16-F10 tumours (p=0.0044, p=0.056; Figure 5.5d, Figure 5.6c).

**Figure 5.6: Tumour burden alters exercise adaptations of skeletal muscle mitochondria.**

Data from Figure 5.5 was normalised to no exercise group for each tumour group to better visualise the effects of exercise. (a) Fold change of the expression of OXPHOS proteins from no exercise control in muscle from tumour-free mice, mice with B16-F10 melanoma or EO771 breast cancer. (b) Fold change in the activity of citrate synthase from no exercise control in quadriceps muscle homogenates from tumour-free mice, mice with B16-F10 melanoma or mice with EO771 breast cancer. Raw data analysed for statistical significance in Figure 5.5. Data shown as mean ± SD. Tumour-Free: n= 9 per group, B16-F10 and EO771: n=11-12 per group.

Taken together, these data indicate that tumour burden alters mitochondrial adaptation to exercise in skeletal muscle, and these effects are different between tumour types.
5.4.3 Correlations between Mitochondrial Markers

In order to determine whether average daily running distance is associated with mitochondrial markers in skeletal muscle (which are closely associated with exercise capacity [12]), I performed correlations between the expression/activity of mitochondrial proteins/enzymes and running distance. I found that average daily running distance was not correlated with any marker, except for a weak correlation with complex IV expression in mice with B16-F10 tumours (Figure 5.7).
Figure 5.7: Expression and activity of mitochondrial proteins/enzymes does not correlate with average daily running distance in tumour-free mice, mice with B16-F10 melanoma or mice with EO771 breast cancer.

Data analysed by Pearson correlation. Data show best fit line with 95% CI. Tumour-Free: n=9; B16-F10: n=12; EO771: n=11.
I next decided to investigate whether complex IV and COX-IV (a subunit of complex IV) expression are associated, as this could provide insight into the assembly and possible functionality of the complex.

Complex IV and COX-IV expression were significantly correlated in muscle from tumour-free mice (r=0.67, p=0.0023; Figure 5.8a and b). In contrast, no significant correlation between these variables was observed in tumour-bearing mice. This may indicate a dysregulation in complex assembly, resulting in impaired functionality of complex IV.

In light of these results, I decided to investigate further by performing correlations between complex IV, COX-IV and cytochrome c, the substrate for complex IV.

In the muscle of tumour free mice, cytochrome c was strongly correlated with complex IV and COX-IV protein expression (r=0.85, p<0.0001 and r=0.87, p<0.0001, respectively; Figure 5.9). In tumour-bearing mice, the correlation between cytochrome c and complex IV expression (although significant) was weaker (B16-F10: r=0.62, p=0.0017; EO771: r=0.49, p=0.019; Figure 5.9a). The correlation between cytochrome c and COX-IV was attenuated in mice with B16-F10 tumours (r=0.54, p=0.0078; Figure 5.9b) and absent in mice with EO771 tumours.
Figure 5.9: Correlations between cytochrome c and complex IV markers are disrupted by tumour burden.

Data analysed by Pearson correlation. Closed circles represent non-exercising mice while open circles represent exercising mice. Data show best fit line with 95% CI. Tumour-Free: n=18; B16-F10: n=24; EO771: n=23.

Taken together, these data indicate that tumour burden may dysregulate complex assembly, which may impact function, in both exercised and non-exercised mice (although this requires validation, as the data is correlative).

5.4.4 Effect of Anti-PD-1 Treatment on Exercise Adaptations of Skeletal Muscle Mitochondria

Local inflammation and the subsequent resolution thereof is an essential stimulus for muscular adaptation to exercise [245]. It is currently unknown whether anti-PD-1 treatment affects exercise adaptations in skeletal muscle. Thus, I investigated how the combination of exercise and anti-PD-1 treatment affected markers of mitochondrial content in the quadriceps muscles of mice with B16-F10 or EO771 tumours.

In mice with B16-F10 tumours, I found that the combination of exercise and anti-PD-1 significantly increased the expression of all five OXPHOS complexes, as well as COX-IV (CI: p=0.0041, CII: p=0.016, CIII: p=0.012, CIV: p=0.030, CV: p=0.015, COX-IV: p=0.0078; Figure 5.10a). In addition, exercise and anti-PD-1 tended to increase cytochrome c expression (p=0.073, Figure 5.10a). No effects of exercise on OXPHOS complex or cytochrome c expression were seen in the mice receiving IgG2a (Figure 5.10a). Intriguingly, anti-PD-1 treatment alone tended to reduce expression of
cytochrome c and COX-IV, leading to a borderline significant interaction effect between exercise and anti-PD-1 (p=0.056 and p=0.075, respectively; Figure 5.10a).

Figure 5.10: Exercise normalises anti-PD-1-induced suppression of skeletal muscle mitochondria. (a) Relative protein expression of cytochrome c, OXPHOS complexes I-V and COX-IV in quadriceps muscle homogenates from exercising (Ex) or non-exercising (No Ex) B16-F10 melanoma-bearing mice treated with anti-PD-1 (aPD-1) or an isotype control antibody (IgG2a). Samples were normalised to a positive control (run on all blots) and GAPDH. (b) Activity of citrate synthase in quadriceps muscle homogenates from exercising or non-exercising mice with B16-F10 melanoma treated with anti-PD-1 or an isotype control antibody. *indicates statistically significantly different from No Ex (same treatment group). Data analysed by two-way ANOVA with Sidak’s multiple comparisons post-test. p<0.05*; p<0.01**. Data shown as individual data points and mean with 95% CI. n=9-10 per group.

In mice with EO771 tumours, I observed that exercise increased the expression of cytochrome c and complexes I, III, IV, and V to a similar degree in both IgG2a and anti-PD-1 treated groups (cytochrome c: IgG2a p=0.0007, aPD-1 p=0.0011; complex I: IgG2a p=0.0040, aPD-1 p=0.0059; complex III: IgG2a p=0.0008, aPD-1 p=0.0091; complex IV: IgG2a p<0.0001, aPD-1 p=0.0010; complex V: IgG2a p=0.0035, aPD-1 p=0.045; Figure 5.11a). In addition, exercise increased the expression of complex II and COX-IV in mice receiving IgG2a but not those receiving anti-PD-1 (complex II: IgG2a p=0.0007, aPD-1 p=0.096; COX-IV: IgG2a p=0.0029, aPD-1 p=0.65; Figure 5.11a). Anti-PD-1
alone did not affect the expression of any of the OXPHOS complexes or cytochrome c in mice with EO771 tumours (Figure 5.11a).

Citrate synthase activity was unaffected by anti-PD-1 treatment and exercise in mice with B16-F10 or EO771 tumours (Figure 5.10b, Figure 5.11b).

Figure 5.11: Exercise increases expression of cytochrome c and OXPHOS complexes in mice with EO771 tumours treated with anti-PD-1 or isotype control antibody.
(a) Relative protein expression of cytochrome c, OXPHOS complexes I-V and COX-IV in quadriceps muscle homogenates from exercising (Ex) or non-exercising (No Ex) EO771 tumour-bearing mice treated with anti-PD-1 (aPD-1) or an isotype control antibody (IgG2a). Samples were normalised to a positive control (run on all blots) and GAPDH. (b) Activity of citrate synthase in quadriceps muscle homogenates from exercising or non-exercising mice with EO771 tumours treated with anti-PD-1 or an isotype control antibody. *indicates statistically significantly different from No Ex (same treatment group). Data analysed by two-way ANOVA with Sidak’s multiple comparisons post-test. $p<0.05*$; $p<0.01**$; $p<0.001***$; $p<0.0001****$. Data shown as individual data points and mean with 95% CI. n=8-10 per group.

These results suggest that IgG2a and anti-PD-1 treatment differentially affect the expression of markers of mitochondrial content in mice with B16-F10 or EO771 tumours.
5.5 Discussion

In this chapter, I aimed to determine whether exercise adaptations of skeletal muscle mitochondria are altered by cancer and immunotherapy. I found that skeletal muscle mitochondrial responses to exercise are altered by tumour burden, and this varies with tumour type. In addition, tumour burden induces weight loss or inhibits body weight gain (weight gain would be expected in the young mice used in this study), and this is not altered by exercise. However, body weight loss only reached the threshold for cachexia (>5% loss) in a subset of mice, indicating that the observed mitochondrial alterations are occurring in pre-cachectic muscle. Complex IV and COX-IV expression correlated strongly with each other in tumour-free mice, but this correlation was attenuated or abolished by tumour burden. Finally, I found that anti-PD-1 and/or IgG2a treatment altered the exercise responsiveness of skeletal muscle mitochondria in tumour-bearing mice.

Incidence and severity of cachexia in this study: As mentioned above, only 1-3 mice in each tumour-bearing group lost more than 5% body weight, thereby meeting the threshold for cachexia. Importantly, although some mitochondrial alterations become evident with <5% body weight loss, stark impairments are only seen when weight loss exceeds this threshold [239]. In my cohort, only complex IV expression was significantly reduced by tumour burden. Together, this highlights that the mice in this study were largely pre-cachectic.

Correlation between distance run and expression of OXPHOS complexes: Expression of the OXPHOS complexes did not correlate with the average daily running distance in tumour-free mice or untreated mice with either tumour type (Figure 5.7). One possible interpretation is that no training effect occurred. However, given that significant increases in the expression of at least one marker was seen with exercise in all groups (Figure 5.5), this seems unlikely. An alternative explanation is inter-individual variability in the response to exercise training. It is well-established that individuals will not exhibit the same degree of adaptation to a standardised training program [11]. Despite the use of inbred mice in this study (which should reduce variability due to high genetic similarity), I observed large variation in a number of measured parameters, most notably the distance run each day (Figure 5.3). Therefore, it is possible that similar variation exists in the response to training, and this explains the lack of correlation between average daily
running distance and the expression of OXPHOS complexes despite evidence of a training effect on a number of mitochondrial markers.

Effect of tumour burden on skeletal muscle mitochondrial adaptation to exercise: I observed significantly higher expression of cytochrome c and complex I in mice with B16-F10 tumours compared with tumour-free mice (suggesting increased mitochondrial content, Figure 5.5), which was unexpected as previous reports indicate that tumour burden reduces expression of mitochondrial markers, indicating reduced mitochondrial content [239]. However, an increase in content may not translate to an increase in function. For example, accumulation of dysfunctional mitochondria is a hallmark of some myopathies [254]. In the context of cancer cachexia, it has been shown in rats and humans (albeit with very small sample sizes in the human study) that mitochondrial area is increased in cachectic muscle, and a higher proportion of mitochondria exhibit aberrant morphology [255,256]. Therefore, it is possible that in some models, cachexia may negatively affect mitochondrial function without changing or even while increasing overall mitochondrial volume.

At first glance, my results suggest that skeletal muscle mitochondrial adaptation to exercise was intact in mice with B16-F10 melanomas, but not in mice with EO771 tumours. However, there are a few clues to suggest this might not be the case. Although exercise increased the expression of cytochrome c and complexes II-V, it is notable that complex IV expression levels were low compared with tumour-free mice (Figure 5.5). Similar findings were recently reported in the quadriceps muscles of pre-cachectic ApcMin/+ mice, in which the authors found that while expression of cytochrome c, complex I and complex II increased with exercise, complex IV expression was reduced in ApcMin/+ compared with wild-type mice, and this did not increase with exercise [153]. This reduction in complex IV expression appears to be a common feature in cancer cachexia [257].

Given the substantial impact of cachexia on quality of life and risk of death, there is much interest in identifying successful therapies for this condition. A number of preclinical studies have investigated exercise as a treatment for cancer cachexia [7,66,147–149,151–155,242,258,259]. Studies beginning exercise prior to the onset of cachexia (but post-tumour initiation) are able to prevent (in less severe models of cachexia such as 4T1 breast cancer) [153–155] or slow (in more severe models of cachexia such as C26 colon
cancer) the onset and progression of the condition [147,151,259]. Therefore, exercise is likely to be most effective when initiated before cachexia becomes clinically apparent, although the subcellular impairments in muscle seen by myself and others, even after exercise, suggest that exercise may delay rather than prevent onset of cachexia when the tumour remains untreated [151,153,242]. Thus, additional treatments may be required to effectively treat or prevent cachexia.

Effect of immunotherapy on skeletal muscle mitochondrial adaptation to exercise: I found that anti-PD-1, but not IgG2a, improved exercise responses of skeletal muscle mitochondria in mice with B16-F10 melanomas, while anti-PD-1 alone tended to decrease expression of cytochrome c and COX-IV in non-exercising mice (Figure 5.10). This suggests that in mice with B16-F10 tumours, anti-PD-1 has specific actions that affect adaptation of skeletal muscle mitochondria.

Chronic inflammation has been suggested to be a key driver of cancer cachexia [6], and systemic immune dysfunction characterised by impaired effector response has been suggested as an early indicator for the syndrome [260]. This is reminiscent of T cell exhaustion, which commonly occurs in the tumour microenvironment due to persistent antigen exposure/inflammation and is characterised by high expression of inhibitory receptors (such as PD-1) and impaired effector functions [261]. I speculate that cachexia-associated inflammation may be inducing an exhausted phenotype in intramuscular immune cells, which is alleviated by anti-PD-1, allowing the cells to respond to a stimulus (exercise) and induce muscle adaptation.

As discussed in more detail in Chapter 4, inoculation with antibodies from foreign species can induce an immune response against these antibodies. This may result in altered immune responses and may explain why mice with EO771 tumours receiving IgG2a showed an increased expression of OXPHOS proteins with exercise (Figure 5.11), while untreated mice did not (Figure 5.5). Given that the immune system plays an essential role in muscle regeneration following injury (including ultrastructural damage caused by exercise) [244], I speculate that the immune response induced by antibody inoculation synergises with exercise to enhance adaptive responses in skeletal muscle in mice with EO771 tumours, independently of the specific action of anti-PD-1.

It is unclear why IgG2a injections had different effects on mitochondrial adaptation to exercise in skeletal muscle in mice with EO771 compared with B16-F10 tumours, but as
illustrated in Chapter 3 (Figure 3.26, Figure 3.27, Figure 3.28), B16-F10 and EO771 tumours show major differences in tumour cell proliferation, CD31+ vessel density, hypoxia, T cell infiltration and NK cell infiltration. Therefore, it is likely that there are different tumour-derived factors which differentially affect the localised immune response to exercise in muscle and subsequent adaptation, or the two tumour types may differentially affect the ability of leukocytes to mobilise to the bloodstream and redistribute to tissues following acute exercise.

Limitations: A limitation of this study is that the tumour-free cohort of mice was not studied alongside the tumour-bearing mice, but at a later time-point. This could have some effect on results due to cohort differences in mitochondrial adaptation or baseline mitochondrial content. However, I would expect these to be minor given that all mice used in the generation of data for this thesis are from the same stock, bred in-house, and the expression of mitochondrial markers was similar between untreated mice with tumours and tumour-bearing mice receiving IgG2a, which were from experiments run at a later time-point than the untreated mice.

This study was an exploratory investigation into differences in mitochondrial adaptation to exercise in pre-cachectic, tumour-bearing mice. Although my results illustrate differences between tumour-free and tumour-bearing mice, it is unknown whether these are associated with differences in muscle function in vivo, as muscle strength measures were not performed. Although exercise can prevent decline in pre-cachectic muscle function, impairments at the subcellular level remain present and may eventually progress to measurable loss of muscle function [153]. Future work should include functional measures such as grip strength testing pre- and post-exercise intervention in mice with different tumour types.

It is important to remember that mice without wheel access still perform physical activity in the form of general cage activity, including climbing on the bars of the feeder, meaning that they may not fully model sedentary versus active humans, but rather less active versus more active. That being said, I did see higher expression of mitochondrial markers in muscle tissue from tumour-free mice provided with a running wheel compared with those with no access to a wheel, indicating that a training effect occurred. It has also been shown that mice with access to a running wheel perform more activity than those without
[262]. This demonstrates that voluntary wheel running does provide an appropriate exercise model.

**Conclusion:**

These data provide preliminary evidence that tumor burden may alter skeletal muscle adaptation to exercise. In addition, I was able to show for the first time that systemic immune stimulation by IgG2a or anti-PD-1 enhanced the adaptation of skeletal muscle mitochondria to exercise training. Although these results should be viewed as hypothesis-generating and followed up with more comprehensive analyses of training adaptations, they have raised the exciting possibility that immunotherapy, in combination with exercise, may be an effective strategy for the prevention of cancer-associated muscle wasting.
6 Association of Markers of Muscle Mitochondria with Tumour Characteristics in Hyperlipidaemic ApoE⁻/⁻ and Wild-Type Mice

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6.1 Introduction

Obesity is associated with hyperlipidaemia (increased circulating lipids), which represents a significant health burden in the developed world, as it increases the risk of cardiovascular disease [263]. Epidemiological studies indicate that hyperlipidaemia contributes to the disease progression of breast cancer [264–266], but provide conflicting evidence as to the effect on breast cancer risk [267–272]. In line with this, preclinical studies show that hyperlipidaemia increases the growth rate of breast tumours in vivo [166,273–276]. One study has utilised apolipoprotein E knockout (ApoE⁻/⁻) mice as a hyperlipidaemic host for breast cancer and showed that tumours grew faster compared with wild type mice [166]. In addition, breast tumours from mice with diet-induced hyperlipidaemia are more proliferative [273,275,276], have decreased apoptosis [275] and have increased microvessel density [273–275].

It is likely that hyperlipidaemia contributes to breast cancer progression by 1) the increased exposure of tumour cells to cholesterol and 2) the development of chronic inflammation. Cholesterol and its metabolite, 27-hydroxycholesterol, have been shown to directly enhance cancer cell proliferation and metastatic potential by activating the phosphatidylinositol-3-kinase (PI3K)/Akt pathway and inducing epithelial to mesenchymal transition (EMT), respectively [166,277]. Chronic inflammation is well-established as a contributor to breast cancer development and progression [278]. Oxidised low density lipoprotein (LDL) can bind to and activate toll-like receptors on macrophages, thereby initializing a pro-inflammatory signalling cascade [279].
Therefore, in hyperlipidaemia, the abnormal elevation of LDL may lead to chronic inflammation and disease progression.

*ApoE*−/− mice are characterised by hyperlipidaemia and associated atherosclerotic plaque development due to impaired clearance of plasma lipids. Plaque development progresses with age, beginning with monocyte adhesion to endothelial cells of the blood vessel wall at 8-10 weeks of age, foam cell lesions from 10 weeks of age, intermediate lesions from 15 weeks and fibrous plaques from approximately 20 weeks of age [280]. Although primarily used for atherosclerosis research, *ApoE*−/− mice have been used in a range of other settings, including for cancer research [166,281,282].

ApoE is involved in lipid transport through the bloodstream and lipid uptake into cells, hence *ApoE*−/− mice are hyperlipidaemic [283]. However, it is important to note that ApoE has additional functions outside of lipid transport. In particular, ApoE has roles in immune regulation. For example, ApoE deficiency enhances MHC-II-dependent antigen presentation by dendritic cells, resulting in greater activation of CD4+ T cells [284]. Similarly, NK cells from *ApoE*−/− mice show greater cytotoxicity than those from wild-type mice, which is associated with reduced incidence of carcinogen-induced lung tumours and IV injected B16-F10 lung ‘metastases’ [285]. These results indicate that ApoE plays an immune modulatory role and as such, any alteration of tumour characteristics in *ApoE*−/− mice compared with wild-type mice should be interpreted with this consideration in mind, as well as considering the lipid transport effects.

During my Honours project, I investigated whether exercise affected tumour growth rate and the tumour microenvironment in *ApoE*−/− mice, and this data has been published [282]. I found that exercise did not affect primary tumour growth rate, but it did reduce the incidence of internal metastases [282]. Exercise did not affect the number of infiltrating T cells, tumour perfusion or hypoxia.

The degree of adaptation to regular exercise is highly variable across a population, in both mice and humans, with some individuals even showing no improvement in exercise capacity with moderate training [159,286]. There are no published studies investigating whether exercise adaptability (aka exercise responsiveness) is associated with cancer outcomes, and as such, it is largely unknown what role exercise adaptability plays in cancer patients’ response to exercise, and specifically, in exercise-induced changes in the TME. There are two possibilities: either the adaptability of non-tumour tissues (such as
muscle) to exercise is linked to the degree of effect seen in the tumour microenvironment (e.g. the effect on tissue oxygenation or immune cell infiltration), or the effect of exercise on the tumour is independent of its effect on other tissues. Association of markers of muscle adaptation to exercise, such as mitochondrial markers, with tumour characteristics could provide insight into this relationship.

I have found previously that exercise did not affect EO771 tumour growth rate, perfusion, hypoxia or T cell infiltrate in ApoE/$^\text{-}$ mice [282]. However, mice were housed in pairs with access to just one running wheel, and we noted (based on running distances of a single mouse after its cage partner was euthanised) that one mouse in each pair seemed to be running substantially more. I hypothesised that this might be confounding exercise effects on the tumour and that a marker of exercise adaptation, or ‘fitness’ (such as a muscle mitochondrial marker), could be utilised to attenuate this confounder. Therefore, in this chapter I investigated the association of muscle mitochondrial markers (as proxies for exercise adaptation) with tumour characteristics in that same cohort of ApoE/$^\text{-}$ mice, as well as in WT mice for comparison (although the two genotypes were not directly compared).

### 6.2 Chapter Aim and Experimental Approach

**Hypothesis:** That muscular COX-IV expression (as a proxy for mouse ‘fitness’) would be inversely associated with tumour growth rate.

Adaptive exercise responses are highly variable in both mice and humans [159,286] and it is possible that individual response to exercise plays a role in the degree of protection against tumour progression. In Chapter 5 I observed that not all mice exhibited increased expression of mitochondrial markers following exercise. Therefore, the aim of this chapter was to conduct an exploratory analysis to determine whether markers of muscle mitochondria (as a proxy for mouse ‘fitness’) are associated with tumour characteristics.

This analysis included the wild-type mice from Chapters 3-5 and a cohort of ApoE/$^\text{-}$ mice, which were from a prior study conducted during my Honours. I obtained muscle samples and performed TME analysis on tumour samples from these mice during my Honours project but analysed for muscular COX-IV expression and performed the correlation analyses outlined in this chapter during my PhD. Both the COX-IV analyses (conducted during my PhD and presented in this chapter) and the results from my Honours thesis are
included in the publication [282]. No analyses from my Honours thesis were included here in this chapter.

In order to be able to compare results from the WT with the ApoE\(^+\) mice, I used COX-IV as the selected marker.

The following specific objectives were addressed:

1. Investigate the association between muscular COX-IV expression and tumour characteristics in ApoE\(^+\) mice.

2. Investigate the association between muscular COX-IV expression and tumour characteristics in WT mice.

3. Investigate the association between muscular COX-IV expression and tumour characteristics in WT mice treated with anti-PD-1 or IgG2a.

### 6.2.1 Study Design

The experimental design for the studies involving the WT mice is outlined in Sections 3.2, 4.2 and 5.2. Briefly, mice were housed in pairs with a cage divider, with one half of each pair receiving a running wheel (exercise) and the other receiving no wheel (non-exercise) from the date of tumour implant. Mice received either B16-F10 (subcutaneous) or EO771 (orthotopic) tumour cells and were euthanised when tumours reached maximum ethical size. Tumour samples were investigated for hypoxia (Section 2.2.11), perfusion (Section 2.2.11), vessel density (Section 2.2.13), T cell (Section 2.2.11.1) and NK cell infiltrate (Section 2.2.13), and muscle samples were investigated for markers of muscle mitochondria (Sections 2.2.6 and 2.2.7).

Quadriceps muscle samples were obtained from ApoE\(^+\) mice during a study conducted for my Honours project. The experimental design for that study is shown in Figure 6.1 and was as follows:

ApoE\(^+\) mice were orthotopically inoculated with \(2 \times 10^5\) EO771 breast tumour cells at 6-10 weeks of age and randomised to either no exercise, low exercise (LEx, wheel access every other day) or high exercise (HEx, wheel access every day). The two exercise groups were used to attempt to investigate different ‘doses’ of exercise. When tumours reached 600 mm\(^3\), mice were injected IP with pimonidazole (a hypoxia marker) 90 minutes prior to euthanasia, and IV with Hoechst 33342 (to label perfused blood vessels) 1 minute prior
to euthanasia. Tumours were analysed for growth rate (Section 2.2.2.5), perfusion (Section 0), hypoxia (Section 2.2.13) and T cell infiltrate (Section 2.2.11.2). The left quadriceps femoris muscles were removed and analysed for COX-IV expression (as in Section 2.2.6, but detected by chemiluminescence).

Results comparing tumour characteristics in No Ex, LEx and HEx groups were reported in my Honours dissertation and have been published [282]. Tumour characteristics were reanalysed for their association with muscular COX-IV expression in this chapter.

![Figure 6.1](image)

**Figure 6.1:** Experimental design for the study investigating the effect of exercise on the tumour microenvironment in *ApoE<sup>-/-</sup>* mice.

Female mice aged 6-10 weeks were orthotopically inoculated with EO771 breast cancer cells, or served as tumour-free controls. Mice were then randomised to either no exercise control, low exercise or high exercise (with access to a modified running wheel with revolution counter). Mice were housed at two per cage. Mice were euthanised when tumours reached 600 mm<sup>3</sup> after which point muscle samples were taken from the left quadriceps femoris muscles and analysed for expression and activity of COX-IV. In addition, tumours were analysed for growth rate, perfusion, hypoxia and T cell infiltrate.

### 6.3 Acknowledgements

I would like to acknowledge Dr Abel Ang for performing the analysis of plasma triglycerides in this chapter.
6.4 Results

6.4.1 Association of Muscular COX-IV Expression with EO771 Tumour Characteristics in ApoE\(^{-/-}\) Mice

Hyperlipidaemia may increase tumour growth rate and metastatic potential [166]. Exercise may be more effective at slowing tumour growth in the presence of metabolic abnormalities [287]; therefore, I investigated whether muscular COX-IV expression was associated with tumour characteristics in a cohort of hyperlipidaemic ApoE\(^{-/-}\) mice with EO771 breast cancer, from which muscle samples were obtained during my Honours project.

6.4.1.1 Effect of exercise on muscular COX-IV expression

Exercising (LEx and HEx) mice tended to have increased COX-IV expression compared with non-exercising mice, but this was not significant due to the large spread of the data (Figure 6.2a and b). This spread may have been due to unequal running by cage partners, i.e. in each cage there seemed to be one mouse doing the majority of the running, while the other did very little. This was based on the observation that running distances from a single mouse (after its cage partner had been euthanised) were often similar to those obtained for that pair. When mice were grouped according to measured/predicted distance run of more or less than 7 km/48 h, COX-IV expression in mice exercising more than average was significantly higher compared to sedentary mice or mice running less than average (Figure 6.2c; \(p=0.03\)). This cut-off was based on half the average distance run per 48 h when HEx and LEx groups were combined. LEx mice ran on average 8 km/day per pair when provided with a wheel, and HEx mice ran on average 10 km/day per pair [282]. This translated to an average running distance of 20 km/48 h per pair for HEx mice, and 8 km/48 h per pair for LEx mice (as LEx mice only had wheel access for 1 of every 2 days) [282]. When HEx and LEx groups were combined, I therefore
estimated the average distance run per mouse per 48 h to be 7 km.

![Image: Representative Western blot probed for COX-IV and GAPDH in tumours from non-exercising, LEx and HEx mice. (b) Densitometric quantification of results in (a). No Ex: n=5, LEx: n=9, HEx: n=6. (c) Quantification of muscular COX-IV expression in mice with inferred average running distance of more or less than ½ mean pair running distance across LEx and HEx groups. No Ex: n=5, <7 km/48 h: n=7, >7 km/48 h: n=4. p<0.05*. Data are expressed as individual data points and mean with 95% CI. Data analysed by one-way ANOVA with Tukey’s multiple comparison’s test. LEx, running wheel provided every other day, HEx, wheel provided on continual basis.

Figure 6.2: Muscular COX-IV expression is increased by wheel running in ApoE<sup>−/−</sup> mice with EO771 tumours.

6.4.1.2 Association of muscular COX-IV expression with plasma triglycerides in ApoE<sup>−/−</sup> mice

In order to confirm the phenotype of ApoE<sup>−/−</sup> mice, serum triglycerides were measured and compared with wild type mice. I found that non-exercising ApoE<sup>−/−</sup> mice had significantly elevated triglycerides compared to non-exercising wild type mice (Figure 6.3a; p<0.001), thus confirming their hyperlipidaemic phenotype. There was no difference in non-exercising compared with exercising ApoE<sup>−/−</sup> mice (Figure 6.3b), showing that wheel running did not significantly reduce plasma triglycerides in
genetically hyperlipidemic mice. In addition, muscular COX-IV expression was not associated with the level of plasma triglycerides (Figure 6.3c and d).

![Figure 6.3](image)

**Figure 6.3: Plasma triglycerides are not associated with muscular COX-IV expression.**

(a) Analysis of plasma triglyceride levels in non-exercising wild-type (WT) compared with non-exercising ApoE<sup>−/−</sup> mice. WT: n=9, ApoE: n=13. (b) Analysis of plasma triglyceride levels in non-exercising, LEx and HEx ApoE<sup>−/−</sup> mice. Sed: n=13, LEx: n=14, HEx: n=12. (c) Analysis of plasma triglyceride levels in mice with low or high muscular COX-IV expression, according to the median of 1.65. Low COX-IV: n=10, high COX-IV: n=9, p<0.001***. Data are expressed as individual data points and mean with 95% CI.

### 6.4.1.3 Association of muscular COX-IV expression with EO771 tumour growth rate and proliferation in ApoE<sup>−/−</sup> mice

Mice with high muscular COX-IV expression had a small, non-significant increase in overall survival (p=0.075; Figure 6.4a). In addition, I observed a marked tumour growth delay in ApoE<sup>−/−</sup> mice with high compared with low muscular COX-IV expression (Figure 6.4b). It is unclear from this data whether this overall difference reflected differences in the lag and/or exponential phases of tumour growth and these phases were therefore analysed separately. Mice with high muscular COX-IV expression had significantly slower lag phase growth compared with those with low COX-IV expression (Figure 6.4c;
p=0.008). COX-IV expression correlated significantly with the time taken for the tumour to reach 100 mm$^3$ (Figure 6.4d; p=0.014, r=0.60). Exponential phase growth remained unchanged between mice with low compared with high COX-IV expression (Figure 6.4e, f).

![Figure 6.4: High COX-IV expression is associated with improved survival and slower EO771 tumour growth rate in ApoE$^{-/-}$ mice.](image)

(a) Survival curves for mice with high or low muscular COX-IV expression, according to the median of 1.65. Data analysed by Log-rank (Mantel-Cox) test. n=5-6 per group. (b) Analysis of the lag phase of tumour growth in mice with low compared with high muscular COX-IV expression. n=8 per group. Data analysed by unpaired, two-tailed student’s t test. p<0.01**. (c) Pearson correlation of the lag phase of tumour growth and muscular COX-IV expression. n=16. (d) Analysis of the exponential phase of tumour growth in mice with low compared with high muscular COX-IV expression. n=7 per group. (e) Pearson correlation of the exponential phase of tumour growth and muscular COX-IV expression. n=14. Data in scatter plots are shown as individual data points and mean with 95% CI. Correlation data are shown as individual data points with best fit line and 95% CI.
Proliferation of tumour cells was estimated by staining for the mitotic marker pHH3 and calculating the percentage of pHH3 positive cells within the tumour. On average, 2% of tumour cells were proliferating, but there was no difference in pHH3 staining between ApoE<sup>-/-</sup> mice with high compared with low COX-IV expression (Figure 6.5).

![Figure 6.5: Muscular COX-IV expression is not associated with EO771 tumour cell proliferation in ApoE<sup>-/-</sup> mice.](image)

(a) Representative immunohistochemical staining for pHH3 in EO771 tumours from mice with low or high COX-IV expression. Scale bar represents 200 µm. (b) Analysis of tumour cell proliferation from ApoE<sup>-/-</sup> mice with low compared with high muscular COX-IV expression according to the percentage of pHH3 positive cells. n=8-10 per group. Data are shown as individual data points and mean with 95% CI. (c) Pearson correlation of muscular COX-IV expression with the percentage pHH3<sup>+</sup> cells in EO771 tumours from ApoE<sup>-/-</sup> mice. Data are shown as individual data points with best fit line and 95% CI.

Taken together, these data suggest that muscular COX-IV expression is associated with slower EO771 tumour establishment but not exponential growth of established tumours.

### 6.4.1.4 Association of muscular COX-IV expression with tumour perfusion, hypoxia and vascularity in ApoE<sup>-/-</sup> mice

Tumour hypoxia was significantly reduced in mice with high compared with low muscular COX-IV expression (Figure 6.6a and b; p=0.01). Furthermore, hypoxia showed a strong inverse correlation with COX-IV expression (Figure 6.6c; p=0.007, r=-0.61).
Figure 6.6: High muscular COX-IV expression is associated with lower levels of tumour hypoxia in EO771 tumours from ApoE−/− mice.

(a) Representative immunohistochemical images of EO771 tumours stained for pimonidazole (a hypoxia marker). (b) Quantification of immunohistochemical staining for pimonidazole in tumours from mice with low compared with high muscular COX-IV expression. Low COX-IV: n=10; high COX-IV: n=8. Intensity and percentage of staining were quantified by H score (for detail refer to Section 2.2.13.3). Data are shown as individual data points and mean with 95% CI. Data were analysed by unpaired, two-tailed student’s t test. p<0.05*. (c) Pearson correlation of hypoxia H score with muscular COX-IV expression. n=18. Data shown as individual data points with best fit line and 95% CI.

In contrast, I observed no significant differences in Hoechst 33342 staining in tumours from ApoE−/− mice with high compared with low muscular COX-IV expression (Figure 6.7).
Figure 6.7: Muscular COX-IV expression is not associated with tumour perfusion in EO771 tumours from ApoE^{-/-} mice.

(a) Representative image of Hoechst 33342 fluorescence in EO771 tumours from ApoE^{-/-} mice. Scale bar represents 200 µm. (b) Quantification of Hoechst 33342 perfused sections by the average number of vessels per field in tumours from mice with low compared with high muscular COX-IV expression, according to the median of 1.65. Low COX-IV: n=10; high COX-IV: n=8. Data shown as individual data points and mean with 95% CI. (c) Spearman correlation of muscular COX-IV expression with tumour perfusion in EO771 tumours from ApoE^{-/-} mice. n=18. Data shown as individual data points and best fit line with 95% CI.

Vessel density was estimated by immunohistochemical staining for CD31 and subsequent counting of the number of CD31^{+} vessels in hotspot areas of vessel density. Vessel density varied greatly across each tumour section, with the majority of hotspot areas localised near the edge of the tumour. Muscular COX-IV expression was not associated with the number of CD31^{+} vessels in hotspot areas (Figure 6.8).
Figure 6.8: Muscular COX-IV expression is not associated with vessel density in EO771 tumours from ApoE−/− mice.

(a) Representative immunohistochemical staining for CD31 in EO771 tumours from ApoE−/− mice. Arrows indicate CD31+ vessels. Scale bar represents 100 µm. (b) Quantification of CD31 staining in EO771 tumours from mice with low compared with high muscular COX-IV expression. Sections were scored by calculating the average number of CD31+ vessels in 5 hotspot areas. Low COX-IV: n=10; high COX-IV: n=8. Data are shown as individual data points and mean with 95% CI. (c) Pearson correlation of CD31+ vessel density with muscular COX-IV expression. n=18. Data shown as individual data points with best fit line and 95% CI.

These results indicate that muscular COX-IV expression is not associated with tumour perfusion or CD31+ vessel density, but is associated with reduced tumour hypoxia in ApoE−/− mice.

6.4.1.5 Association of muscular COX-IV expression with T cells in EO771 tumours from ApoE−/− mice.

I observed no association between muscular COX-IV expression and the average number of CD3+, CD3+Foxp3+ or CD3+CD8+ T cells in EO771 tumours from ApoE−/− mice (Figure 6.9).
Figure 6.9: Muscular COX-IV expression is not associated with T cell density in EO771 tumours from ApoE−/− mice.

(a) Representative immunofluorescent images stained for CD3 (red) and Foxp3 (green) or CD8 (green; CD3+ CD8+ cells are yellow). DAPI stained nuclei are visible in blue. White arrows indicate CD3+ Foxp3− or CD3+CD8+ cells; yellow arrows indicate CD3+ Foxp3+ or CD3+ CD8+ cells. Scale bar represents 50 µm. Quantification of the number of intratumoural CD3+ (b), CD3+Foxp3+ (c) and CD3+CD8+ T cells per field in mice with low compared with high muscular COX-IV expression, according to the median of 1.65. Cells were counted in 5 random fields. Data expressed as individual data points and mean with 95% CI. Low COX-IV: n=6, high COX-IV: n=8. Pearson correlation of muscular COX-IV expression with CD3+ (e), CD3+Foxp3+ (f) or CD3+CD8+ (g) intratumoural T cells. Data shown as individual data points with best fit line and 95% CI.

Similarly, the proportion of intratumoural Foxp3+ and CD8+ T cells was not associated with muscular COX-IV expression (Figure 6.10).
Figure 6.10: The proportion of Foxp3+ and CD8+ T cells in EO771 tumours from ApoE−/− mice is not associated with muscular COX-IV expression.

Percentage Foxp3+ (a) or CD8+ (b) of total T cells in EO771 tumours from mice with low compared with high muscular COX-IV expression. Low COX-IV: n=6, high COX-IV: n=8. Data expressed as individual data points and mean with 95% CI. Pearson correlation of muscular COX-IV expression with the %Foxp3+ (c) or %CD8+ (d) intratumoural T cells. Data shown as individual data points with best fit line and 95% CI.
6.4.2 Association of Muscular COX-IV Expression with B16-F10 and EO771 Tumour Characteristics in WT Mice

6.4.2.1 Association of muscular COX-IV expression with tumour growth rate

Mice were grouped according to high or low COX-IV expression based on the median. I found no differences in mouse survival or tumour growth rate in mice with low compared with high muscular COX-IV expression (Figure 6.11) for either B16-F10 or EO771 tumours.

Figure 6.11: Muscular COX-IV expression is not associated with growth rate of B16-F10 or EO771 tumours.

Survival curves for mice with high or low muscular COX-IV expression (according to the median) bearing B16-F10 (a) or EO771 (b) tumours. Data analysed by Log-rank (Mantel-Cox) test. B16-F10: n=12 per group; EO771: n=8-11 per group. Analysis of the lag phase of B16-F10 (c) or EO771 (d) tumour growth in mice with low compared with high muscular COX-IV expression. B16-F10: n=12 per group; EO771: n=8-11 per group. Analysis of the exponential phase of B16-F10 (e) or EO771 (f) tumour growth in mice with low compared with high muscular COX-IV expression. B16-F10: n=8-11 per group; EO771: n=7-10 per group. Data are shown as individual data points and mean with 95% CI.
6.4.2.2 Association of muscular COX-IV expression with tumour characteristics

In order to determine whether muscular COX-IV expression is associated with altered tumour microenvironmental features, I performed correlation analysis of muscular COX-IV expression with tumour cell proliferation (%pHH3+ cells), tumour perfusion, tumour hypoxia, CD31+ vessel density, intratumoural NK cells, intratumoural CD3+ T cells, intratumoural CD3+Foxp3+ T cells and intratumoural CD3+CD8+ T cells (Table 6.1).

No significant correlations were found (Table 6.1).

Table 6.1: Correlation of muscular COX-IV expression with tumour microenvironmental features.

<table>
<thead>
<tr>
<th></th>
<th>B16-F10</th>
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<th>EO771</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td></td>
<td>r</td>
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</table>

Data were analysed using 1Pearson’s or 2Spearman’s correlation. B16-F10: n=18-24; EO771: n=19-20.

6.4.3 Association of Muscular COX-IV Expression with Tumour Characteristics in Mice Receiving Anti-PD-1 or IgG2a

I investigated whether muscular COX-IV expression was associated with tumour characteristics in mice receiving IgG2a or anti-PD-1.

6.4.3.1 Association of muscular COX-IV expression with tumour growth rate

Tumour growth rate and mouse survival were not associated with muscular COX-IV expression in mice with either tumour type, receiving either IgG2a or anti-PD-1 (Figure 6.12).
6.4.3.2 Association of muscular COX-IV expression with tumour microenvironmental features

Muscular COX-IV expression was negatively associated with the number of CD3+ T cells in B16-F10 tumours treated with anti-PD-1 (r=-0.73, p=0.0087; Table 6.2). This simply reflects results reported in previous chapters: that anti-PD-1 treatment increased T cell numbers in B16-F10 tumours from non-exercising mice, but exercise reduced these numbers (Figure 4.11), and exercise significantly increased muscular COX-IV expression in anti-PD-1 treated mice (Figure 5.10 and Figure 5.11). No other significant associations were found.
Table 6.2: Correlation of muscular COX-IV expression with tumour microenvironmental features from mice receiving IgG2a or anti-PD-1.

<table>
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<td>aPD-1</td>
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Data were analysed using <sup>1</sup>Pearson’s or <sup>2</sup>Spearman’s correlation. B16-F10: n=11-20; EO771: n=13-20.

### 6.5 Discussion

In this chapter, I conducted an exploratory analysis to investigate whether markers of muscle mitochondria (as a proxy for mouse ‘fitness’) were associated with tumour growth rate and microenvironmental characteristics in WT and hyperlipidaemic ApoE<sup>-/-</sup> mice. I found no correlations in WT mice. Of interest, however, is the finding that ApoE<sup>-/-</sup> mice with high muscular COX-IV expression had longer survival time and slower tumour establishment time. In addition, tumour hypoxia was negatively correlated with COX-IV expression in the muscle of hyperlipidaemic mice. These changes were independent of changes in circulating triglycerides.

**ApoE<sup>-/-</sup> mice as a model of hyperlipidaemia:** Hyperlipidaemia in ApoE<sup>-/-</sup> mice is induced by knockout of ApoE, which impairs clearing of plasma lipoproteins [288]. ApoE is a circulating glycoprotein that mediates uptake of plasma lipoproteins via receptor-mediated endocytosis [289]. Hence, although the ApoE<sup>-/-</sup> phenotype results in a greater...
exposure of the EO771 tumour cells to cholesterol (and triglycerides), it likely also results in reduced, not increased, lipid uptake. However, cholesterol in cell culture (in the absence of ApoE as a mediator of uptake) has also been reported to support EMT, indicating that other uptake mechanisms can still provide the cell with cholesterol and induce a pro-tumour phenotype [277].

Association of muscular COX-IV expression with tumour growth rate: Interestingly, muscular COX-IV expression in ApoE\(^{-/-}\) mice was only associated with significant growth impairment in the lag, or tumour establishment, phase of growth, but was not associated with exponential phase growth (Figure 6.4). I speculate that higher levels of physical activity (as inferred from higher muscular COX-IV expression) may pre-condition tissues to be less receptive to tumour cell seeding, which would slow tumour establishment time but not exponential growth. In support of this, Pedersen et al. showed that 4 weeks of pre-implant exercise slowed tumour growth by delaying tumour establishment, but post-implant exercise did not [29].

I found that high muscular COX-IV expression was associated with a slower tumour growth rate in ApoE\(^{-/-}\) (Figure 6.4) but not WT mice (Figure 6.12). A previous study found that high fat diet feeding accelerated growth of subcutaneous B16-F10 tumours, and exercise reduced this back to control levels [287]. However, exercise did not affect tumour growth rate in normal chow fed mice. Similarly, ApoE\(^{-/-}\) mice with low COX-IV expression had a maximum time to euthanasia of just 16 days, whereas in WT mice this was more than 30 days. This suggests that exercise may be more effective at slowing tumour growth rate in a background of metabolic dysregulation, bringing the growth rate back to 'normal' levels.

Association of mitochondrial markers with characteristics of the tumour microenvironment: Despite similar perfusion parameters, tumours from ApoE\(^{-/-}\) mice with high muscular COX-IV showed a significant reduction in hypoxia compared to those with low COX-IV (Figure 6.6). These seemingly discrepant findings of reduced hypoxia and unchanged perfusion may be explained by aberrations in haemodynamic control. Variations in red blood cell flux and oxygen consumption rate of the tumour alter the efficacy of oxygen transport to tumour cells [64], which is not detected by Hoechst 33342 labelling. Therefore, the reduced hypoxia seen in tumours from ApoE\(^{-/-}\) mice with high muscular COX-IV expression may be due to improved oxygen delivery to the tissue.
Exercise and muscular COX-IV expression in ApoE/– mice: Skeletal muscle mitochondrial content and function is not well characterised in ApoE/– mice; however, patients with peripheral artery disease exhibit skeletal muscle mitochondrial dysfunction [290]. In addition, ApoE/– mice show impaired muscle regeneration after injury, indicating dysfunction in at least one aspect of muscle health [291]. Therefore, it is possible that ApoE/– mice show mitochondrial dysfunction in skeletal muscle, which likely progresses with age along with the atherosclerotic phenotype, which becomes fully apparent from around 20 weeks of age [280].

If the above hypothesis is correct, low COX-IV expression (indicating mitochondrial loss) could reflect more rapid progression of the ApoE/– phenotype, whereas those with high COX-IV expression may be progressing more slowly. The mice used in my study were young (6-10 weeks of age) and would have been at varying early stages of plaque development [280]. As such, the slower tumour growth rate seen in mice with high compared with low muscular COX-IV expression may simply be reflective of the severity of the ApoE/– phenotype rather than of the effect of physical activity. However, this is purely speculative and would need to be investigated by characterising skeletal muscle mitochondrial content and function in ApoE/– mice at various stages of phenotype development.

It should be noted that mice that ran further than the average had significantly higher muscular COX-IV expression that those that did not exercise or ran less than the average (Figure 6.2). This indicates that even if mitochondrial dysfunction exists in the skeletal muscle of ApoE/– mice, exercise can increase mitochondrial content when it is performed at sufficiently high volumes. In addition, complex III and citrate synthase activity in skeletal muscle were significantly increased by 8 weeks of treadmill exercise in ApoE/– mice [292,293], illustrating that mitochondrial function can also increase with exercise. Therefore, alternatively to the above hypothesis that muscular COX-IV expression simply reflects the severity of the ApoE/– phenotype, it remains possible that higher COX-IV expression reflects better adaptation to exercise.

Limitations: The primary limitation of this chapter is that it was an exploratory, correlative analysis. As such, no definitive conclusions as to causation can be drawn and interpretation of results should be treated as speculative and hypothesis-generating.
Slightly different staining methods (details in Section 2.2.13) were used to obtain tumour microenvironmental data from ApoE−/− and WT mice, as the experiments on the ApoE−/− mice were conducted as part of my Honours project and the methods used therein were further optimised for my PhD work. Due to this, results (on tumour hypoxia, CD31+ vessel density, proliferation and T cell infiltrate) between WT and ApoE−/− mice are not directly comparable.

**Future directions:** Given that the analyses in this chapter are correlative and do not demonstrate causation, future work should aim to address some of the hypotheses arising from this chapter. In order to determine whether muscle adaptation to exercise contributes to changes in the tumour microenvironment and subsequent growth rate, a mouse model with skeletal muscle specific PGC-1α overexpression could be used. PGC-1α is often termed the master regulator of exercise adaptations [294]. One previous study observed that mice with muscle specific PGC-1α overexpression had larger tumours at endpoint in WT mice [295]; however, the authors also noted that mice deemed in too poor of a condition to survive until endpoint were euthanised early and excluded from the analysis. This was the case for three WT mice and one PGC-1α overexpressing mouse. Given that a higher number of WT mice (with presumably large tumours) were excluded, the result indicating that PGC-1α overexpression increased tumour size may not be accurate. In addition, effects on the tumour microenvironment were not investigated. Therefore, a future study using mice with muscle specific PGC-1α overexpression could investigate whether muscle adaptation contributes to altered tumour characteristics.

**Conclusion:** Although exploratory in nature, the analyses in this chapter have highlighted some intriguing associations between muscle mitochondrial markers and tumour characteristics. In particular, the negative association of COX-IV expression with the lag phase of tumour growth in ApoE−/− mice suggests that mouse ‘fitness’ is protective against tumour establishment in a hyperlipidaemic background.
7 Discussion

In this chapter I will first summarise the main findings of this thesis (Figure 7.1). I will then discuss the implications for clinical translation, limitations of the work, and finally discuss possibilities for future research.

7.1 Summary of Findings

In Chapter 3, I investigated the effect of exercise as a sole therapy on tumour growth and the tumour microenvironment. Here, the major findings were that exercise did not affect tumour growth rate, hypoxia or perfusion, suggesting that post-implant exercise (mimicking post-diagnosis exercise) alone may not be particularly efficacious at modifying tumour biology. However, the number of intratumoural CD8$^+$ T cells was reduced by exercise in mice with EO771 breast tumours, indicating that immune modulation may be occurring.

In light of this, Chapter 4 investigated the combination of exercise and anti-PD-1 treatment on tumour growth and the tumour microenvironment. Intriguingly, the combination of exercise and anti-PD-1 had different effects on the tumour microenvironment in B16-F10 and EO771 tumours. In B16-F10 melanomas, exercise prevented the anti-PD-1-induced influx of CD8$^+$ T cells. In EO771 tumours, the combination of anti-PD-1 and exercise (but not either alone) increased the proportion of CD8$^+$ T cells, suggesting that the combination of exercise and anti-PD-1 may improve anti-tumour immunity against these tumours.

Importantly, comparison of untreated mice (Chapter 3) with IgG2a and anti-PD-1 treated mice (Chapter 4) revealed that mice receiving either antibody had longer time to euthanasia, higher numbers of intratumoural NK cells and reduced numbers of intratumoural CD8$^+$ T cells. This was true for both tumour types. This finding is integral to accurately interpreting results, as it suggests that induction of an immune reaction can have significant effects on tumour characteristics independently of the targeted effect of anti-PD-1.

In Chapter 5, I examined the effect of tumour burden on skeletal muscle mitochondrial adaptation to exercise, and its modulation by anti-PD-1 treatment. Here, I found that tumour burden altered skeletal muscle mitochondrial responses to exercise, and these
Effects varied with tumour type. EO771 tumours appeared to have a greater systemic effect leading to the inhibition of mitochondrial exercise adaptations. This finding has important implications for the use of exercise in the management or prevention of cancer cachexia, as it suggests that tumour burden may limit the effectiveness of exercise training. This reveals an important avenue for future research, aiming to develop strategies to improve the effectiveness of exercise training for the prevention of cancer cachexia.

Figure 7.1: Graphical summary of findings.
Exercise alone reduced the number and proportion of CD8$^+$ tumour-infiltrating T cells (CTLs), while exercise in combination with anti-PD-1 (aPD-1) either increased (EO771) or decreased (B16-F10) the percent CTL of the total T cell population depending on tumour type. Tumour hypoxia and perfusion were not affected by exercise or anti-PD-1 treatment. Furthermore, skeletal muscle mitochondrial adaptation to exercise was altered by tumour burden, showing variability in which mitochondrial markers increased their expression in response to exercise. Treatment with anti-PD-1 improved the skeletal muscle mitochondrial exercise response in mice with either tumour type, and IgG2a showed similar effects only in mice with EO771 tumours. Furthermore, anti-PD-1 and IgG2a produced similar changes in the immune composition of the TME (increased NK cell numbers, decreased proportion of CTLs and decreased (B16-F10) or unchanged (EO771) proportion of T$_{reg}$ cells), suggesting that both antibodies are capable of inducing an immune response. Together, this work provides novel insights into the immunological effects of both exercise alone, and exercise in combination with anti-PD-1, on the TME and skeletal muscle. In addition, it reveals that the isotype control antibody used here is not inert, and has substantial effects on both the TME and the muscle mitochondrial response to exercise.

Furthermore, anti-PD-1 improved the exercise adaptation of skeletal muscle mitochondria in mice with either tumour type (although it should be acknowledged that
in mice with EO771 tumours, IgG2a had the same effect). This is an exciting finding, as it highlights the key role of the immune system in muscle adaptation to exercise and reveals a possible new strategy to address the tumour-induced impairment of exercise responses in muscle.

Chapter 6 comprised an exploratory analysis to determine whether markers of skeletal muscle mitochondria (as a proxy for exercise capacity) were associated with tumour characteristics in wild-type or ApoE⁻/⁻ mice. The purpose of this was to glean insights into whether training adaptations, rather than exercise per se, might be important for the potential of exercise to alter tumour growth rate and the tumour microenvironment. ApoE⁻/⁻ mice with high muscular COX-IV expression had significantly longer tumour establishment times and reduced tumour hypoxia compared with mice with low muscular COX-IV expression. This same effect was not seen in wild-type mice. This suggests that in a metabolically abnormal background, high skeletal muscle mitochondrial content may modify muscle-tumour cross-talk to reduce tumour aggressiveness.

### 7.2 Implications for Clinical Translation

Understanding the biological mechanisms underlying the exercise-cancer relationship is essential for informing the development of effective interventions and new treatment strategies. As such, the work in this thesis has provided some important insights which may have implications for clinical translation.

I had hypothesised that exercise would boost immunity and enhance tumour infiltration by CD8⁺ T cells and NK cells, based on prior preclinical work [29,122]. However, in EO771 tumours, the opposite was seen – exercise reduced numbers of T cells overall, and specifically of CD8⁺ T cells. I also observed a greater inhibition of skeletal muscle mitochondrial adaptation to exercise in mice with EO771 tumours than those with B16-F10 tumours, suggesting that EO771 tumours may have a greater systemic impact.

Importantly, tumour burden creates a tremendous demand for energy. It has recently been suggested that individual energetic capacity (“the amount of energy that can be generated and used by an individual on a sustained basis”, including the ability to upregulate energy output on demand) may determine whether or not exercise is beneficial in a cancer setting [296]. The authors highlighted that exercise and immune reactions both create a substantial energy demand, and if exercise is performed in the midst of a strong immune
reaction it may compromise the efficacy of that reaction due to energetic deficit. This provides a possible explanation for the unexpected reduction of T cell numbers in EO771 tumours following exercise training when considered together with the observation that EO771 tumours appear to have a greater systemic effect than B16-F10 tumours (as evidenced by greater inhibition of skeletal muscle mitochondrial adaptation to exercise) – the tumour and exercise together may be creating an energetic demand close to the limits of what the mouse can sustain, thereby negatively affecting anti-tumour immunity.

If the above hypothesis is true, it greatly emphasises the need for exercise prescription in oncology to occur on a personalised basis (by referral to an exercise professional, such as an exercise physiologist, for individual assessment) especially given the large inter-individual variability in exercise responses [11]. As seen in this thesis, even mice from the same inbred mouse strain (which are thus almost genetically identical) show great variability in their running behaviour. Furthermore, it is important to note that in some studies, exercise was not only ineffective at modulating tumour growth, but even accelerated it or worsened cachexia [4,297]. In addition, despite the frequent assertion that exercise is safe [298–302], there are acute risks associated with the increased stress it places on the body, not least injury. Moreover, people with cancer require the consideration of unique issues when being prescribed exercise - for example, peripheral neuropathy may negatively impact balance and preclude the use of certain equipment [303]. Therefore, any exercise intervention should be carefully monitored at the level of the individual (by both the treating oncologist and an exercise physiologist) to identify any possible adverse outcomes early.

Individual exercise responses, the amount of exercise required for an effect to be seen and individual motivation to exercise are highly variable [304], and as such a one-size-fits-all approach is unlikely to be successful. This variability may even be higher in cancer populations, based on my data showing that COX-IV expression in muscle is much more variable in tumour-bearing than tumour-free mice. It has been suggested that the way forward to translating exercise prescription into clinical practice in oncology is by treating exercise as a drug and implementing a typical phase I, II, III trial design [305]. I suggest that this may not be the right approach for a number of reasons. First, as mentioned above, exercise responses are highly variable, making it difficult to determine a universal, optimal ‘dose’ – how then, will it be possible for initial dose-finding trials to be successful? Second, exercise differs from a drug in two key aspects: it is more difficult
to apply, as it requires substantial effort on the part of the recipient, and it has a vast multitude of effects on the body, many of which are likely to still be unknown. Regarding the first aspect, factors such as individual enjoyment of exercise (and therefore the motivation to perform exercise) become extremely important. Mice generally have a high motivation to perform exercise, but even among the inbred mice in this study, running behaviour was highly variable. This is likely to be even greater in a more genetically heterogeneous population.

Despite these issues, exercise does have the potential to become a standard of oncology care. Exercise re- or prehabilitation is now embedded in the care of other chronic, non-communicable diseases, such as heart disease [306], emphasising that it is feasible to implement in clinical practice. The key will likely be to determine a desired outcome as a measure of success (e.g. improvement of muscular strength by X%), tailor the exercise program to achieve this and carefully monitor for adverse outcomes.

### 7.3 Future Directions

*The role of the immune system in skeletal muscle adaptation to exercise:* The work in this thesis raises several exciting possibilities for future research in exercise oncology. First: the novel finding that anti-PD-1 enhances exercise responses of skeletal muscle mitochondria. The vital role of the immune system in muscle regeneration and adaptation to exercise has only recently emerged [245]. As such, how the tight regulation of the local immune reaction to exercise in muscle is affected by additional factors such as tumour burden or cancer treatment remains unclear. This is important to understand if exercise is to become a standard of oncology care, particularly to aid in the prevention of cancer cachexia. Moreover, identification of the pathways involved in the immune-muscle relationship and their effect on the tumour may lead to the development of novel treatment strategies.

A possible approach to investigate the role of immune-modulating drugs in the prevention of cancer cachexia is to expand on the preliminary work already conducted in this thesis. In order to better determine the potential of anti-PD-1 in boosting exercise responses to prevent cachexia, a mouse model more inclined to develop cachexia could be used. Possibilities for this include the C26 subcutaneous colon tumour model or the *Ape*<sup>Min/+</sup> model of spontaneous intestinal carcinogenesis, both of which are widely used in preclinical cancer cachexia research [307]. Alternatively, emerging models of
spontaneous or inducible pancreatic cancer exist [308]. This route is of high clinical relevance, as pancreatic cancer has one of the highest rates of cachexia, with approximately 80% of patients developing the condition [309]. Additionally, it is important to include in vivo functional measures such as strength (grip strength meter, in which the mouse is held by the base of the tail, encouraged to grasp the meter and gently pulled away until it releases to measure strength [139]) and exercise performance tests (treadmill test, akin to an exercise tolerance test in humans, in which mice run at progressively higher intensities until fatigue [208]) as primary outcomes to ascertain the effect of anti-PD-1 on functional exercise adaptation. This would provide a valuable first step in the testing of immune modulating drugs in combination with exercise as a potential strategy for the prevention of cancer cachexia.

Moreover, understanding the mechanisms behind the effects of anti-PD-1 on muscular exercise responses may identify new avenues for drug development. Given that the local immune reaction in muscle is tightly temporally regulated [245], characterisation of local immune cell subsets in muscle over time, following exercise, would provide a key piece in the puzzle. This could involve multiplex immunohistochemical staining of immune cells in muscle at different time-points post-exercise. The inflammatory reaction begins rapidly following exercise and peaks around two days later before beginning to switch to a more anti-inflammatory phenotype [245]. This peaks around 4 days post-insult but can continue much longer [245]. Therefore, possible time-points to investigate could be immediately following, and 2, 4 and 7 days after an acute exercise session.

**Understanding whether energetic capacity affects the response of the tumour to exercise:**

As discussed in the previous section, the dual challenge of exercise and tumour induction may create an energy (ATP) demand close to or exceeding what the mouse can sustain, leading to weight loss and impaired anti-tumour immunity. This hypothesis would provide an explanation for both the early weight loss seen in most mice in the days following tumour implant (Figure 3.6), and for the observation that exercise reduced T cell infiltration into EO771 tumours (Figure 3.19). In addition, this could explain why exercise often has little effect on tumour growth when initiated post-tumour implant (discussed in Section 3.5), but can have substantial growth inhibitory effects when initiated prior to tumour implant [29]. Exercise prior to tumour implant would be expected to increase energetic capacity, as the mouse is healthy and unchallenged by additional stressors, whereas the dual challenge of tumour induction (which would likely
induce an immediate immune response, as well as energy costs to sustain the tumour as it becomes established) and exercise may create excessive energy demand.

Further evidence for the role of energetic availability in defining tumour growth and anti-tumour immunity comes from studies comparing mice housed below thermoneutrality (approx. 22°C, as done in the present study) with those housed at thermoneutrality (approx. 30°C, thermoneutrality is the temperature zone in which a warm-blooded organism does not need to expend any energy on thermoregulation). In these studies, the authors found that tumour growth of four different transplantable tumours (B16-F10, 4T1, CT26 and Pan02) was significantly reduced (by approx. 100 to 600 mm³ at endpoint) by housing at thermoneutrality, and this was associated with increased proportions of CD8⁺ intratumoural T cells and decreased proportions of Foxp3⁺ intratumoural T cells [310]. In addition, CD8⁺ cell depletion nullified the protective effect of thermoneutral housing, as did implant into immune-deficient mouse models [310]. In a second study, thermoneutral housing enhanced the effect of anti-PD-1 treatment [311]. These studies provide evidence that decreased energy expenditure (in this case on thermoregulation) can substantially improve anti-tumour immunity, presumably as there is more energetic capacity for immune responses. It is possible that housing at 22°C affected immune infiltration and tumour growth outcomes in my studies, and it would be of interest to compare the results obtained in this thesis with future studies in which mice are housed at thermoneutrality.

A second possibility to address the role of energetic capacity in defining tumour growth rate and anti-tumour immune responses could be to use rats with high and low inherent exercise capacity (indicative of high and low energetic capacity [160,296]). Rats would be challenged with exercise and tumour implant at the same time, as in the current study, and monitored for body weight change, metabolic rate (using metabolic cages) and changes in VO₂ max (by testing prior to study begin and at endpoint). At endpoint, tumours would be excised and analysed for immune cell infiltrate. If energetic capacity plays a role in the strength of the immune response that is able to be mounted, we would expect the high exercise capacity rats to show improved immune cell infiltration into the tumour and reduced tumour growth rate compared with the low exercise capacity rats. Additional studies could investigate whether starting exercise prior to implant increases energetic capacity in low inherent exercise capacity rats to a large enough degree that
they exhibit reduced tumour growth rate similar to that seen in high inherent exercise capacity rats.

**Understanding how variability in the exercise response affects tumour outcomes:** In this thesis, I found that exercise did not alter tumour growth rate. This is in accord with some preclinical studies, but not others (reviewed in [18]), highlighting the heterogeneous nature of the field. Given that 50% of the variation in the exercise response may be genetically determined [304], it is possible that genetic variation has a considerable effect on the response of the tumour to exercise (although it is unlikely that this had a significant effect within my study, as I used inbred C57BL/6 mice which are genetically identical). Single nucleotide polymorphisms (SNP) are among the most well-studied type of genetic variant and have been associated with diseases such as asthma or Crohn’s disease [312]. Thus, a first approach could be to test for the effect of a specific SNP (that has been associated with a change in a relevant phenotype) on the ability of exercise to modulate tumour growth, by generating mice with each possible genotype for the SNP.

**Characterising the effect of anti-drug immune reactions on tumour characteristics in preclinical studies:** Intriguingly, I observed that mice receiving IgG2a had a significantly altered intratumoural immune profile compared with untreated mice. As discussed in Chapters 4 and 5 (Sections 4.5 and 5.5), this may be due to an anti-drug immune reaction stemming from the fact that the IgG2a antibody is derived from a foreign species (rat). In order to determine whether this is occurring, plasma from untreated mice and mice receiving IgG2a could be tested for the presence of antibodies against rat immunoglobulins via an ELISA. If it is shown that anti-drug antibodies are present, this would have extensive implications for required controls when testing the effect of monoclonal antibody drugs in preclinical research. An untreated (vehicle) control group should always be included in addition to the isotype control group, to determine whether an anti-drug immune reaction or other off-target effects are occurring.

**Investigating the effect of exercise on metastatic spread:** In the clinic, around 90% of cancer deaths occur from metastatic disease [313]. Therefore, prevention of metastasis is an important strategy in cancer treatment. The effect of exercise on metastasis was not able to be investigated in this study as no metastases were found in any group (although a small number of mice did develop EO771 tumours in the peritoneal cavity, but as these were not accompanied by a tumour in the mammary fat pad, it is likely that these resulted
from implant error rather than metastatic spread). Previous work has suggested that exercise may inhibit metastasis [31,35,36], but again, results are heterogeneous, with some studies showing the opposite [24,41]. Therefore, similar approaches to those described above (e.g. the use of mice with a specific genetic variant) could be used to investigate the mechanisms behind the role of exercise in the prevention of metastasis. Ideally, a spontaneously arising and metastasising tumour model would be used, such as the KPC model of pancreatic cancer, in order to properly recapitulate tumour development and progression [314]. Alternative models of metastasis include tail vein injections of tumour cells [40], which do not model tumour cell escape from the primary site, or implantable models which are then resected when they reach maximum size in order to prolong mouse lifespan and allow time for metastatic spread [315]. The effect of exercise could then be assessed by quantifying the number and size of metastases. Given the high mortality caused by metastatic disease, the role of exercise in its prevention is an important avenue of research, not least because it may also identify druggable pathways.

*Studying the effect of exercise on tumour growth in metabolically abnormal populations:* In Chapter 6, I found that muscular COX-IV expression was inversely associated with tumour growth rate and tumour hypoxia in hyperlipidaemic ApoE−/− mice but not wild-type mice. This is in accord with previous work showing that mice on a high-fat diet, but not those on a normal chow diet, had slower tumour growth with exercise [287]. Specifically, the growth rate in the exercised mice on a high fat diet was similar to that of mice on a normal chow diet, suggesting that exercise may ‘normalise’ tumour growth in metabolically abnormal individuals. Given that a high proportion of the population, particularly in Western societies, is overweight or obese, it would be of great clinical relevance to incorporate groups with metabolic abnormalities into future preclinical exercise research.

### 7.4 Conclusion

Although exercise has been reported to improve survival in cancer patients and slow tumour growth in mice, these benefits do not occur universally, the underlying mechanisms are still poorly understood and the interaction of exercise with immunotherapy is unknown. This thesis aimed to contribute to these knowledge gaps by identifying physiological mechanisms whereby exercise might improve cancer
outcomes, and how the interaction of exercise with immunotherapy might further modulate these outcomes. Based on characterisation of the tumour microenvironment and skeletal muscle mitochondria following exercise and/or anti-PD-1 treatment, it can be concluded that exercise and anti-PD-1 interact to produce unique effects on the tumour and muscle in mice, and these effects vary with tumour type. These results highlight the important role of the immune system in mediating the effects of exercise in tumour-bearing mice. In addition, this work has generated the novel hypothesis that immunotherapies may serve as a novel treatment for cancer cachexia when combined with exercise.
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9 Appendix

9.1 Supplementary Material to Chapter 2

9.1.1 Image J Macro Code

*To count total T cells (+ subtypes) per field and T cells (+subtypes) in hypoxic area:

*Start*

```plaintext
file1 = getDirectory("Choose a Directory");
list1 = getFileList(file1);
list1 = Array.sort(list1);
outfile = replace(list1[0],"555 x20 1", "mouse");
outfile = replace(outfile,"tif", "csv");
small=(lengthOf(list1)/5)-1;
for(i = 0; i <= small; i++) {
    open(file1+list1[i]);
    run("Subtract Background...", "rolling=5");
    run("Enhance Contrast...", "saturated=0.5");
    setAutoThreshold("Default no-reset");
    //run("Threshold...");
    setThreshold(7, 255);
    //setThreshold(7, 255);
    setOption("BlackBackground", false);
    run("Convert to Mask");
}
```
run("Make Binary", "thresholded remaining black");

run("Watershed");

run("Analyze Particles...", "size=10-250 show=Outlines summarize");

open(file1+list1[i+5]);
run("Subtract Background...", "rolling=15");
run("Enhance Contrast...", "saturated=0.5");
setAutoThreshold("Default dark no-reset");
//run("Threshold...");
setThreshold(7, 255);
//setThreshold(7, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Watershed");
run("Analyze Particles...", "size=50-1000 show=Outlines summarize");

open(file1+list1[i+10]);
run("Subtract Background...", "rolling=15");
run("Enhance Contrast...", "saturated=0.5");
setAutoThreshold("Default dark no-reset");
//run("Threshold...");
setThreshold(10, 255);
//setThreshold(10, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Watershed");
run("Analyze Particles...", "size=50-1000 show=Outlines summarize");
open(file1+list1[i+20]);
run("Subtract Background...", "rolling=50");
run("Enhance Contrast...", "saturated=0.3");
setAutoThreshold("Default no-reset");
//run("Threshold...");
setThreshold(7, 255);
//setThreshold(7, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Analyze Particles...", "size=5-Infinity show=Outlines summarize");
run("Analyze Particles...", "size=5-Infinity record add");

if (roiManager("count") > 0) {
open(file1+list1[i]);
run("Subtract Background...", "rolling=5");
run("Enhance Contrast...", "saturated=0.5");
setAutoThreshold("Default no-reset");
//run("Threshold...");
setThreshold(7, 255);
//setThreshold(7, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Make Binary", "thresholded remaining black");
run("Watershed");
run("Show Overlay");
roiManager("Deselect");

if (roiManager("count") > 1) {
roiManager("Combine");
} else {
roiManager("Select", 0);
}
run("Analyze Particles...", "size=10-250 show=Outlines summarize");

open(file1+list1[i+5]);
run("Subtract Background...", "rolling=15");
run("Enhance Contrast...", "saturated=0.5");
setAutoThreshold("Default dark no-reset");
//run("Threshold...");
setThreshold(7, 255);
//setThreshold(7, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Watershed");
run("Show Overlay");
roiManager("Deselect");
if (roiManager("count") > 1) {
  roiManager("Combine");
} else {
  roiManager("Select", 0);
}
run("Analyze Particles...", "size=50-1000 show=Outlines summarize");

open(file1+list1[i+10]);
run("Subtract Background...", "rolling=15");
run("Enhance Contrast...", "saturated=0.5");
setAutoThreshold("Default dark no-reset");
//run("Threshold...");
setThreshold(10, 255);
//setThreshold(10, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Watershed");
run("Show Overlay");
roiManager("Deselect");
if (roiManager("count") > 1) {

roiManager("Combine");

} else {

roiManager("Select", 0);

}

run("Analyze Particles...", "size=50-1000 show=Outlines summarize");

roiManager("Delete");

}

}

selectWindow("Summary")

saveAs("Results", "/C:/Users/busli309/Syncplicity Folders/PhD/IF-IHC/Image analysis/Hotspots/C417 and C116/CD8 and Foxp3/Hypoxia/" +outfile);

End

To count number of T cells (+subtypes) in perfused area:

Start

file1 = getDirectory("Choose a Directory");

list1 = getFileList(file1);

list1 = Array.sort(list1);

outfile = replace(list1[0],"555 x20 1", "mouse");

outfile = replace(outfile,"tif", "csv");
small=(lengthOf(list1)/5)-1;

for(i = 0; i <= small; i++) {

open(file1+list1[i+15]);
run("Subtract Background...", "rolling=25");
run("Threshold..."); // open Threshold tool
title = "WaitForUserDemo";
msg = "If necessary, use the \"Threshold\" tool to\nadjust the threshold, then click \"OK\"."
waitForUser(title, msg);
run("Convert to Mask");
run("Remove Outliers...", "radius=40 threshold=10 which=Dark");
run("Fill Holes");
run("Analyze Particles...",
    "size=0-Infinity show=Nothing summarize");
    run("Analyze Particles...", "record add");

if (roiManager("count") > 0) {

open(file1+list1[i]);
run("Subtract Background...", "rolling=5");
run("Enhance Contrast...", "saturated=0.5");
setAutoThreshold("Default no-reset");
//run("Threshold...");
setThreshold(7, 255);

//setThreshold(7, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Make Binary", "thresholded remaining black");
run("Watershed");
run("Show Overlay");
roiManager("Deselect");

if (roiManager("count") > 1) {
roiManager("Combine");
} else {
roiManager("Select", 0);
}
run("Analyze Particles...", "size=10-250 show=Outlines summarize");

open(file1+list1[i+5]);
run("Subtract Background...", "rolling=15");
run("Enhance Contrast...", "saturated=0.5");
setAutoThreshold("Default dark no-reset");

//run("Threshold...");
setThreshold(7, 255);

//setThreshold(7, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Watershed");
run("Show Overlay");
roiManager("Deselect");
if (roiManager("count") > 1) {
    roiManager("Combine");
} else {
    roiManager("Select", 0);
}
run("Analyze Particles...", "size=50-1000 show=Outlines summarize");

open(file1+list1[i+10]);
run("Subtract Background...", "rolling=15");
run("Enhance Contrast...", "saturated=0.5");
setAutoThreshold("Default dark no-reset");
//run("Threshold...");
setThreshold(10, 255);
//setThreshold(10, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Watershed");
run("Show Overlay");
roiManager("Deselect");
if (roiManager("count") > 1) {
    roiManager("Combine");
} else {
    roiManager("Select", 0);
}
run("Analyze Particles...", "size=50-1000 show=Outlines summarize");

roiManager("Delete");

selectWindow("Summary")
saveAs("Results", "/C:/Users/busli309/Synepicity Folders/PhD/IF-IHC/Image analysis/Hotspots/C417 and C116/CD8 and Foxp3/Perfusion/" +outfile);

End

To count haematoxylin-stained nuclei (for analysis of %pHH3+ cells)

Start
file1 = getDirectory("Choose a Directory");
list1 = getFileList(file1);
list1 = Array.sort(list1);
outfile = replace(list1[0],"555 x20 1", "mouse");
outfile = replace(outfile,"tif", "csv");
small=(lengthOf(list1))-1;
for(i = 0; i <= small; i++) {
    open(file1+list1[i]);
    img1 = getTitle();
    run("RGB Color");
    run("Colour Deconvolution", "vectors=[H DAB]");
    selectWindow(img1+" (RGB)-(Colour_1)");
    setOption("BlackBackground", false);
    run("Make Binary");
    run("Watershed");
    run("Analyze Particles...", "size=500-infinity show=Outlines summarize");
}

selectWindow("Summary")
saveAs("Results", "/C:/Users/Linda/Syncplicity/PhD/IF-IHC/Image analysis/pHH3 nuclei count/" +outfile)

End
9.1.2 Examples of pimonidazole staining intensity

Figure 9.1: Representative pimonidazole staining intensities 1-3.
Intensities used alongside percent stained area (modified H score as described in section 2.2.13.3) to quantify staining in Chapter 6. Scale bar represents 200 µm.
9.2 Supplementary Material to Chapter 3

9.2.1 Cage Dividers and Cage Setup

Figure 9.2: Representative cage setup (left) and custom cage divider (right).
Cage setup shows the section for the exercising mouse to the left of the divider, with modified saucer wheel, feeder access and housing. To the right of the divider is the section for the non-exercising mouse, with feeder access and housing. The divider is fitted with numerous small holes to enable air flow and made of clear plastic to enable mice to see, smell and hear each other.

9.2.2 Fluorescence Image Analysis

9.2.2.1 Image Analysis Workflows

Figure 9.3: Representative images of workflow for hypoxic area analysis.
(a) Original image of hypoxic area (white/grey). (b) Binary mask of hypoxic area after processing (background subtraction, contrast enhancing, thresholding). (c) Outline of analysed area. Individual ROIs are labelled with red numbers. This was overlaid on the sister T cell images to identify T cells in hypoxic areas as in Figure 9.5.
Figure 9.4: Representative images of workflow for perfused area analysis.
(a) Original image of perfused area (white/grey). (b) Binary mask of perfused area after processing (background subtraction, thresholding, removing outliers and filling holes). (c) Outline of analysed area. Individual ROIs are labelled with red numbers. This was overlaid on the sister T cell images to identify T cells in perfused areas as in Figure 9.5.

Figure 9.5: Representative images of workflow for T cell analysis.
In this example, CD3⁺ T cells are shown. Analysis occurred analogously for Foxp3⁺ and CD8⁺ T cells. (a) Original image of CD3⁺ T cells (white/grey). (b) Binary mask of CD3⁺ T cells after processing (background subtraction, contrast enhancing, thresholding). (c) Outline of analysed particles for total CD3⁺ T cells in that field. (d) Hypoxic area overlay from Figure 9.3 on mask in (b). (e) Outline of particles analysed for CD3⁺ T cells present in hypoxic area. Analysis of T cells in perfused area occurred analogously to the workflow in (d) and (e). Individual ROIs are labelled with red numbers.

9.2.2.2 T Cell Macro Validation
In order to validate the accuracy of the macros used for image analysis I conducted a comparative analysis of manual versus automated image analysis on a subset of images. For B16-F10 tumours, I had previously manually analysed images of the tumours on a Zeiss AxioObserver Z1 microscope fitted with an ApoTome.2, so I compared the results obtained from these to images of the same tumours taken on a Nikon Ni-U microscope and analysed using a macro in Image J. Despite using different microscopes and images,
I was able to obtain the same results regarding T cell localisation patterns (although the absolute numbers did differ slightly) (Figure 9.6).

![Figure 9.6: Comparison of manual and automated image analysis for the quantification of T cell density in discrete microenvironmental areas in B16-F10 tumours.](image)

Quantification in (a) was done on images taken on a Zeiss AxioObserver Z1 microscope equipped with an ApoTome.2, while quantification in (b) used images taken on a Nikon Ni-U microscope. Images in each analysis were taken of sections from the same tumours. Data presented as individual data points and mean ± 95% CI. Data analysed by two-way ANOVA with Sidak’s multiple comparison’s test. p<0.05*, p<0.01**, p<0.001***. *indicates statistically significantly different from perfusion, same exercise group. n=9-12 per group.

For EO771 tumours, I selected 6 representative samples: 3 from exercising and 3 from non-exercising mice, which had been stained and imaged in different batches. I then directly compared manual and automated analysis on the same images. There were no differences between the mean number of CD3+, Foxp3+ or CD8+ cells counted per field (Figure 9.7a-c). In addition, pairwise analysis showed that although the absolute numbers counted with the two methods did differ slightly, the trends between samples were similar, with both methods detecting whether the sample had comparatively low or high infiltrate (Figure 9.7d-f).
Figure 9.7: Comparison of manual and automated image analysis for the quantification of T cell subset abundance in EO771 tumours.

Images were taken on a Nikon Ni-U microscope and manual and automated image analysis was done on the same images for 6 representative EO771 tumours. Comparison of mean numbers of CD3⁺ (a), Foxp3⁺ (b) and CD8⁺ (c) T cells in manually or automatically (macro) analysed images. Pairwise comparison of numbers of CD3⁺ (d), Foxp3⁺ (e) and CD8⁺ (f) T cells in manually or automatically analysed images. n=6.
Figure 9.8: Exercise and anti-PD-1 treatment do not alter growth rate of B16-F10 or EO771 tumours.
Survival curves for mice bearing B16-F10 (a) or EO771 (b) tumours (endpoint due to tumour size only). Lag phase of tumour growth for mice bearing B16-F10 (c) or EO771 (d) tumours. B16-F10: n=10 per group; EO771: n=8-10 per group. Exponential phase of tumour growth for mice bearing B16-F10 (e) or EO771 (f) tumours. B16-F10: n=6-10 per group; EO771: n=8-10 per group. Data are shown as individual data points and mean with 95% CI.
Figure 9.9: IgG2a and Anti-PD-1 do not alter growth rate of B16-F10 or EO771 tumours.

Survival curves for untreated, non-exercising compared with exercising mice, or mice treated with IgG2a or anti-PD-1, bearing B16-F10 (a) or EO771 (b) tumours (endpoint due to tumour size only). Lag phase of tumour growth for mice bearing B16-F10 (c) or EO771 (d) tumours. B16-F10: n=9-12 per group; EO771: n=8-10 per group. Exponential phase of tumour growth for mice bearing B16-F10 (e) or EO771 (f) tumours. B16-F10: n=6-11 per group; EO771: n=7-10 per group. Data are shown as individual data points and mean with 95% CI. Data analysed by two-way ANOVA.

9.4 Supplementary Material to Chapter 5

In order to optimise protein loading amount for Western blotting (to avoid signal saturation and maintain a reproducible ratio to the loading control) I loaded the same sample on a blot 5 times at different amounts (10, 20, 30, 40 and 50 µg). The linear range for COX-IV was found to be between 10 and 40 µg (Figure 9.10a) and the linear range for GAPDH was between 10 and 30 µg (Figure 9.10b). The ratio of COX-IV to GAPDH was constant at 20 and 30 µg, but it was underestimated at 10 µg (due to very low COX-IV signal) and started to be overestimated above 40 µg as signal saturation occurred for
GAPDH (Figure 9.10c). The linear range for complex I, II and IV was between 10 and 40 µg, while complex III and V did not reach saturation at the loading amounts tested (Figure 9.10d). Based on these results, 25 µg protein was selected as the amount to load, as it fell within the linear range of all proteins tested.

Figure 9.10: Optimisation of protein loading amount for Western blot to detect OXPHOS complexes in mouse skeletal muscle.
Area under the curve values obtained from densitometry of a blot probed for COX-IV (a) and GAPDH (b). The same sample was loaded at 5 different amounts: 10 µg, 20 µg, 30 µg, 40 µg and 50 µg. (c) Ratio of COX-IV to GAPDH at each of the 5 protein loading amounts. (d) Area under the curve values obtained from densitometry of a blot probed for the 5 OXPHOS complexes.

As GAPDH is an enzyme involved in the glycolytic pathway, it is conceivable that it might be changed with exercise. In order to confirm this was not the case and it could be used as a loading control, densitometric analysis was done on Western blots probed for GAPDH, of muscle samples from non-exercising (sedentary, Sed), low exercise (LEx,
wheel access every other day) and high exercise (HEx, wheel access every day) ApoE−/− mice. No difference in GAPDH expression was seen across groups (Figure 9.11).

![Figure 9.11: Representative whole blot image probed for GAPDH (left) and densitometric analysis (right).](image)

Densitometry normalised to the same positive control present on all blots. Sedentary (sed): n=5, low exercise (LEx): n=9, high exercise (HEx): n=6. Data are shown as means ± SD. Quadriceps muscle tissue from ApoE−/− mice was used.

Each sample for Western blot analysis was run in duplicate, with the two samples being run on separate gels. Variation between 9 representative duplicates is shown in Figure 9.12. Overall, little variation was observed within samples, allowing confidence that the difference between animals is biological variation and not technological variation.
Figure 9.12: Variation between Western blot duplicates.

Nine randomly selected muscle homogenate samples were assessed for variation between Western blot duplicates. Each column represents a different sample; the same samples were used for complexes I-V but not cytochrome c and COX-IV. Data are shown as individual data points and mean.