The Influence of Exercise and Hyperlipidaemia on Breast Cancer

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Abstract

Exercise reduces the risk of breast cancer development, and improves survival in breast cancer patients. However, the underlying mechanisms of this protective effect remain to be fully elucidated. It is unclear whether exercise can attenuate or modify the pro-tumour effects of obesity and related conditions, such as hyperlipidaemia, on breast cancer growth. The main aims of this study were (1) to develop a relevant, tumour-bearing mouse exercise model and (2) to determine the effect of exercise and hyperlipidaemia on the breast tumour microenvironment. We hypothesise that exercise attenuates the negative effect of hyperlipidaemia through ‘normalisation’ of the tumour microenvironment.

Hyperlipidaemic ApoE−/− and wild-type (WT) C57BL/6 mice with orthotopic EO771 breast tumours were randomly assigned to intermittent or continuous voluntary wheel running or sedentary control. When tumours reached maximum size, mice were sacrificed and the serum, organs and tumours removed for analysis of tumour cell proliferation, immune infiltrate, circulating inflammatory factors, perfusion, microvessel density, hypoxia, HIF-1α protein level and GLUT-1 protein expression. This was done by immunohistochemistry, immunofluorescence, ELISA and Western blotting.

Although exercise and hyperlipidaemia did not significantly impact tumour growth rate, exercising mice had significantly reduced body weights. Tumour-bearing mice showed a significant increase in serum monocyte chemoattractant protein 1 (MCP-1) compared to non-tumour-bearing mice (p<0.05) and this was further increased in mice bearing internal tumours (p<0.001). Serum MCP-1 was drastically reduced in exercising mice bearing internal tumours.

In addition, analysis of immunofluorescent images revealed that CD3+CD8+ cytotoxic T lymphocytes as a percentage of total T cells was unchanged by either exercise or hyperlipidaemia. The percentage of CD3+FoxP3+ regulatory T cells was significantly reduced in sedentary ApoE−/− compared to sedentary WT mice (p<0.05), and this reduction tended to be attenuated in exercising mice. Furthermore, intratumoral Treg cell percentage inversely correlated with individual running distance in WT mice (p<0.05). Analysis of CD31+ vessels revealed that sedentary ApoE−/− mice had a significant reduction in microvessel density compared to sedentary WT mice (p<0.05). Hypoxia was also significantly reduced in ApoE−/− compared to WT mice (p<0.05), but perfusion was not significantly altered. Further studies are necessary to clarify and confirm these results, as this study was limited both by a short exercise and tumour-bearing time period.
This study identifies a number of key considerations in the design of future preclinical exercise studies in tumour-bearing mice. In addition, our results provide evidence for the potential value of MCP-1 as an exercise-regulated, prognostic biomarker in mouse models, and indicate that hyperlipidaemia normalises the microenvironment of the tumour.
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# Abbreviations

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<th>Explanation</th>
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<tbody>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees celcius</td>
</tr>
<tr>
<td>µ</td>
<td>Micro ($10^{-6}$)</td>
</tr>
<tr>
<td>25HC</td>
<td>25-hydroxycholesterol</td>
</tr>
<tr>
<td>27HC</td>
<td>27-hydroxycholesterol</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ApoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Apolipoprotein E knock-out</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CRF</td>
<td>Cardiorespiratory fitness</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>dH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Deionised water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EE</td>
<td>Environmental enrichment</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>Ex</td>
<td>Exercise</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HE&lt;sub&gt;x&lt;/sub&gt;</td>
<td>High exercise</td>
</tr>
<tr>
<td>HR</td>
<td>Hormone receptor</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>IHC</td>
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<tr>
<td>Abbreviation</td>
<td>Explanation</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LDS</td>
<td>Lithium dodecyl sulfate</td>
</tr>
<tr>
<td>LEx</td>
<td>Low exercise</td>
</tr>
<tr>
<td>m</td>
<td>Milli (10^-3)</td>
</tr>
<tr>
<td>M</td>
<td>Molar (mol/litre)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Michigan cancer foundation 7</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NPC</td>
<td>Nasopharyngeal carcinoma</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with tween-20</td>
</tr>
<tr>
<td>PIC</td>
<td>Protease inhibitor cocktail</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-Page</td>
<td>Sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sed</td>
<td>Sedentary</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour associated macrophage</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with tween-20</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple negative breast cancer</td>
</tr>
<tr>
<td>T_{reg} cell</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>VO_{2peak}</td>
<td>Maximum rate of oxygen consumption during exercise</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Explanation</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>CCL2</td>
<td>CC chemokine ligand 2</td>
</tr>
<tr>
<td>CD3</td>
<td>Cluster of differentiation 3</td>
</tr>
<tr>
<td>CD31</td>
<td>Cluster of differentiation 31</td>
</tr>
<tr>
<td>CD8a</td>
<td>Cluster of differentiation 8a</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte associated protein 4</td>
</tr>
<tr>
<td>CYP27A1</td>
<td>cytochrome P450 oxidase, sterol 27-hydroxylase</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FIH</td>
<td>Factor inhibiting HIF</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead box protein 3</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>Glucose transporter 1</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>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed death ligand 1</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain protein</td>
</tr>
<tr>
<td>pHH3</td>
<td>Phosphohistone H3</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein acidic and rich in cysteine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel Lindau protein</td>
</tr>
</tbody>
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1 Introduction

Cancer is a disease characterised by the uncontrolled proliferation of an organism’s own cells. This can be fatal when either the primary tumour or metastases colonise tissues that are essential to the organism’s survival, such as the brain, lungs or liver. This uncontrolled proliferation is underpinned by key functional capabilities present in all cancers, known as the hallmarks of cancer (1). The six original hallmarks of cancer consist of sustained proliferative signalling, evasion of growth suppressors, activation of invasion and metastasis, replicative immortality, induction of angiogenesis and resistance to cell death (1). Recently, two further hallmarks have been added to this list (deregulation of cellular energetics and avoidance of immune destruction), as well as two characteristics enabling the acquisition of the hallmarks of cancer (genome instability/mutation and tumour-promoting inflammation) (1).

Breast cancer is a global health concern and is the most common cancer diagnosed in New Zealand women (2). It is the third largest contributor to cancer deaths in New Zealand (2). As such, understanding the underlying mechanisms of disease progression is vital to providing better therapies and prevention.

Breast cancer is broadly classified into in situ (non-invasive) and invasive breast cancer (3). It is further classified according to the tissue of origin (ductal or lobular), giving 4 subtypes: ductal carcinoma in situ, invasive ductal carcinoma, lobular carcinoma in situ and invasive lobular carcinoma (4).

Invasive carcinomas are the most common forms of breast cancer and can be classified into different subtypes according to the expression of cell surface receptors (4). The most common type is hormone receptor (HR) positive breast cancer, which expresses the estrogen receptor (ER) and/or the progesterone receptor (PR). ER+ and PR+ tumours grow in response to estrogen or progesterone, respectively, and as such can be treated with hormone therapy (5). In addition, ER+ breast cancer can be further classified based on gene expression profiles to give luminal A and B subtypes (3).

The second type of invasive breast cancer is human epidermal growth factor receptor 2 (HER2) positive breast cancer. HER proteins are activated by the binding of epidermal growth factor (EGF)-like ligands (6). HER2 has no known ligand and initiates an intracellular signalling cascade when activated by heterodimerisation with other ligand-bound HER receptors, which promotes cellular proliferation (6, 7). However, overexpression of HER2 can lead to increased
activation by hetero or homodimerisation and thus stimulate uncontrolled proliferation (7). HER2+ breast cancer can be treated with HER2 antagonistic antibodies such as trastuzumab (8).

The least common invasive breast cancer type is triple negative breast cancer (TNBC), which does not express any of the three receptors mentioned above. TNBC is highly aggressive and difficult to treat due to the absence of targetable receptors (9). In addition, TNBC is of the basal subtype according to classification by gene expression profile (3).

Hyperlipidaemia is the abnormal elevation of serum lipid levels. It is associated with increased risk of cardiovascular disease and is more prevalent in developed countries (10, 11). Although the association is unconfirmed in epidemiological studies as results have been contradictory (12-22), preclinical studies provide strong evidence that hyperlipidaemia may also increase breast cancer risk and worsen prognosis (23). Hyperlipidaemia in the context of breast cancer is discussed further in section 1.3.

Physical activity is important for physical and mental well-being. It can prevent excess weight gain or aid in weight loss, lower serum lipid levels and reduce the risk of cardiovascular disease (24). Furthermore, it is increasingly recognised as an effective, well-tolerated adjunct to cancer therapy (25). It has been associated with reduced breast cancer risk and improved survival of breast cancer patients (26, 27). Types of exercise include resistance training, aerobic exercise and training that includes a mindfulness component such as Tai chi. For the purpose of this introduction the different training types used in epidemiological studies will not be differentiated between; rather the effect of exercise in general will be assessed (section 1.4).

The current study investigates the effect of hyperlipidaemia and exercise on the tumour microenvironment using hyperlipidaemic apolipoprotein E knockout (ApoE−/−) mice with orthotopic breast cancer. These mice are allowed to exercise freely on running wheels and are compared to wild-type (WT) and sedentary controls.

1.1 Tumour Microenvironment

A tumour consists not only of transformed tumour cells, but also of a variety of other cell types including immune cells, cells of the vascular and lymphatic systems, and other stromal cells such as fibroblasts and adipocytes (28). As such, the tumour microenvironment encompasses these different cell types, the presence of hormones and growth factors, as well as physical and chemical factors such as hypoxia, pH and the structure of the microenvironment (29-31). The
interactions between malignant and non-malignant cells within the tumour play a key role in the tumour phenotype and disease progression.

1.1.1 Immune Microenvironment

The immune system comprises a vital part of mammalian organisms. It serves to protect the host from infection, and must be able to adapt to meet challenges from constantly evolving pathogens. 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 (32). It can be divided into two major arms: the innate and the adaptive immune system.

![Image](image-url)

**Figure 1.1: Overview of the immunosuppressive tumour microenvironment.**

Tumour cells employ diverse strategies to promote an immunosuppressive microenvironment. Crosstalk between tumour cells and immune cells results in a range of tumour-resident, immunosuppressive cell types such as M2 tumour-associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs) and regulatory T cells (T_{reg} cells). These cells suppress the tumour-killing capabilities of cytotoxic cells such as natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) (33, 34).
The innate immune system provides a rapid, unspecific response to an invader. Phagocytes such as macrophages destroy pathogens through phagocytosis after recognition of pathogen associated molecular patterns (35). Activation of the adaptive immune system (primarily B and T cells) is achieved through the recognition of specific antigens presented on major histocompatibility complexes (MHC) on the surface of antigen-presenting cells (primarily dendritic cells) (36). This enables a pathogen-specific response.

The immune microenvironment of a tumour is largely immunosuppressive (Figure 1.1; (33)). Tumour cells employ strategies such as the secretion of immunosuppressive cytokines, the expression of inhibitory molecules such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed death ligand 1 (PD-L1), and the downregulation of MHC-I molecules to evade immune recognition (33, 37). This promotes an immunosuppressive phenotype of infiltrating innate and adaptive immune cells, resulting in large proportions of M2 tumour-associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs) and regulatory T cells (T\textsubscript{reg} cells) (38). These cell types in turn inhibit the cytotoxic capabilities of cells such as natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) (36).

1.1.2 Hypoxia and HIF Transcription Factors

Hypoxia is the deficiency of oxygen within a tissue and has been associated with a more aggressive tumour phenotype (39). This is due in large part to the activation of a group of hypoxia sensitive transcription factors, the hypoxia inducible factors (HIF) 1-3. HIF transcription factors consist of two subunits, the oxygen-sensitive alpha (α) and the constitutively expressed beta (β) subunit (40). When sufficient oxygen is present, HIF-α is hydroxylated at two proline residues by prolyl hydroxylase domain proteins (PHDs) (Figure 1.2) and at an asparagine residue by factor inhibiting HIF-1 (FIH-1) (41). Hydroxylation of the proline residues enables binding of the von Hippel Lindau protein (VHL), which mediates ubiquitination and degradation of HIF-α, and hydroxylation of the asparagine residue blocks recruitment of the coactivator p300/CBP (41). These hydroxylation reactions require oxygen and α-ketoglutarate as substrates (41); thus, enzyme activity is inhibited when oxygen is low, leading to the stabilisation of HIF-α. HIF-α then dimerises with HIF-1β in the nucleus and binds to hypoxia response elements (HRE) in the genome, resulting in the transcription of target genes (41). In addition, iron and ascorbate are required as cofactors for the HIF hydroxylases (42, 43). HIF-1α and HIF-2α are better characterised than HIF-3α and, despite sharing many target genes, induce subtly different responses to hypoxia (43). Nevertheless, they have both been implicated
in increased tumour cell proliferation and survival, as well as increased angiogenesis and metastasis (41).

Figure 1.2: Regulation of HIF-1α.
In normoxia, HIF-1α is hydroxylated by PHDs and bound by the VHL protein. This is followed by polyubiquitination and proteosomal degradation. In hypoxic conditions, the enzymatic activity of the PHDs is inhibited, resulting in the stabilisation and nuclear translocation of HIF-1α. In the nucleus, HIF-1α forms a complex with HIF-1β and p300/CBP. This complex binds hypoxia response elements (HREs), leading to the transcription of genes responsible for cellular adaptation to hypoxia. Figure adapted from (44).

HIF-1 directly targets more than 100 genes (45). These include genes responsible for cell proliferation and survival, tumour metastasis, angiogenesis and cell metabolism (Figure 1.2 (44, 45)). Key target genes of HIF-1 involved in tumour growth and metastasis include glucose transporter 1 (GLUT1), vascular endothelial growth factor (VEGF) and matrix metalloproteinase 2 (MMP2) (46-49). In addition, HIF-1 downregulates E-cadherin expression (49).
GLUT-1 supports tumour growth by mediating glucose transport into the cell to provide fuel for rapidly proliferating tumour cells, which rely largely on glycolysis for their energy needs (50). VEGF is involved in the induction of angiogenesis and the aberrant development of tumour vasculature (51-53). This is discussed in more detail in the next section. Increased expression of MMP-2 and decreased expression of E-cadherin contribute to epithelial to mesenchymal transition (EMT), resulting in increased invasiveness of cells and metastasis (49, 54).

1.1.3 Tumour Vascularisation and Angiogenesis

Just like any other tissue, tumours require adequate blood flow in order to receive the oxygen and nutrients necessary for cell growth and proliferation. As such, when tumours reach a size which exceeds the oxygen diffusion distance (100-200 µm), regions of hypoxia develop (55). This stimulates the induction of pro-angiogenic genes such as VEGF, a HIF-1 target gene (56). VEGF plays a major role in the induction of angiogenesis. It binds to the VEGF receptors 1 and 2 on endothelial cells, thereby stimulating angiogenesis (51, 53, 57). However, the overexpression of different VEGF isoforms by tumour cells results in a chaotic, abnormal vasculature system, which often perpetuates hypoxic regions rather than alleviating them as controlled vascularisation would (52). This disorganised vasculature system consists of vessels which are not mature or structurally sound, resulting in leaky, dysfunctional vessels with large intercapillary distances (58, 59). This results in hypoxia and chronic overexpression of HIF-1α and thus VEGF, further aggravating abnormal vascularisation (exemplified by hotspots of high microvessel density) and other hypoxic, pro-tumorigenic responses.

1.2 Obesity and Breast Cancer

Obesity is an increasing health problem in developed countries worldwide. It has been associated with an increased risk of developing breast cancer, as well as with a poorer prognosis (26, 27, 60). It has been shown that adipocytes in the immediate vicinity of the tumour (cancer associated adipocytes) interact with breast cancer cells, causing them to become more invasive as well as providing them with metabolites (61). In addition, obese adipose tissue is characterised by chronic low-grade inflammation. Hypertrophic adipocytes in obese adipose tissue can grow to a size of 150-200 µm in diameter, thus reaching or exceeding the maximum oxygen diffusion distance (62). This results in a hypoxic state, leading to the activation of HIF-1, subsequent tissue fibrosis and the increased secretion of inflammatory adipokines, as well as
macrophage infiltration (62). This inflammation has been associated with further metabolic
dysregulation (62).

1.3 Hyperlipidaemia and Breast Cancer

Hyperlipidaemia is commonly comorbid with obesity, but its implications as an independent
risk or prognostic factor in breast cancer are much less clear. Epidemiological studies have
produced contradictory results regarding the effect of hyperlipidaemia, particularly
hypercholesterolaemia, on breast cancer risk and progression (12-22). In connection with this,
the role of statins and other lipid lowering drugs on breast cancer risk and progression is unclear
(13, 16, 63-67), although two recent meta-analyses of observational studies found that statin
use was associated with reduced breast cancer recurrence and/or mortality (68, 69). These
discrepancies may be due to the inherent limitations of epidemiological studies, as well as
differences in methodology, influences of different cancer treatments and possible differences
in the roles of high density lipoprotein cholesterol (HDL-C) and low density lipoprotein
cholesterol (LDL-C). Indeed, a recent retrospective study found that patients with
nasopharyngeal carcinoma (NPC) with pre-treatment serum LDL-C greater than 3.64 mmol/L
had reduced distant metastasis-free and overall survival, while abnormal HDL-C and total
cholesterol were not correlated with prognosis (70). Furthermore, serum cholesterol is reduced
in cancer patients, as it is rapidly utilised by proliferating tumour cells (71, 72). Thus, it is
possible that some results from studies examining the role of serum cholesterol in breast cancer
risk may be confounded by undiagnosed malignancies (73).

The role of triglycerides is clearer. The majority of studies suggest that ω-3 fatty acids are
protective, while ω-6 polyunsaturated fatty acids are pro-tumorigenic (74-81).

On the other hand, preclinical studies show great consensus in that hyperlipidaemia increases
breast tumour growth rate, incidence and metastasis (82-86). In addition, breast tumours from
hyperlipidaemic mice are more proliferative (83, 85, 86), have reduced apoptosis (85) and show
increased microvessel density (83-85). Increased tumour incidence has also been demonstrated
in hyperlipidaemic models of prostate and colorectal cancer (87, 88), and increased
proliferation, rate of pulmonary metastasis and microvessel density have been corroborated in
a prostate cancer model (87).

A number of mechanisms to explain the observed pro-tumorigenic effect of hyperlipidaemia in
rodent studies have been proposed, mainly focussing on the effect of hypercholesterolaemia
(Figure 1.3). Firstly, Alikhani et al. demonstrated that cholesterol induces Akt signalling, and suggested that cholesterol-mediated activation of the PI3K/Akt pathway may be causative for the increased proliferation of breast tumour cells (82). However, the concentration of cholesterol required for activation of the PI3K/Akt pathway was much higher than that required for an increase in cellular proliferation, making it unlikely that this is the primary mechanism responsible for cholesterol-mediated cancer cell proliferation.

**Figure 1.3: Potential mechanisms of cholesterol in breast cancer progression.**

It has been postulated that cholesterol exerts a pro-tumorigenic effect in a number of ways. It promotes proliferation through the action of its metabolite, 27-hydroxycholesterol (27HC), on the estrogen receptor (ER). In addition, it may increase macrophage recruitment, activate the liver X receptor (LXR) through 27HC, induce androgen signalling and/or induce PI3K/Akt signalling. ? denotes unverified or, in the case of PI3K/Akt signalling, potentially non-physiological mechanisms. Schematic summarises findings or hypotheses of published works (82, 85, 89).

Secondly, it has been reported that 27-hydroxycholesterol (27HC), an abundant primary cholesterol metabolite generated by the cytochrome P450 oxidase, sterol 27-hydroxylase (CYP27A1), exerts pro-tumorigenic effects (23, 90). Two independent studies have shown that 27HC promotes MCF-7 breast cancer cell xenograft growth (89, 91), and one of these studies
also showed that 27HC promotes tumour growth in an immunocompetent model (89). Two mechanisms have been proposed to explain this. First, 27HC can function as an ER agonist (92). ER signalling is an important driver of ER+ breast cancer growth, and as such activation of this pathway by 27HC could provide an explanation for the increased growth rate of ER+ breast tumours in a hyperlipidaemic environment. Second, Nelson et al. postulate that 27HC stimulates liver X receptor (LXR) signalling, thereby promoting EMT and metastasis (89). However, their data is inconclusive (and as yet unconfirmed), as a synthetic LXR agonist did not promote metastasis to the same extent as 27HC in ER- breast tumour xenografts (89).

Thirdly, 25HC has been implicated in a similar way to 27HC in increased breast cancer cell proliferation through the activation of ER-signalling (93).

The above ER-mediated effect of 27HC and 25HC does not provide an explanation for the observed pro-tumorigenic effect of cholesterol on ER+ breast tumours in a study conducted by Pelton et al. (85). The authors of that study suggest that cholesterol-induced tumour progression could be mediated by androgen signalling or monocyte/macrophage recruitment (85); however both hypotheses are yet to be validated.

Taken together, 27HC-mediated ER-signalling provides the most solid mechanism for increased proliferation of ER+ breast tumour cells in a hyperlipidaemic environment, but does not explain cholesterol-mediated proliferation of ER- breast tumour cells. Thus, further studies are necessary to elucidate the mechanism(s) behind the more rapid tumour growth and increased metastasis occurring in a hyperlipidaemic host.

1.4 Exercise and Cancer

1.4.1 Epidemiological Studies

Exercise has been associated with a decreased risk of developing breast cancer (26, 27). In addition, numerous epidemiological studies have investigated the association between exercise and cancer prognosis (including breast, colorectal, prostate, ovarian, non-small cell lung cancer and glioma) (94-113). The majority have found that exercise improves survival (94, 96, 98-113). The magnitude of this decrease in mortality ranged from 15-57% for all-cause mortality and 20-67% for cancer-specific mortality. Both studies that found no association between exercise and survival were observational studies (95, 97), which are prone to bias by over-reporting of exercise frequency/intensity (114); this may have skewed the results. In addition,
the volume and intensity of physical activity required for the improved prognosis vary between studies. As such, the optimal therapeutic exercise dose remains to be elucidated.

Emerging evidence indicates that cardiorespiratory fitness (CRF) may be an important prognostic marker (102, 112, 115-118). A single study has specifically investigated the CRF-prognosis relationship in breast cancer patients, finding a non-significant improvement in survival in patients with metastatic disease with VO2peak > 1.09 L/min compared to those with VO2peak < 1.09 L/min (VO2peak is the maximum rate of oxygen consumption during exercise) (116). Multiple studies have found that breast cancer patients have reduced CRF compared to healthy individuals, placing them at increased risk of cardiovascular disease (reviewed in (119)). Moreover, CRF can be improved by exercise training, thereby reducing this risk (119). Therefore, it seems clear that improvement of CRF by exercise training can improve survival by reduction of the risk of cardiovascular events, but the relationship between CRF and breast cancer-specific mortality warrants further research.

1.4.1.1 Biomarkers in Cancer Patients

Exercise is well known to modulate levels of blood-based biomarkers. As such, it is of interest whether an association can be found between biomarkers modulated by exercise and cancer prognosis. A number of studies have found changes in the levels of metabolic and/or inflammatory biomarkers in breast cancer survivors following exercise training, as described below (120-137).

The main metabolic biomarkers that have been investigated to date are factors of the insulin-glucose axis. Insulin-like growth factor 1 (IGF-1) has mitogenic and anti-apoptotic effects, while IGF binding protein 3 (IGFBP-3) regulates the activity and bioavailability of IGF-1 (138). As such, high serum levels of IGF-1 and/or low levels of IGFBP-3 have been associated with an increased risk of developing breast cancer, as well as aiding breast cancer progression (122, 139, 140). Therefore, modifying levels of these factors could be important for cancer outcome. Studies investigating the effect of exercise on levels of these factors have indicated that exercise reduces serum IGF-1 and/or increases IGFBP-3 in breast cancer survivors (123, 127-129); however, these results are inconsistent between studies, with some reporting opposite effects or no change in one or both markers (127, 128, 134). Nevertheless, a meta-analysis of randomised controlled trials in breast cancer survivors concluded that exercise was significantly associated with a reduction in circulating IGF-1 levels, despite non-significant results in some of the primary studies (141). In addition, high levels of IGF-1 and an increased IGF-1:IGFBP-3 ratio
(indicative of free IGF-1) have been correlated with decreased survival in breast cancer patients (142, 143). Taken together, modulation of serum IGF-1 and the IGF-1:IGFBP-3 ratio by exercise may be of prognostic value in breast cancer patients.

A second metabolic biomarker that has been shown to be modulated by exercise is leptin. Leptin is an adipokine that has a wide array of physiological roles and is secreted by white adipose tissue, which functions as an energy storage site and endocrine organ (138). Leptin is present in higher levels in obese or overweight individuals (127, 135, 144). High levels of leptin are associated with an increased breast cancer risk (138, 145), and leptin has been shown to promote breast cancer cell growth both in vitro and in vivo (146, 147). A number of studies have found that exercise decreases circulating leptin levels in breast cancer survivors (121, 127, 135, 137). However, some studies have found no reduction from baseline in circulating leptin levels following an exercise intervention (131, 133, 136). This could be explained by no or only a small decrease in body mass index (BMI), suggesting that weight loss rather than exercise itself is more important for the reduction of serum leptin. This is supported by a significant decrease in circulating leptin in 3 studies where weight loss was the goal (121, 137, 144). In addition, a recent study has demonstrated that alteration of leptin levels by exercise training is dependent on body fat changes (148).

C reactive protein (CRP) is a common marker of systemic inflammation and is associated with an increased risk of cardiovascular disease (149). In addition, CRP has been associated with decreased overall and disease-free survival in breast cancer patients (150). The majority of studies investigating the effect of exercise on CRP levels in breast cancer survivors found a decrease in CRP levels in exercise groups (120, 124-126, 132, 137). Two studies found no difference, but of these one had a baseline level of CRP comparative to that of healthy individuals (136), suggesting that CRP levels may not have been sufficiently elevated for exercise to cause a reduction, and the other identified that their exercise dose may not have been high enough to elicit a response (130). Taken together, there is strong evidence that exercise reduces CRP in breast cancer survivors. Therefore, CRP is an important prognostic biomarker that can be modulated by exercise training.

Interleukin 6 (IL-6) is a myokine released from skeletal muscle during exercise, resulting in up to a 100-fold increase in serum IL-6 levels (151). It is postulated to mediate some exercise-induced anti-inflammatory effects by inhibiting tumour necrosis factor α (TNF-α) and IL-1 production, as well as inducing IL-1 receptor antagonist (IL-1ra) and IL-10 (151). On the other
hand, increased serum IL-6 has also been associated with a poor prognosis in breast cancer (152). Studies investigating the effect of exercise on serum IL-6 levels in breast cancer survivors have reported no significant changes (126, 129, 130, 133, 136, 137). However, a recent meta-analysis has indicated that exercise reduces serum IL-6, despite non-significant results in a number of the initial studies (153). Taken together, the association between exercise, breast cancer and IL-6 is unclear and warrants further research.

Monocyte chemoattractant protein 1 (MCP-1), also known as CC chemokine ligand 2 (CCL2), is the primary chemokine responsible for attracting monocytes and immature macrophages to peripheral sites (154). As such, it plays an important role in the recruitment of monocytes and macrophages to the tumour, where they are programmed by factors in the microenvironment to take on either a pro-tumour M2 phenotype or an anti-tumour M1 phenotype (155). In general, a large number of TAMs is associated with poor prognosis in breast cancer (155). Similarly, intratumoral MCP-1 expression is linked to increased macrophage infiltration and poorer prognosis (reviewed in (156)). To our knowledge, no epidemiological studies have investigated the influence of exercise on serum levels of MCP-1 in breast cancer patients, and data on serum MCP-1 levels in breast cancer patients are inconclusive, with some reporting an increase and others reporting no change (reviewed in (156)). However, recent preclinical mouse studies indicate that serum MCP-1 is significantly elevated in tumour-bearing animals compared to non-tumour-bearing controls (157, 158). In addition, exercise may attenuate this increase (157). Taken together, MCP-1 shows promise as a prognostic biomarker that may be modulated by exercise, but further studies, in both animal models and humans, are required to confirm this.

The abovementioned biomarkers are among those most commonly investigated with regards to exercise and cancer, and may prove to be of prognostic value. However, none of these were robust predictors of survival, and as such it would be of value to identify other biomarkers that are regulated by exercise and may play a role in breast cancer outcome.

1.4.2 Preclinical Studies

1.4.2.1 Effect of Exercise on Tumour Growth

Numerous preclinical studies have attempted to elucidate the role of exercise in tumour progression and/or the impact on the tumour microenvironment (159-182). However, these studies have produced conflicting results with regards to the effect of exercise on tumour growth, with some reporting inhibited tumour growth (159, 162, 163, 165, 166, 169, 173, 176, 179, 181), some reporting mixed results (164, 168, 174) and others reporting no inhibition of
growth (160, 161, 167, 170-172, 175, 177, 178, 180). These discrepancies may be explained by differences in animal model (immunocompetent versus immunodeficient), mode of exercise (forced versus voluntary), and other confounding factors such as stress caused by individual housing. It is therefore important that a model system be developed that minimises stress factors and mimics the clinical situation as closely as possible.

Forced exercise paradigms, such as treadmill running, have been identified as a source of stress for rodents, increasing levels of corticosteroids and changing normal circadian rhythm (183-186). This may be a confounding factor in studies using forced exercise to investigate the effect of exercise on tumour progression. Indeed, of the 13 studies found using forced exercise (164, 168-174, 176, 178, 180-182), only 4 reported an inhibition of tumour growth (169, 173, 176, 181), whereas of the 10 studies found using voluntary exercise (159-163, 165, 166, 175, 178, 179), 6 reported an inhibition of tumour growth (159, 162, 163, 165, 166, 179). This suggests that stress caused by forced exercise may confound beneficial effects caused by exercise training.

### 1.4.2.2 Effect of Exercise on the Tumour Microenvironment

Despite the discrepant results regarding tumour growth, some progress has been made towards determining changes in the tumour microenvironment following exercise training (Figure 1.4; (159-182)). Jones et al. have shown in multiple studies that exercise increases intratumoral perfusion, thereby normalising the tumour microenvironment (normalisation: remodelling of the microenvironment to more closely resemble that of normal tissue) (159-161). These results were consistent across different cancer types (breast and prostate), immunocompetent and immunodeficient mice, and despite varying impact on tumour growth. Furthermore, McCullough et al. have reported increased intratumoral perfusion in prostate tumours of Copenhagen rats during acute exercise (182); however, they failed to investigate whether this effect was maintained beyond exercise duration. Nevertheless, an increase in perfusion is likely to cause a reduction in hypoxia, thereby reducing the aggressiveness of the tumour. Indeed, some of the abovementioned studies have reported reduced hypoxia alongside increased perfusion (159, 182). Increased perfusion may also improve the delivery of therapeutic agents.

However, the effect of exercise on intratumoral perfusion may differ depending on tumour location. Garcia et al. conducted a recent study in which they compared the effect of exercise on intratumoral blood flow in orthotopic and ectopic (subcutaneous) prostate tumours, and found directly opposing effects in that blood flow was increased to the orthotopic tumour, but
decreased to the ectopic tumour during exercise (187). This raises important considerations for the study design of preclinical exercise studies, as results from studies using an ectopic model may not reflect true physiological results. Additionally, the blood flow and perfusion effects of exercise may vary according to tumour type, even in orthotopic models.

Figure 1.4: Proposed Mechanisms for the Effects of Exercise on the Tumour Microenvironment.

Exercise has been reported to have multiple effects on tumour growth and the tumour microenvironment. It induces increased perfusion through increased microvessel density and maturity, which results in decreased hypoxia and a less aggressive tumour as well as improved drug delivery. In addition, it promotes anti-tumour immunity and stimulates the release of myokines from skeletal muscle. This reduces proliferation and increases apoptosis of tumour cells. Moreover, exercise may upregulate the estrogen receptor (ER). Taken together, exercise induces favourable effects in the tumour microenvironment, resulting in inhibited tumour growth and metastasis. Figure summarises results from published studies (159, 162, 180, 188, 189).

Two studies by the same group have shown an increase in HIF-1α protein levels in tumours of exercising animals (160, 161), while a further study from a different group indicates a decrease in intratumoral HIF-1α mRNA following exercise training (181). Unfortunately, none of these studies assessed intratumoral hypoxia itself, and as such it cannot be conclusively stated that the expression of HIF-1α reflected tumour hypoxia as HIF-1α expression can also be regulated independently of oxygen tension (190). As such, the effect of exercise training on intratumoral hypoxia and HIF-1α expression remains to be confirmed.

Further modifications of the tumour microenvironment caused by exercise include increased apoptosis and increased microvessel density and maturity, providing potential mechanisms for reduced tumour growth and improved perfusion, respectively (159, 160, 166, 168). A single
study reports contradictory results, showing a reduction in apoptosis and blood vessel density in the tumours of exercising mice (164). However, this study used forced treadmill running to exhaustion in order to investigate the effect of intense, prolonged exercise on tumour growth and the tumour microenvironment, and the method of detection of apoptosis and vessel density was suboptimal (quantification of haematoxylin and eosin (H&E) stained slides without a specific marker for the structures of interest). Thus, these results may not accurately reflect the effect of therapeutic exercise on the tumour microenvironment.

A recent study in Sprague-Dawley rats has found that exercise increases tumoral expression of the ER (180). The authors suggest that this may be a favourable change as it would make tumours easier to treat (via endocrine therapy). However, this is a preliminary result that requires validation and further research into its implications.

Physical activity may also induce favourable changes in the immune microenvironment of the tumour. Studies have shown that exercise training may cause a phenotypic shift in TAMs from a pro-tumour M2 to an anti-tumour M1 phenotype (reviewed in (155)). In addition, recent data suggests that NK cells are mobilised in response to exercise, resulting in increased infiltration of the tumour and suppression of tumour growth (162). Exercise training may also cause an increase in the number of intratumoral CTLs and a reduction in immunosuppressive Treg cells (165, 191). Taken together, exercise seems to alleviate the immunosuppressive microenvironment present in tumours, thereby enhancing anti-tumour immunity.

Furthermore, secretion of exercise-induced myokines from skeletal muscle may impact tumour growth. It has been demonstrated that a number of different myokines reduce tumour growth or tumour cell proliferation by as yet unidentified mechanisms (189, 192, 193). Identified myokines include secreted protein acidic and rich in cysteine (SPARC), oncostatin M (OSM) and irisin.

### 1.4.2.3 Validity of Wheel Running as an Experimental Model

A concern with using wheel running in an experimental setting is the question whether this reflects natural behaviour or is an artefact of captivity. It has been argued that wheel running is unnatural and may reflect neurosis or stereotypy, thus providing a suboptimal model for ‘normal’ exercise (194-196). In order to answer this question, Meijer et al. investigated whether wild animals would use an exercise wheel (197). They found that mice voluntarily used exercise wheels, with and without the lure of food, and that bout length was similar to that of C57BL/6 mice in the laboratory (197). This indicates that running in an exercise wheel is not solely an
artefact of captivity. However, Mason and Würbel comment that laboratory wheel running may still be indicative of pathology, as abnormal behaviours can arise from normal behaviours, particularly in a stressful or poor quality environment (198). Nevertheless, the results of the study performed by Meijer et al. provide evidence that wheel running is a natural behaviour, and as such can be used reliably as an exercise model, provided stress is minimised.

1.4.2.4 Environmental Enrichment

Environmental enrichment (EE) provides a varied, stimulating cage setup for laboratory mice. EE has been shown to have an anti-tumour effect by decreasing tumour size, inducing favourable changes in serum biomarkers and hypothalamic gene expression, reducing tumour cell proliferation and increasing apoptosis (177, 199-201). Of relevance to the current study, EE setups tend to include running wheels. This provides a concern in experimental investigation of the effect of exercise on tumour growth that results are confounded by EE. Cao et al. have attempted to address this by directly comparing EE with wheel running in a tumour model (177). They found that EE mice ran approximately 66% less than running mice, and that the serum biomarker and hypothalamic gene expression profiles differed between the two groups. This suggests that EE and exercise-mediated effects are physiologically distinct. Furthermore, all studies investigating the effect of EE on tumour growth used a comprehensive, varied cage setup with large numbers of animals per cage, whereas voluntary exercise setups house animals individually or in small groups, and differ from standard housing solely by the addition of a running wheel (and a larger cage to accommodate the wheel). These differences make it unlikely that EE would play a major role in any effects observed in exercise studies.

1.5 Apolipoprotein E Knockout Mice

Apolipoprotein E (ApoE) is a protein that plays a major role in lipid transport around the body (202, 203). It forms complexes with other lipid transport proteins in order to bind lipids and transport them through the bloodstream as part of a lipoprotein complex (203). ApoE binds to a variety of receptors on the cell surface (including the LDL receptor and its family members), thereby initiating receptor-mediated endocytosis leading to lipid uptake from the bloodstream (203).

ApoE\(^{-/-}\) mice are deficient in ApoE. As a result, these mice exhibit elevated serum cholesterol and triglyceride levels due to inhibited cellular lipid uptake, without accompanying insulin resistance or hyperglycaemia (204).
1.6 Study Objectives

A number of preclinical studies have investigated the effect of exercise on the tumour microenvironment (159-161, 164, 166, 168, 182). However, to our knowledge none have done so in a hyperlipidaemic model. Numerous preclinical studies have indicated that hyperlipidaemia, and high cholesterol in particular, increase tumour growth rate and metastasis (82-86). Furthermore, there is increasing evidence that exercise may improve survival of breast cancer patients (26, 27, 94, 96, 98-101, 110, 113). Therefore, it is of interest to determine whether exercise can ameliorate the negative effects caused by hyperlipidaemia, thereby reducing tumour growth rate and inducing favourable microenvironmental changes.

The two major aims of this study were (1) to develop a relevant mouse exercise model that mimics the clinical situation of increasing exercise post-diagnosis and (2) to determine the impact of exercise on the breast tumour microenvironment in hyperlipidaemic ApoE−/− mice. These included the following specific objectives (comparing WT to ApoE−/− mice and sedentary to exercising mice):

- To characterise the mouse exercise model with regard to mouse and organ weights, distance run, and tumour growth and proliferation;
- To determine changes in the immune microenvironment of the tumour, specifically measuring infiltrate of selected T cell subsets;
- To identify a possible serum biomarker of exercise in tumour-bearing mice
- To determine changes in the vascular and oxic microenvironment of the tumour.

Hypothesis

We hypothesise that exercise normalises the tumour microenvironment in both WT and hyperlipidaemic mice, resulting in a reduction in tumour growth rate or signs of tumour aggression.

Overview of experimental design

The current study used ApoE−/− mice as a model for hyperlipidaemia. This model is briefly described in section 1.5.

ApoE−/− and WT mice were randomly assigned to three activity groups: sedentary, low exercise and high exercise (n=10-15 per group), in order to obtain graded running groups to attempt to
narrow down the minimum exercise dose required for a response. Exercise began directly after tumour cell inoculation and continued until sacrifice. Mice were implanted orthotopically with syngeneic EO771 breast tumour cells and sacrificed when tumours reached a maximum size of 600 mm$^3$. Tumours, organs and plasma were harvested for analysis. Specifically, mouse body weight, organ weight and running distance were measured. In addition, we investigated the following microenvironmental aspects: tumour cell proliferation, T cell infiltrate, perfusion, microvessel density and hypoxia, as well as serum levels of circulating inflammatory factors.
2 Material 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% 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 BoltTM transfer buffer</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
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<tr>
<td>20x NuPAGE MOPS SDS running buffer</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
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<tr>
<td>Absolute ethanol</td>
<td>LabServ, Thermo Fisher Scientific, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>Amersham Enhanced Chemiluminescence Prime Western blotting Detection Reagent</td>
<td>GE healthcare, Auckland, New Zealand</td>
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<tr>
<td>Amphotericin</td>
<td>Thermo Fisher Scientific, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>Bicinchoninic acid solution</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
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<td>Sigma-Aldrich, Auckland, NZ</td>
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<tr>
<td>BSA</td>
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</tr>
<tr>
<td>Copper II sulfate solution</td>
<td>Sigma-Aldrich, Auckland, NZ</td>
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<td>DMEM with GlutaMax and L-D glucose</td>
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<td>DTT</td>
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<tr>
<td>Dual Endogenous Enzyme Block</td>
<td>Dako, Copenhagen, Denmark</td>
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<tr>
<td>FCS</td>
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</tr>
<tr>
<td>KCl</td>
<td>BDH VWR, Radnor, PA, USA</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>BDH VWR, Radnor, PA, USA</td>
</tr>
<tr>
<td>LDS sample buffer</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Methanol</td>
<td>LabServ, Thermo Fisher Scientific, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>BDH VWR, Radnor, PA, USA</td>
</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>
</tr>
<tr>
<td>Name</td>
<td>Supplier</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>Penicillin/streptomycin</td>
<td>Thermo Fisher Scientific, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>ProLong Diamond antifade mountant</td>
<td>Thermo Fisher Scientific, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>ProLong Gold antifade mountant with DAPI</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>Sigma-Aldrich, Auckland, NZ</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>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>
</tbody>
</table>

### 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</td>
<td>100 mM</td>
</tr>
<tr>
<td>10x PBS</td>
<td>NaCl, KCl, Na₂HPO₄, KH₂PO₄</td>
<td>1.37 M, 27 mM, 101 mM, 18 mM</td>
</tr>
<tr>
<td>10x TBS, pH 7.6</td>
<td>Tris, NaCl</td>
<td>200 mM, 1.37 M</td>
</tr>
<tr>
<td>1x citrate buffer</td>
<td>10x citrate buffer, Tween-20</td>
<td>1x (10 mM), 0.05% (v/v)</td>
</tr>
<tr>
<td>1x PBS</td>
<td>10x PBS</td>
<td>1x</td>
</tr>
<tr>
<td>1x PBST</td>
<td>10x PBS, Tween-20</td>
<td>1x 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>
</tr>
<tr>
<td>1x running buffer</td>
<td>20x NuPAGE MOPS SDS running buffer dH₂O</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 dH₂O</td>
<td>1x 10% (v/v)</td>
</tr>
<tr>
<td>Culture media +/- antibiotics/antimycotics</td>
<td>DMEM with GlutaMax and L-D glucose FCS Penicillin/streptomycin Amphotomycin</td>
<td>1x 10% (v/v) 1% (v/v) 0.1% (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>Mild stripping buffer, pH 2.2</td>
<td>Glycine SDS Tween-20 dH₂O</td>
<td>0.2 M 0.1% (v/v) 1% (v/v)</td>
</tr>
<tr>
<td>RIPA buffer, pH 8.0</td>
<td>NaCl Tris NP-40 (IGEPAL CA-630) Sodium deoxycholate SDS dH₂O</td>
<td>150 mM 50 mM 1% (v/v) 0.5% (v/v) 0.1% (v/v)</td>
</tr>
</tbody>
</table>

### 2.1.3 Kits

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EnVision™ G/2 System/AP Rabbit/Mouse (Permanent Red) kit</td>
<td>Dako, Copenhagen, Denmark</td>
</tr>
<tr>
<td>Hypoxyprobe™ 1-1000 kit</td>
<td>Hypoxyprobe, Inc., Massachusetts, USA</td>
</tr>
<tr>
<td>Mouse C reactive protein ELISA kit (PTX1) (ab157712)</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Mouse MCP-1 ELISA kit (ab100721)</td>
<td>Abcam, Cambridge, UK</td>
</tr>
</tbody>
</table>
2.1.4 Antibodies

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

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalogue Number</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3</td>
<td>ab21703</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-CD31</td>
<td>ab124432</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-CD8a</td>
<td>14-0808-80</td>
<td>eBioscience, San Diego, CA, USA</td>
</tr>
<tr>
<td>Anti-FoxP3</td>
<td>14-5773-80</td>
<td>eBioscience, San Diego, CA, USA</td>
</tr>
<tr>
<td>Anti-GLUT-1</td>
<td>ab14683</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-HIF-1α</td>
<td>AF1935</td>
<td>R&amp;D Systems, Minneapolis, USA</td>
</tr>
<tr>
<td>Anti-pHH3</td>
<td>ab5176</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-Pimonidazole</td>
<td>HP1-1000 kit</td>
<td>Hypoxyprobe, Inc., Massachusetts, USA</td>
</tr>
<tr>
<td>Anti-β-actin</td>
<td>AF4000</td>
<td>R&amp;D Systems, Minneapolis, USA</td>
</tr>
<tr>
<td>Donkey anti-rabbit Alexa Fluor 594</td>
<td>ab150076</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Donkey anti-rat Alexa Fluor 488</td>
<td>ab150153</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Goat-anti-mouse-HRP</td>
<td>P044701</td>
<td>Dako, Copenhagen, Denmark</td>
</tr>
<tr>
<td>Rabbit-anti-goat-HRP</td>
<td>P044901</td>
<td>Dako, Copenhagen, Denmark</td>
</tr>
</tbody>
</table>

2.1.5 Mouse Strains and Cell Lines

Table 2.5: List of mouse strains and cell lines and their origin

<table>
<thead>
<tr>
<th>Mouse strain/cell line</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE&lt;sup&gt;−/−&lt;/sup&gt; (mouse)</td>
<td>Bred in house (originally generated by Piedrahita &lt;i&gt;et al.&lt;/i&gt; (205))</td>
</tr>
<tr>
<td>C57BL/6 (mouse)</td>
<td>Bred in house</td>
</tr>
<tr>
<td>EO771 (cell line)</td>
<td>Dr Andreas Moeller, QIMR Berghofer, Australia</td>
</tr>
</tbody>
</table>

2.2 Methods

2.2.1 Cell Culture

EO771 cells are a medullary breast adenocarcinoma cell line originally derived from a C57BL/6 mouse (206), found to be ER<sup>−</sup> in a previous study by our group (207). Cells were brought up
from liquid nitrogen and initially cultured in DMEM culture media (Table 2.2) with antibiotics/antimycotics for 24 hours at 37°C and 5% CO₂ in a 75 cm² flask. Following this, the media was changed to culture media without antibiotics/antimycotics (as antibiotics/antimycotics are undesired for injection into mice). Cells were split as required by washing with PBS and then detaching from the flask by adding 1-5 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 were prepared for injection as follows. When they reached approximately 80% 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 counted using a haemocytometer and spun down again. Finally, cells were resuspended in PBS at a concentration of 10x10⁶ cells/mL.

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 Scientific, Waltham, MA, USA) placed at -80°C before transfer to liquid nitrogen.

### 2.2.2 General in vivo Methods

#### 2.2.2.1 Ethical Approval

Ethical approval for this study was obtained from the University of Otago Animal Ethics Committee (C01/16). International guidelines on animal welfare in experimental neoplasia were strictly followed (208). 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 Setup

Mice were housed in either a standard mouse or rat cage in pairs or groups of three (see below for details) under a 12:12 hour light-dark cycle. The temperature was maintained around 22°C. Cages were swapped out for fresh cages as required or every 2 weeks at the latest. Mice were kept on a normal chow diet, provided ad libitum along with water.

Sedentary control mice were housed in a Safesealplus greenline IVC GM500 mouse cage with a floor area of 501 cm² (Tecniplast, Buguggiate, Italy). Exercising mice were housed in a Safesealplus greenline IVC GR 900 rat cage with a floor area of 904 cm² (Tecniplast, Buguggiate, Italy) containing 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 (Architectural Design Engineer, Tait Communications). 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. Mice were housed in pairs or groups of three (the latter only in the sedentary control group when uneven numbers of mice were available). Mice in the high exercise group were allowed constant access to a running wheel, while those in the low exercise group had access every other day.

### 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 EO771 Cells

EO771 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), and 20 µL of cell suspension (2x10⁵ cells) were injected into the 4th mammary fat pad of 6-10 week old female C57BL/6 or ApoE⁻/⁻ mice using a 29-31 gauge needle. Mice were ear notched for identification and allowed to recover before being placed into a cage. Mice were monitored daily for tumour growth and general well-being. Tumour size was measured with callipers at its longest and widest points and the volume estimated using the following formula:

\[
\text{Tumour volume} = \text{width}^2 \times \frac{\text{length}}{2}
\]

### 2.2.2.5 Euthanasia and Organ Harvest

When tumour volume reached the ethical limit of 600 mm³ 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 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 10 000 rpm for 10 minutes and the supernatant (serum) transferred to a fresh cryovial. Serum was stored at -80°C until analysis. In addition, the tumour, liver, kidneys, heart and spleen were removed for
analysis, as well as internal tumours if present. Internal tumours are highly aggressive tumours established from tumour cells that have escaped either from the primary tumour or from initial implantation, and approximate the condition of peritoneal carcinomatosis in breast cancer patients (209). All organs were immediately placed on ice and promptly weighed. The heart and kidneys were discarded following weighing. The tumour was cut into three portions (if big enough) – one third was frozen at -80°C for Western blot analysis, 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. Portions of the liver and whole spleens were similarly processed, and the remainder discarded.

2.2.3 Tissue Lysate Preparation

In order to analyse the protein content of tissues by Western blotting, the tissues first need to be lysed. To this effect frozen tumour samples were ground into a fine powder using a mortar and pestle on dry ice before lysis in 200 µL ice cold RIPA buffer (Table 2.2) with freshly added protease inhibitor cocktail (PIC). Samples were then sonicated for 10-15 seconds and spun down at 10 000 rpm for 10 minutes at 4°C. The supernatant was transferred to a new tube and the pellet discarded. Samples were stored at -80°C.

2.2.4 Quantification of Protein Content in Tissue Lysates by BCA Assay

Reagent A (bicinchoninic acid solution) and reagent B (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 (1:10) 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 the Wallac 1420 Victor3 microplate reader (PerkinElmer Life and Analytical Sciences, Massachusetts, USA) at 544 nm. Protein concentrations of the lysates were estimated from the BSA standard curve.

2.2.5 Separation of Proteins by SDS-Page

Samples were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) by taking 40 µg of tissue lysate (as determined by the BCA test) and adding 5 µL of LDS sample buffer, 2 µL of DTT and RIPA buffer up to 20 µL before incubating for 10 minutes at 70 °C. Next, 5 µL (10 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 running buffer (Table 2.2) until the protein marker reached the end of the gel.

### 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 75 minutes at 25 V in transfer buffer (Table 2.2) with constant cooling. Blots were cut into two to enable detection of HIF-1α and GLUT-1 without the need for stripping. Following this the membrane was blocked in either 3% bovine serum albumin (BSA) or 5% skim milk in TBST at room temperature for 1h. The blocking solution was antibody dependent (Table 2.6). The membrane was then incubated overnight at 4°C with the primary antibody at the appropriate dilution (Table 2.6), with the exception of β-actin which was incubated for 1h at room temperature. Next, the membrane was washed three times in TBST for 10 minutes before incubation with the secondary antibody in the appropriate blocking buffer (HIF-1α and GLUT-1) or TBST (β-actin) for 1h at room temperature. After a further three washing steps in TBST the membrane was developed using the Amersham Enhanced Chemiluminescence Prime Western blotting Detection Reagent according to the manufacturer’s instructions before imaging with the UVItec Alliance 4.7 (Uvitec, Cambridge, UK). Automatic exposure times were used in order to prevent saturation of the signal.

In order to detect β-actin, the membrane was stripped using mild stripping buffer (Table 2.2) for 5 minutes at room temperature before washing 3x for 5 minutes in TBST. The membrane was then blocked and reprobed.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution (primary antibody)</th>
<th>Secondary antibody</th>
<th>Dilution (secondary antibody)</th>
<th>Blocking Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>1:800</td>
<td>Rabbit-anti-goat-HRP</td>
<td>1:10 000</td>
<td>3% BSA in TBST</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>1:10 000</td>
<td>Goat-anti-mouse-HRP</td>
<td>1:10 000</td>
<td>5% skim milk in TBST</td>
</tr>
<tr>
<td>β-actin</td>
<td>1:10 000</td>
<td>Goat-anti-mouse-HRP</td>
<td>1:10 000</td>
<td>5% skim milk in TBST</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 (10 µg EO771 tumour lysate) and between samples within the same blot by the use of β-actin as a loading control. Specifically, the sample band intensity was first divided by the control band intensity. This value was then divided by the β-actin band intensity for that sample.

2.2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

2.2.7.1 Detection of Serum MCP-1

The amount of MCP-1 in serum samples was determined using the Mouse MCP-1 ELISA kit (Table 2.3) according to the manufacturer’s instructions. Specifically, 100 µL of each protein standard or diluted serum sample (1:5) were added to the appropriate wells of a 96 well plate pre-coated with MCP-1 antibody and incubated for 2.5 hours at room temperature with gentle shaking. Plates were then washed 4x with the wash solution provided with the kit. 100 µL of biotinylated MCP-1 detection antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking before 4 further washes. Next, 100 µL of HRP-streptavidin solution was added to each well and incubated for 45 minutes at room temperature with gentle shaking. The plate was then washed 4x. 100 µL of TMB One-Step substrate reagent was added to each well and incubated for 30 minutes at room temperature, in the dark, with gentle shaking. Finally, 50 µL of stop solution was added to each well and the absorbance measured immediately at 450 nm by the Wallac 1420 Victor³ microplate reader (PerkinElmer Life and Analytical Sciences, Massachusetts, USA). MCP-1 protein concentration was calculated by interpolation from a standard curve ranging from 2.74 pg/mL-2000 pg/mL using the MCP-1 protein standard supplied with the kit.

2.2.7.2 Detection of Serum CRP

The amount of CRP in serum samples was determined using the Mouse C reactive protein ELISA kit (PTX1) (Table 2.3) according to the manufacturer’s instructions. Specifically, 100 µL of each protein standard or diluted serum sample (1:10) were added to the appropriate wells of a 96 well plate pre-coated with CRP antibody and incubated for 10 minutes at room temperature with gentle shaking. Plates were then washed 4x with the wash solution provided with the kit. 100 µL of enzyme-antibody conjugate was added to each well and incubated for 10 minutes at room temperature with gentle shaking before 4 further washes. Next, 100 µL of TMB substrate solution was added to each well and incubated for 5 minutes at room
temperature, in the dark, with gentle shaking. Finally, 100 µL of stop solution was added to each well and the absorbance measured immediately at 450 nm by the Wallac 1420 Victor³ microplate reader (PerkinElmer Life and Analytical Sciences, Massachusetts, USA). CRP protein concentration was calculated by interpolation from a standard curve ranging from 0.78 ng/mL-25 ng/mL using the CRP protein standard supplied with the kit.

2.2.8 Cryosectioning

OCT embedded samples were cut to a thickness of 8 µm and adhered to either Superfrost Plus adhesion slides (Thermo Fisher Scientific, Sunnyvale, CA, USA) or Polysine adhesion slides (Thermo Fisher Scientific, Sunnyvale, CA, USA). Adhered sections were stored at -20°C in the dark (to protect Hoechst 33342 from photobleaching) prior to analysis or further processing.

2.2.9 Immunofluorescence

2.2.9.1 Perfusion

Intratumoral perfusion was assessed using Hoechst 33342 as a perfusion marker. Frozen sections were coverslipped manually using ProLong Diamond Antifade mountant. Slides were left at 4°C overnight in the dark to allow the mountant to set before imaging with a Zeiss AxioObserver Z1 microscope (equipped with an ApoTome.2 to enable optical sectioning).

Sections were quantified by counting the number of Hoechst perfused vessels per field at 10x magnification for 5 random fields and then calculating the average.

A second method of quantification was used in order to enable comparison of vessel number and perfused area as measures of perfusion for the same images. The percent perfused area per field was quantified using Image J software and averaged over 5 fields.

2.2.9.2 T Cells

Intratumoral T cell infiltrate was analysed by immunofluorescent staining for CD3⁺CD8⁺ CTLs and CD3⁺FoxP3⁺ Treg cells as follows.

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 2.7) 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 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).

Sections were quantified by counting the total number of CD3+ T cells and number of either CD3+CD8+ CTLs or CD3+FoxP3+ Treg cells per field at 40x magnification. An average number of cells per field was obtained for 5 random fields. The relative proportion of CTLs and Treg cells of total T cells was obtained by calculating the percentage of CTLs or Treg cells of total T cells.

Table 2.7: Antibody dilution scheme for immunofluorescence

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3</td>
<td>Prediluted</td>
<td>Donkey anti-rabbit Alexa Fluor 594</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-CD8a</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>

2.2.10 Immunohistochemistry

2.2.10.1 Method

FFPE blocks were cut into 10 consecutive sections at 3-5 µm and the first section H&E stained by Gribbles Veterinary, Christchurch. Tumour sections were stained using primary antibodies (Table 2.8) and the EnVision™ G/2 System/AP Rabbit/Mouse (Permanent Red) kit. 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 washed 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. Slides were then washed for 5 minutes in PBST before incubation with the primary antibody (Table 2.8) overnight at 4°C. Next, slides were washed 3x for 5 minutes in PBST before incubation with the secondary antibody for 30 minutes at room temperature. Following this, slides were washed twice for 5 minutes in PBST before incubation with AP Enzyme (enhancer) for 10 minutes at room temperature. This was followed by a further two 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 2.8). Slides were then placed in distilled water for at least 5 minutes and counterstained by incubation in haematoxylin for 45 seconds, followed by a rinse in tap water, brief wash in acid/alcohol and incubation in Scott’s tap water (a blueing reagent) for 20 seconds. Slides were then dehydrated by graded alcohol washes (80%, 95%, 95% and 100%) and cleared in xylene. Coverslipping was performed by an automated system (Leica Autostainer XL, Leica Biosystems, Nussloch, Germany).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Developing Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-pHH3</td>
<td>1:100</td>
<td>13</td>
</tr>
<tr>
<td>Anti-CD31</td>
<td>1:200</td>
<td>10</td>
</tr>
<tr>
<td>Anti-pimonidazole</td>
<td>1:1000</td>
<td>14</td>
</tr>
</tbody>
</table>

2.2.10.2 Quantification

All scoring was done in a blinded manner by one (pHH3, CD31 and necrosis) 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 at 40x magnification 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 at 40x magnification.
CD31
Angiogenic potential was estimated by counting the number of CD31 positive vessels in 5 hotspot areas at 40x magnification 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. Specifically, the percent area of the tumour which was positive for pimonidazole was multiplied by the intensity of the staining (scored from 1-3; supplementary Figure 7.4), giving a score between 0 and 300.

Necrosis
Necrosis was estimated as percentage tumour area covered by tissue with typical histological signs of necrosis from H&E stained FFPE sections.

2.2.11 Statistical Analysis
All data were analysed using GraphPad Prism 5. The Kolmogorov-Smirnov normality test was used to determine if data was from a Gaussian distribution. Correlations were determined using Pearson correlation. Comparison between more than two groups was performed using one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison post-hoc test to determine differences between individual groups. Comparison between two groups was done using an unpaired, two-tailed student’s t test. P values less than 0.05 were considered significant.
3 Mouse Model Development

3.1 Introduction

Epidemiological evidence indicates that exercise has a protective effect against breast cancer development, and slows tumour progression (96, 98). Data from preclinical studies, however, remain inconclusive, likely due to discrepancies in tumour, animal and exercise model (158-163, 165, 166, 175, 178, 179). This highlights the need for a clinically relevant preclinical exercise model, to enable further research into the mechanisms behind the protective effect observed in epidemiological studies.

The effect of hyperlipidaemia, on the other hand, remains controversial due to conflicting evidence from epidemiological studies (13, 21). In contrast, data from preclinical work uniformly indicates that hyperlipidaemia causes accelerated tumour growth (82-86).

To our knowledge, the current study is the first to investigate the effect of exercise on breast cancer in conjunction with hyperlipidaemia, and the first to investigate the effect of hyperlipidaemia on breast cancer using ApoE<sup>-/-</sup> mice on a chow diet.

3.2 Chapter Aim and Experimental Approach

The primary aim of this chapter was to develop a clinically relevant mouse model for research in exercise oncology.

The specific objectives of this chapter were as follows:

- To characterise differences in running behaviour between WT and hyperlipidaemic ApoE<sup>-/-</sup> mice;
- To determine the effect of exercise and hyperlipidaemia on the body and organ weight of mice;
- To determine the effect of exercise and hyperlipidaemia on tumour growth rate and proliferation.

Running behaviour was characterised by measurement of running distance per cage per 24 hours. Running wheels were equipped with a magnetic sensor and digital counter to measure revolutions; these were then used to calculate running distance per 24 hours (section 2.2.2.2).
Mice were weighed at tumour cell inoculation and daily for the duration of the study. The heart, liver and kidneys were removed immediately after sacrifice and promptly weighed (section 2.2.2.5).

Tumour growth rate was estimated by daily calliper measurement of tumour length and width followed by calculation of tumour volume according to the following formula:

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

Proliferation was assessed by immunohistochemical analysis of pHH3 (2.2.10). pHH3 stained sections were quantified by counting pHH3 positive cells in 10 random fields at 40x magnification and dividing the average by the average total number of cells per field (calculated from total cell count of 4 random fields). The result was then multiplied by 100 to give the average percentage of proliferating cells per field.

3.3 Results

3.3.1 Characterisation of Running Behaviour in WT and ApoE\(-/\) Mice

To our knowledge, this is the first study to investigate the effect of exercise on breast cancer in a hyperlipidaemic model. We therefore wished to determine whether hyperlipidaemia would impact the distance run per day. In addition, we wished to investigate whether tumour-burden would affect running behaviour in mice.

![Figure 3.1: Tumour burden does not affect running behaviour of WT or ApoE\(-/\) mice.](image)

Box and whiskers plot of average running distance of exercising tumour-bearing (T) and non-tumour-bearing (NT) WT and ApoE\(-/\) mice. Data are distance in kilometres per cage of two mice. WT-T and ApoE-T: n=6; WT-NT and ApoE-NT: n=2.
Pairs of WT mice ran an average of $14 \pm 0.7$ km/day and ApoE$^{-/-}$ mice ran an average of $10 \pm 0.5$ km/day. We observed no difference in average daily running distance between tumour-bearing and non-tumour-bearing mice (Figure 3.1). In addition, daily running distances were similar to reported values of 4-10 km for female C57BL/6 mice (162, 210). This indicates that tumour-burden does not significantly impact the running behaviour of WT or ApoE$^{-/-}$ mice.

In contrast, pairs of high exercising WT mice ran significantly more ($14 \pm 0.7$ km/day) than pairs of high exercising ApoE$^{-/-}$ mice ($10 \pm 0.5$ km/day) (Figure 3.2a; p<0.01). In addition, pairs of low exercising WT mice tended to run more ($12 \pm 0.9$ km/day) than pairs of low exercising ApoE$^{-/-}$ mice ($8 \pm 0.6$ km/day) (Figure 3.2b; p=0.07).

Taken together, these results indicate that while mice are unhindered by orthotopic EO771 tumour burden (to a maximum volume of 600 mm$^3$), hyperlipidaemia influences the running behaviour of mice by reduction of average daily running distance.

![Graphical representation of average daily running distance of high exercising (HEx, a) and low exercising (LEX, b) WT and ApoE$^{-/-}$ mice over the course of the experiment. Data are distance per cage of two mice. Distance for HEx are displayed as the average of two consecutive days, and the distance for LEx is the distance run on the days where a wheel was present in the cage. n=6 for all groups except ApoE-LEx, where n=7. Data are shown as individual data points and mean ± SEM, p<0.01**. Data were analysed by unpaired, two-tailed student’s t test.](image)

We were able to obtain individual running data for some mice due to differing tumour growth rates resulting in the earlier sacrifice of one mouse in a cage of two. Of the 14 mice outliving their cage mate long enough to obtain running data, 10 ran similar distances to cages containing two mice, while 4 ran less (Table 3.1). These mice were regrouped accordingly into high exercising (H) and low exercising (L) individuals for further analysis. Genotype and original exercise grouping were disregarded due to low numbers and lack of difference between genotypes or the two original exercise groupings. Where a genotypical difference in results using original groupings was observed, the genotypes were separated. The new groupings were defined according to the average daily running distance for each genotype: L was defined as

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running less than or equal to half of the average daily running distance ± SEM for the appropriate genotype, and H as running more than half of the average daily running distance ± SEM for the appropriate genotype (i.e. L≤5±0.5 km/day for ApoE⁻/⁻ and ≤7±0.7 km/day for WT; H>5±0.5 km/day for ApoE⁻/⁻ and >7±0.7 km/day for WT). A similar distribution of both genotype and original exercise grouping was present in the new groupings. It is important to note that the average measured running distance for some individual mice was higher than the average pair distance (Table 3.1). This may be due to stress caused by individual housing resulting in increased running activity.

Table 3.1: Summary of running data from individual mice. Measured individual running data was compared to data calculated from pairs. Mice were reclassified into below or at (L) vs above (H) ½ mean ± SEM running distance for pairs of their genotype (WT: 7±0.7 km/day; ApoE⁻/⁻: 5±0.5 km/day).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Assumed average distance per mouse (calculated as ½ of pair distance) in km/day</th>
<th>Average distance per individual mouse (measured) in km/day</th>
<th>Original Exercise Grouping</th>
<th>New classification of individual mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6.8</td>
<td>4.1</td>
<td>HEx</td>
<td>L</td>
</tr>
<tr>
<td>WT</td>
<td>5.4</td>
<td>6.2</td>
<td>LEx</td>
<td>L</td>
</tr>
<tr>
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<td>7.4</td>
<td>8.7</td>
<td>LEx</td>
<td>H</td>
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<td>9.9</td>
<td>HEx</td>
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</tr>
<tr>
<td>WT</td>
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<td>11.0</td>
<td>HEx</td>
<td>H</td>
</tr>
<tr>
<td>WT</td>
<td>4.5</td>
<td>11.4</td>
<td>LEx</td>
<td>H</td>
</tr>
<tr>
<td>WT</td>
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<td>HEx</td>
<td>H</td>
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<tr>
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<td>19.1</td>
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<td>H</td>
</tr>
<tr>
<td>ApoE</td>
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<td>LEx</td>
<td>L</td>
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<td>5.9</td>
<td>15.2</td>
<td>HEx</td>
<td>H</td>
</tr>
</tbody>
</table>

3.3.2 Analysis of Body and Organ Weights

Exercise causes favourable changes in body fat composition and can cause weight loss (211). ApoE⁻/⁻ mice are leaner than WT mice, although overall body weight often remains unchanged.
This is likely due to the impaired uptake of plasma lipids into adipose tissue, caused by loss of apolipoprotein E (212).

In order to determine body weight change over the study period, mice were weighed daily following tumour cell inoculation. We observed an increase in weight in sedentary WT mice compared to exercising mice; this was significant for sedentary vs low exercising mice (Figure 3.3, p<0.001). Low exercising WT mice lost more weight over the course of the study than high exercising WT mice (p<0.001).

Figure 3.3: Change in body weight of mice over the study period.
Body weight was measured daily following tumour cell inoculation in high exercising (HEx), low exercising (LEX) and sedentary (Sed) WT and ApoE−/− mice. Data are presented as percent change in body weight from initial body weight at tumour cell inoculation. WT-HEx, WT-LEX and ApoE-HEx: n=12; WT-Sed: n=9; ApoE-LEX: n=13, ApoE-Sed: n=14. Data are shown as individual data points and mean ± SEM. WT-LEX vs WT-Sed p<0.001***; WT-HEx vs WT-LEX p<0.001***; ApoE-HEx vs ApoE-Sed p<0.001***; ApoE-HEx vs ApoE-LEX p<0.001***; ApoE-LEX vs ApoE-Sed p<0.05*, WT-HEx vs ApoE-HEx p<0.001***. Data were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison test to detect differences between individual groups.

Sedentary ApoE−/− mice gained significantly more weight than both high exercising (p<0.001) and low exercising (p<0.05) mice (Figure 3.3). In addition, low exercising ApoE−/− mice gained significantly more weight than high exercising ApoE−/− mice (p<0.001). Our results show that exercise can prevent weight gain and/or cause weight loss.

We observed a significant decrease in weight over the study period in high exercising ApoE−/− mice compared to high exercising WT mice (Figure 3.3; p<0.001). In addition, sedentary ApoE−/− mice tended to gain less weight than sedentary WT mice. However, these differences between genotypes was not observed in the low exercising groups.
Changes in the weights of different organs can give an indication of the health of that organ. We chose to investigate whether exercise and hyperlipidaemia cause weight changes in the kidneys, liver, heart and spleen (spleen weight presented in Chapter 4) of tumour-bearing mice.

![Figure 3.4: Kidney weight is unaltered by tumour burden, exercise, hyperlipidaemia or tumour location.](image)

(a) Analysis of kidney weight in high exercising tumour-bearing (T) and non-tumour-bearing (NT) WT and ApoE⁻/⁻ mice. WT-T: n=11; ApoE-T: n=12; WT-NT and ApoE-NT: n=4. (b) Analysis of kidney weight in high exercising (HEx), low exercising (LEx) and sedentary (Sed) WT and ApoE⁻/⁻ mice. WT-HEx and WT-LEx: n=11; WT-Sed: n=10; ApoE-HEx: n=12; ApoE-LEx: n=13; ApoE-Sed: n=7. (c) Analysis of kidney weight from exercising WT and sedentary ApoE⁻/⁻ mice with orthotopic or internal EO771 tumours. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. WT-Ex: n=22; WT-Internal/Ex: n=2; ApoE-Sed and ApoE-Internal/Sed: n=7. All Data are shown as individual data points and mean ± SEM.

There was no difference in kidney weight between exercising tumour-bearing and non-tumour-bearing mice (Figure 3.4a). In addition, we observed no differences between high exercising, low exercising and sedentary WT and ApoE⁻/⁻ mice (Figure 3.4b). Furthermore, kidney weight was unaltered in mice bearing internal tumours compared to mice bearing orthotopic tumours.
Finally, there were no differences in kidney weight between WT and ApoE⁻/⁻ mice (Figure 3.4a and b). Therefore, kidney weight remains unaffected by tumour burden, exercise, hyperlipidaemia and tumour location.

Similarly, we found no change in liver weight in exercising tumour-bearing compared to non-tumour-bearing mice (Figure 3.5a), between exercising groups or genotypes (Figure 3.5b), or between mice bearing internal tumours compared to those bearing orthotopic tumours (Figure 3.5c). Liver weight is unaltered by tumour burden, exercise, hyperlipidaemia or tumour location.

**Figure 3.5: Liver weight is unaltered by tumour burden, exercise, hyperlipidaemia or tumour location.**

(a) Analysis of liver weight in high exercising, tumour-bearing (T) and non-tumour-bearing (NT) WT and ApoE⁻/⁻ mice. WT-T: n=11; ApoE-T: n=12; WT-NT and ApoE-NT: n=4. (b) Analysis of liver weight in high exercising (HEx), low exercising (LEx) and sedentary (Sed) WT and ApoE⁻/⁻ mice. WT-HEx and WT-LEx: n=11; WT-Sed: n=10; ApoE-HEx: n=12; ApoE-LEx: n=13; ApoE-Sed: n=7. (c) Analysis of liver weight from exercising WT and sedentary ApoE⁻/⁻ mice with orthotopic or internal EO771 tumours. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. WT-Ex: n=22; WT-Internal/Ex: n=2; ApoE-Sed and ApoE-Internal/Sed: n=7. All Data are shown as individual data points and mean ± SEM.
These results indicate that liver weight is not affected by tumour burden, exercise, hyperlipidaemia or tumour location.

**Figure 3.6: Hyperlipidaemia increases heart weight in non-tumour-bearing mice.**

(a) Analysis of heart weight in high exercising, tumour-bearing (T) and non-tumour-bearing (NT) WT and ApoE−/− mice. WT-T: n=11; ApoE-T: n=12; WT-NT and ApoE-NT: n=4. (b) Analysis of heart weight in high exercising (HEx), low exercising (LEx) and sedentary (Sed) WT and ApoE−/− mice. WT-HEx and WT-LEx: n=11; WT-Sed: n=10; ApoE-HEx: n=12; ApoE-LEx: n=13; ApoE-Sed: n=7. (c) Analysis of heart weight in exercising WT and sedentary ApoE−/− mice with orthotopic or internal EO771 tumours. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. WT-Ex: n=22; WT-Internal/Ex: n=2; ApoE-Sed and ApoE-Internal/Sed: n=7. All Data are shown as individual data points and mean ± SEM. p<0.01**: p<0.001***. Data were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison test to detect differences between individual groups.

The heart is required to work harder during exercise than at rest, and as such elite athletes and high exercising individuals may develop a significantly larger heart (213). In addition, hyperlipidaemia can cause the heart to work harder due to the development of atherosclerotic
plaques, likewise causing cardiac hypertrophy (214). We decided to measure heart weight in order to determine whether either exercise or hyperlipidaemia caused cardiac hypertrophy in this model.

Heart weight was significantly increased in non-tumour-bearing ApoE\(^{-/-}\) compared to tumour-bearing ApoE\(^{-/-}\) mice (p<0.001) and compared to non-tumour-bearing WT mice (p<0.01; Figure 3.6a). Unfortunately, we did not obtain heart weight data for low exercising or sedentary non-tumour-bearing ApoE\(^{-/-}\) mice. No difference was observed between tumour-bearing and non-tumour-bearing WT mice (Figure 3.6a), between exercising groups or their genotypes (Figure 3.6b), or between mice bearing orthotopic vs internal tumours (Figure 3.6c).

Taken together, these results demonstrate that neither exercise, hyperlipidaemia, tumour burden nor tumour location influence kidney or liver weight, indicating that there is no major impact on these organs caused by the mouse model used in this study. Furthermore, heart weight is unaffected by tumour location or exercise. However, increased heart weight was observed in non-tumour-bearing ApoE\(^{-/-}\) mice, indicating that hyperlipidaemia may cause cardiomegaly.

### 3.3.3 Analysis of Tumour Growth Rate and Tumour Cell Proliferation

The effect of exercise on tumour growth rate in preclinical models remains controversial due to greatly varying and contradictory results obtained in different studies (157-166, 168-176, 178-182, 215). Hyperlipidaemia, on the other hand, was shown to greatly increase growth rate and proliferation in preclinical models (82, 84, 85), although only one of these used ApoE\(^{-/-}\) mice (82). Data from epidemiological studies remain inconclusive (reviewed in (216)).

Tumour length and width was measured daily by calliper in order to estimate tumour volume, and proliferation was analysed by immunohistochemical staining for the mitotic marker pHH3.

Tumour growth was variable between individual mice, with 72/74 mice inoculated with EO771 cells producing orthotopic tumours, 9/74 mice containing internal tumours, and 2/74 mice containing both primary and secondary cancers. Upon visualisation of the individual tumour growth curves of high exercising, low exercising and sedentary WT and ApoE\(^{-/-}\) mice, we observed no obvious differences either in tumour growth rate or growth rate variability between exercising groups or by genotype (Figure 3.7).
Figure 3.7: Individual tumour growth curves in high exercising, low exercising and sedentary WT and ApoE\(^{-/-}\) mice.

Tumour growth was measured daily by calliper and the length and width used to calculate tumour volume. WT-HEx and ApoE-LEx: n=12; WT-LEx and WT-Sed: n=9; ApoE-HEx and ApoE-Sed: n=10.

Tumour growth rate was further quantified by separation into lag and log phase data. The lag phase was defined as the time for orthotopic tumours to reach 100 mm\(^3\), and the log or exponential phase as the time taken to triple in volume after reaching 100 mm\(^3\).

Figure 3.8: Tumour growth rate is unaffected by exercise and hyperlipidaemia.

(a) Lag phase of tumour growth in high exercising (HEx), low exercising (LEx) and sedentary (Sed) WT and ApoE\(^{-/-}\) mice, defined as the time taken to reach 100 mm\(^3\). WT-HEx and ApoE-LEx: n=12; WT-LEx, ApoE-HEx and ApoE-Sed: n=10; WT-Sed: n=9. (b) Log phase of tumour growth in HEx, LEx and Sed WT and ApoE\(^{-/-}\) mice, defined as the time taken to triple in volume after the lag phase. WT-HEx: n=11; WT-LEx: n=9; WT-Sed: n=7; ApoE-HEx and ApoE-LEx, n=10; ApoE-Sed: n=8. Data are shown as individual data points and mean ± SEM.
No difference was observed in the time taken to reach 100 mm$^3$ in exercising compared to sedentary groups, nor were any differences observed between WT or ApoE/- mice (Figure 3.8a). The same was true for log phase growth (Figure 3.8b). This suggests that tumour growth rate in this model is unaffected by either exercise or hyperlipidaemia.

In order to determine whether differences in tumour growth rates due to exercise were perhaps masked by unequal running distances by individual mice in a cage, we analysed tumour lag and log phase growth in the mice for which we had individual running data (Table 3.1). Mice were regrouped into H or L according to distance run (WT-H>7±0.7 km/day, WT-L<7±0.7 km/day, ApoE-H> 5±0.5 km/day, ApoE-L<5±0.5 km/day). Due to low overall numbers and lack of genotypical difference in growth rate in the original groupings, WT and ApoE/- mice were combined for this analysis.

![Figure 3.9: High exercise tends to increase lag phase of tumour growth.](image)

Individual mice were separated into high exercising (H, n=9) and low exercising (L, n=4) groups according to average distance run per day (Table 3.1), regardless of genotype or original exercise grouping. (a) Lag phase of tumour growth in H and L individual mice, defined as the time taken to reach 100 mm$^3$. Sed: n=19. (b) Log phase of tumour growth in H and L individual mice, defined as the time taken for the tumour to triple in volume. Sed: n=14. Data are shown as individual data points and mean ± SEM.

There was no significant correlation between running distance and the time for the tumour to reach 100 mm$^3$ (supplementary Figure 7.1). Nevertheless, tumours in high exercising mice tended to take longer to grow to 100 mm$^3$ than low exercising or sedentary mice (Figure 3.9a). There was no difference in log phase data (Figure 3.9b). This suggests that exercise may increase tumour latency, but has no effect on the exponential growth of an established tumour.

Tumour cell proliferation was assessed by immunohistochemical staining for pHH3. The percentage of tumour cells staining positive for pHH3 varied between individual tumours, ranging from 0.1 to 3.3%. We observed no differences in proliferation between exercising and
sedentary groups, or between genotypes (Figure 3.10). This indicates that tumour cell proliferation is unaffected by exercise or hyperlipidaemia.

![Figure 3.10: Proliferation of tumour cells is unaffected by exercise or hyperlipidaemia.](image)

(a) Representative immunohistochemical staining for pH3 in high exercising (HEx), low exercising (LEX) and sedentary (Sed) WT and ApoE<sup>-/-</sup> mice. Scale bar represents 200 µm. (b) Quantification of results in (a). Data are expressed as percentage of proliferating cells per field. WT-HEx, WT-LEX and ApoE-Sed: n=12; WT-Sed: n=10; ApoE-HEx: n=11; ApoE-LEX: n=13. Data are shown as individual data points and mean ± SEM.

Next, we assessed proliferation in internal compared to orthotopic tumours. Proliferation was unaltered (Figure 3.11), indicating that tumour location has no impact on EO771 tumour cell proliferation. In addition, exercise did not alter proliferation of internal tumours.
Figure 3.11: Proliferation of EO771 tumour cells is unaltered by tumour location.
(a) Representative immunohistochemical staining for pHH3 in orthotopic and internal tumours from WT and ApoE-/- mice. Scale bar represents 200 µm. Internal tumours from exercising mice were all from WT animals, while internal tumours from sedentary mice were all from ApoE-/- animals. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. (b) Quantification of results in (a). Data are expressed as percentage of proliferating cells per field. WT-Ex: n=24; WT-Internal/Ex: n=2; ApoE-Sed: n=12; ApoE-Internal/Sed=6. Data are shown as individual data points and mean ± SEM.
3.4 Discussion

Increasing evidence indicates that exercise reduces breast cancer risk and improves survival of breast cancer patients (26). In contrast, hyperlipidaemia is postulated to accelerate breast cancer growth and metastasis, although epidemiological evidence remains inconclusive (21). To our knowledge, this is the first preclinical study to investigate the effect of a combination of exercise and hyperlipidaemia on breast cancer.

Running distances obtained in this study were similar to published distances of 4-10 km/day for individual female C57BL/6 mice (162, 210). To our knowledge, no other study has measured running distance of ApoE<sup>−/−</sup> mice. Our data show that ApoE<sup>−/−</sup> run less than WT mice (Figure 3.2), but daily running distances were nevertheless similar to published data for C57BL/6 mice.

3.4.1 Effect of Exercise and Hyperlipidaemia on Running Behaviour, Body and Organ Weight

We observed that ApoE<sup>−/−</sup> mice ran less than WT mice in both high exercise and low exercise groups (Figure 3.2). Despite this, high exercising ApoE<sup>−/−</sup> mice lost significantly more weight than high exercising WT mice, and sedentary ApoE<sup>−/−</sup> mice tended to gain less weight than sedentary WT mice (Figure 3.3). Apolipoprotein E plays a major role in cellular lipid uptake, causing ApoE<sup>−/−</sup> mice to exhibit an impaired ability to take up lipids from the bloodstream (212). This may cause an inability to keep up with the increased energy demand caused by exercise, resulting in greater weight loss in high exercising ApoE<sup>−/−</sup> compared to WT mice. In contrast, Matsumoto et al. observed no difference in body weight change between sedentary and exercising ApoE<sup>−/−</sup> mice (217). However, these mice did not carry a tumour. This suggests that the weight loss observed in exercising ApoE<sup>−/−</sup> mice in the current study may be due, at least in part, to tumour burden creating an increased demand on the body for energy.

The observed reduction in running distance of ApoE<sup>−/−</sup> compared to WT mice may also be explained by the failure to meet cellular demand for energy due to impaired lipid uptake from the bloodstream. However, Maxwell et al. demonstrated that impaired exercise capacity in hyperlipidaemic mice was true for both genetic (ApoE<sup>−/−</sup>) and diet-induced models, indicating that impaired cellular lipid uptake cannot be the only mechanism for the observed reduction in running distance (218). Maxwell et al. postulate that this is due to a defect in the nitric oxide synthase (NOS) pathway, thereby impairing oxygen transport (218).
Running distance in ApoE<sup>−/−</sup> mice may also be reduced due to impaired angiogenesis reducing oxygen transport to working muscles. A number of studies have shown inhibited angiogenesis in ApoE<sup>−/−</sup> mice or other models of hyperlipidaemia (219-221). However, Maxwell <i>et al.</i> did not observe any difference in microvessel density of skeletal muscle in concurrence with impaired exercise capacity in 12 week old mice (218).

Heart weight was significantly increased in exercising non-tumour-bearing ApoE<sup>−/−</sup> mice compared to exercising tumour-bearing ApoE<sup>−/−</sup> mice and non-tumour-bearing WT mice (Figure 3.6a). Non-tumour-bearing mice were approximately one month older at sacrifice than tumour-bearing mice used in this study (due to stock availability), therefore it may be that the observed increase in heart weight in non-tumour-bearing ApoE<sup>−/−</sup> mice was simply due to the greater age of the mice and thus a more advanced atherosclerotic condition.

### 3.4.2 Effect of Exercise and Hyperlipidaemia on Tumour Growth Rate and Proliferation

We observed no difference in tumour growth rate in exercising compared to sedentary mice, regardless of genotype (Figure 3.8). This is in concurrence with some studies (160, 161, 170-172, 175, 178, 180), and in opposition to others (159, 162, 163, 165, 166, 169, 173, 176, 179, 181). Although we controlled for stressors such as forced running (183-185) and individual housing (222, 223) through the use of voluntary running wheels and housing in pairs, our results may be confounded by one mouse in a cage running much more than the other, thus eliciting different responses in individual mice within the same exercise grouping. This is supported by the trend observed for high exercising individual mice to have an increased lag phase compared to low exercising or sedentary individuals (Figure 3.9). In addition, it may be that the exercising period was too short to significantly influence tumour growth rate, particularly in the fast growing EO771 tumours used in this study. Furthermore, it may be that the tumour growth period may not be long enough for differences to become obvious. Data from Betof <i>et al.</i> using the same model of orthotopic EO771 tumour growth in C57BL/6 mice show a significant decrease in tumour growth rate only when tumour volume becomes larger than 600 mm<sup>3</sup>, the maximum ethical size used in our study (159). This further highlights the difficulty in selecting an appropriate model system to effectively research exercise oncology in a manner that mimics the clinical situation as closely as possible, while maintaining appropriate ethical limits.

In concurrence with growth rate data, no change in proliferation of tumour cells was observed in exercising compared to sedentary mice, regardless of genotype (Figure 3.10). This is
supported by published data, in which exercise had no impact on tumour cell proliferation (168). However, this study did not measure growth rate (168). This makes it difficult to determine the relationship between growth rate and proliferation in exercising mice, as tumour growth rate is influenced by a balance of proliferating and apoptotic cells.

In both our study and the study mentioned above (168), tumour cell proliferation was assessed by mean proliferation. However, when a cut-off of 0.54% pHH3 positive cells was used (according to Gerring et al for human breast cancer (224)), internal tumours contained more tumours in the high proliferation category compared to orthotopic tumours (Figure 3.11). This indicates that tumour location may affect tumour cell proliferation, but requires confirmation due to low numbers. When this cut-off was applied to orthotopic tumours, high exercising WT mice contained more tumours in the low proliferation category compared to low exercise or sedentary groups; this trend was not observed in ApoE−/− groups (Figure 3.10). This suggests that tumour cell proliferation is unaffected by hyperlipidaemia, but may be reduced by high exercise in WT mice. Again, low numbers preclude robust conclusions. Moreover, as EO771 tumours exhibit very rapid growth, a higher cut-off point may be more relevant. Further refinement is required in order to identify an optimal cut-off point for the analysis of proliferation in EO771 tumours.

In contrast to a study by Alikhani et al., we observed no difference in tumour growth rate or proliferation in ApoE−/− compared to WT mice ((82), Figure 3.8). However, Alikhani et al. additionally fed mice a high fat, high cholesterol (HFHC) diet, resulting in serum cholesterol levels well above the physiological range. Thus, the results from that study likely do not reflect physiological processes. Other preclinical studies investigating the effect of hyperlipidaemia on tumour growth utilised WT mice on a HFHC diet in order to induce hyperlipidaemia (83-86), which may have other effects on the body which contribute to tumour growth, such as the expansion of adipose tissue and subsequent change in adipokine release (225).

The young age of mice used in this study, short exercising/tumour-bearing time period and pair housing are all factors that may have affected the results in this chapter. These are discussed more fully in Chapter 6.

Taken together, this chapter highlights important differences between ApoE−/− and WT mice as models for investigating the effect of exercise on tumour growth. A greater reduction in body weight due to exercise was observed in ApoE−/− compared to WT mice, despite a lower average
running distance. However, this did not result in a difference in either tumour growth rate or proliferation between ApoE\(^{-/-}\) and WT mice.
4 The Influence of Exercise and Hyperlipidaemia on Anti-Tumour Immunity and Inflammation

4.1 Introduction

Exercise has been reported to favourably modify the immune microenvironment in both animal models and patient studies, resulting in enhanced anti-tumour immunity (165, 191, 226, 227). However, whether this holds true in the presence of comorbidities such as hyperlipidaemia remains to be established, particularly as hyperlipidaemia has been shown to impair immune responses (228-230).

Tumours often cultivate an immunosuppressive microenvironment, allowing them to evade immune surveillance and grow unhindered (33). Characteristic of this is the presence of high numbers of both innate and adaptive immunosuppressive cells, such as M2 macrophages and T<sub>reg</sub> cells, respectively (33, 34). Both cell types are indicative of poor prognosis in breast cancer (231, 232). On the other hand, an increase in CTLs correlates with an improved prognosis in breast cancer patients (233). Preclinical studies indicate that exercise may result in an increased influx of CTLs into the tumour, and a reduction in the number of T<sub>reg</sub> cells, thus reducing immunosuppression within the tumour and enabling increased killing of tumour cells (191, 227).

It is of clinical interest to see whether a biomarker can be found that is modulated by exercise and is predictive of prognosis. The inflammatory proteins CRP and MCP-1 are promising candidates, with CRP in humans (120) and MCP-1 in mice (157) showing evidence for serum or plasma levels being reduced by exercise.

The spleen is a secondary immunological organ, responsible for eliminating pathogens from the blood. As such, it contains large numbers of both innate and adaptive immune cells. It has been known to grow in size in tumour-bearing mice (234).

We hypothesise that exercise enhances the anti-tumour immune response and alleviates the immunosuppressive microenvironment found within tumours, in both WT and ApoE<sup>−/−</sup> mice. In addition, we hypothesise that exercise reduces levels of the inflammatory biomarkers CRP and MCP-1 in the serum of both WT and ApoE<sup>−/−</sup> mice.
4.2 Chapter Aim and Experimental Approach

The primary aim of this chapter was to determine whether exercise induces favourable microenvironmental and systemic immunological changes in normal and hyperlipidaemic mice. ApoE^{−/−} and WT mice at 6-10 weeks of age were randomised to continuous running (HEx), intermittent running (LEx) or sedentary control following orthotopic inoculation of EO771 tumour cells. Each group contained 10-15 mice. Tumours, organs and serum were harvested when tumours reached a maximum size of 600 mm³. Immunological changes between groups were assessed by analysis of intratumoral CTLs, T_{reg} cells and total T cells, as well as measurement of serum levels of CRP and MCP-1. In addition, the spleens of all mice were weighed.

Intratumoral total T cell, CTL and T_{reg} cell numbers were estimated by the analysis of immunofluorescent images (section 2.2.9.2). Specifically, frozen sections were double-stained for CD3 (a pan T cell marker) and either CD8 (a surface marker for CTLs) or FoxP3 (the master transcription factor responsible for the T_{reg} cell phenotype). DAPI was used as a counterstain. Only double-positive cells were counted. T cells were counted in 5 random fields and the average calculated to obtain total T cell number, CTL number and T_{reg} number. In addition, the proportion of CTLs and T_{reg} cells of total T cells was calculated by the percentage of each subset of total T cells, as this likely represents the immune microenvironment of the tumour more accurately than total cell numbers. Samples were scanned by eye to ensure that the images obtained were representative of the entire section. Scoring was done in a blinded manner.

In order to investigate the value of CRP and MCP-1 as biomarkers of exercise, we used commercially available ELISA kits to measure the levels of CRP and MCP-1 in serum from both tumour-bearing and non-tumour-bearing exercising and sedentary WT and ApoE^{−/−} mice (section 2.2.7).

4.3 Results

4.3.1 Analysis of Intratumoral T Cells

4.3.1.1 Analysis of Total T Cell Numbers

In order to determine whether infiltrating numbers of T cells as a whole are altered by exercise or hyperlipidaemia, we calculated the average number of CD3^{+} T cells per field at 40x
magnification for 5 random fields from sections doublestained for CD3 and CD8, and 5 random fields from sections doublestained for CD3 and FoxP3.

![Image of Figure 4.1](image)

**Figure 4.1:** Total T cell number is unchanged by exercise, hyperlipidaemia or tumour location in EO771 tumours.

Quantification of immunofluorescent images. Total T cells were counted from 5 random fields each from 2 consecutive sections to give an average for each tumour. (a) Comparison of high exercising (HEx), low exercising (LEx) and sedentary (Sed) groups from ApoE<sup>-/-</sup> and WT mice. WT-HEx: n=10; WT-LEx: n=12; WT-Sed and ApoE-HEx: n=9, ApoE-LEx: n=11, ApoE-Sed: n=7. (b) Comparison of orthotopic and internal EO771 tumours in WT and ApoE<sup>-/-</sup> mice. Internal tumours from exercising mice were all from WT animals, while internal tumours from sedentary mice were all from ApoE<sup>-/-</sup> animals. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. WT-Ex: n=22; WT-Internal/Ex: n=2; ApoE-Sed: n=7; ApoE-Internal/Sed: n=3. All Data are shown as individual data points and mean ± SEM.

No significant differences in total T cell number between genotype, exercising vs sedentary animals or tumour location was observed (Figure 4.1). This indicates that infiltrating T cell numbers are unaffected by hyperlipidaemia, exercise and tumour location.

### 4.3.1.2 Analysis of Intratumoral Cytotoxic T Lymphocytes

We observed no difference in the percentage of CTLs of total T cells in EO771 tumours between exercise groups (Figure 4.2b), nor did we observe a difference by genotype between WT and ApoE<sup>-/-</sup> mice. However, when considering total CTL influx into the tumour, tumours from sedentary ApoE<sup>-/-</sup> mice showed a significant increase compared to sedentary WT mice (Figure 4.2c; p<0.05). In addition, tumours from sedentary ApoE<sup>-/-</sup> mice showed a significant increase in total CTL number compared to low exercising ApoE<sup>-/-</sup> mice (Figure 4.2c; p<0.05), and a trend for an increased number of CTLs compared to high exercising ApoE<sup>-/-</sup> mice (Figure 4.2c). These results would indicate that exercise results in fewer intratumoral CTLs and thus less
killing of tumour cells in ApoE\textsuperscript{−/−} but not in WT mice. However as this trend is abolished when data are viewed as a ratio of CTL to total T cells, it is likely that the effect is compensated for by the presence of other T cell subsets.

Figure 4.2: Hyperlipidaemia increases the number of intratumoral CTLs in sedentary mice. (a) Representative immunofluorescent images of frozen tumour sections from high exercising (HEx), low exercising (LEx) and sedentary (Sed) groups for WT and ApoE\textsuperscript{−/−} mice. CD3 is labelled in red; CD8 is labelled in green; nuclei are labelled in blue. Double positive cells are visible as yellow/orange. Images are at 40x magnification. Scale bar represents 50 µm. White arrows denote CTLs. (b) Quantification of results in (a). Total T cells and CTLs were counted in 5 random fields to give an average for each tumour, and the percent CTLs of total T cells calculated. WT-HEx: n=10; WT-LEx: n=12; WT-Sed and ApoE-HEx: n=9, ApoE-LEx: n=11, ApoE-Sed: n=7. Data are shown as individual data points and mean ± SEM. p<0.05*. Data were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison test to detect differences between individual groups.
Figure 4.3: The ratio of CTLs to total T cells is reduced in internal compared to orthotopic breast tumours. 
(a) Representative immunofluorescent images from orthotopic and internal EO771 tumours in exercising (Ex) and sedentary (Sed) mice. Internal tumours from exercising mice were all from WT animals, while internal tumours from sedentary mice were all from ApoE-/- animals. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. CD3 is labelled in red; CD8 is labelled in green; nuclei are labelled in blue. Double positive cells are visible as yellow/orange. Images are at 40x magnification. Scale bar represents 50 µm. White arrows denote CTLs. (b) Quantification of results in (a). Total T cells and CTLs were counted in 5 random fields to give an average for each tumour, and the percent CTLs of total T cells calculated. WT-Ex: n=22; WT-Internal/Ex: n=2; ApoE-Sed: n=7; ApoE-Internal/Sed: n=3. Data are shown as individual data points and mean ± SEM. p<0.01**. Data were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison test to detect differences between individual groups. Low sample numbers precluded analysis of Internal-HEx/WT group.
We next compared the CTL to total T cell ratio of internal tumours from sedentary (all ApoE\(^{-/-}\) mice) and exercising (all WT mice) to the appropriate groups of mice bearing orthotopic breast tumours and to each other. We observed that internal tumours from sedentary ApoE\(^{-/-}\) mice contained a significantly lower percentage of CTLs than their counterpart orthotopic tumours (Figure 4.3; \(p<0.01\)). In addition, there was a trend for internal tumours from exercising mice to have an increased CTL percentage compared to those from sedentary mice (Figure 4.3b), but small numbers preclude statistical conclusions. A similar trend for all results was observed upon analysis of average CTL numbers (Figure 4.3c). These results suggest that internal EO771 tumours are better able to evade immune-mediated killing than orthotopic EO771 breast tumours.

In summary, these results suggest that exercise does not appear to greatly influence the level of CTLs within EO771 tumours. Furthermore, they indicate that tumour location plays a significant role in the T cell composition of the tumour.

### 4.3.1.3 Analysis of Intratumoral Regulatory T Cells

We observed a trend for a reduced percentage of intratumoral T\(_{\text{reg}}\) cells in exercising WT mice compared to sedentary WT mice (Figure 4.4b). This trend was maintained upon analysis of average T\(_{\text{reg}}\) cell numbers per field (Figure 4.4c). However, the opposite trend was observed in ApoE\(^{-/-}\) mice, in that exercise increased the percentage of T\(_{\text{reg}}\) cells (Figure 4.4b). This was not observed upon analysis of average T\(_{\text{reg}}\) cell numbers (Figure 4.4c). In addition, there was a significant reduction in the percentage of T\(_{\text{reg}}\) cells within the tumours of sedentary ApoE\(^{-/-}\) mice compared to sedentary WT mice (Figure 4.4b; \(p<0.05\)). This result was not observed upon analysis of average T\(_{\text{reg}}\) cell numbers (Figure 4.4c). These results suggest that hyperlipidaemia favourably alters the immunoregulatory response of the tumour, particularly in sedentary mice, resulting in a less immunosuppressive microenvironment.

We next compared the T\(_{\text{reg}}\) cell to total T cell ratio of internal tumours from sedentary (all ApoE\(^{-/-}\) mice) and exercising (all WT mice) to the appropriate groups of mice bearing orthotopic breast tumours and to each other. Internal tumours from sedentary ApoE\(^{-/-}\) mice had a significantly higher percentage of intratumoral T\(_{\text{reg}}\) cells than orthotopic tumours from sedentary ApoE\(^{-/-}\) mice (Figure 4.5b; \(p<0.001\)). Internal tumours from exercising mice looked to have a similar percentage of T\(_{\text{reg}}\) cells as those from sedentary animals; however low sample numbers preclude drawing conclusions. These results were not observed upon analysis of average T\(_{\text{reg}}\) cell numbers per field (Figure 4.5c).
Figure 4.4: Hyperlipidaemia reduces the ratio of T_{reg} cells to total T cells in orthotopic EO771 tumours.

(a) Representative immunofluorescent images from high exercise (HEx), low exercise (LEx) and sedentary (Sed) groups for WT and ApoE^{-/-} mice. CD3 is labelled in red; FoxP3 is labelled in green; nuclei are labelled in blue. Images are at 40x magnification. Scale bar represents 50 µm. White arrows denote T_{reg} cells. (b) Quantification of results in (a). Total T cells and T_{reg} cells were counted in 5 random fields to give an average for each tumour, and the percent T_{reg} cells of total T cells calculated. WT-HEx: n=10; WT-LEx: n=12; WT-Sed and ApoE-HEx: n=9, ApoE-LEx: n=11, ApoE-Sed: n=7. Data are shown as individual data points and mean ± SEM. p<0.05*. Data were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison test to detect differences between individual groups.
Figure 4.5: The ratio of T\(_{\text{reg}}\) cells to total T cells is increased in internal EO771 tumours compared to orthotopic EO771 breast tumours.

(a) Representative immunofluorescent images from orthotopic and internal EO771 tumours in exercising (Ex) and sedentary (Sed) mice. Internal tumours from exercising mice were all from WT animals, while internal tumours from sedentary mice were all from ApoE\(^{-/-}\) animals. CD3 is labelled in red; FoxP3 is labelled in green; nuclei are labelled in blue. Images are at 40x magnification. Scale bar represents 50 \(\mu\)m. White arrows denote T\(_{\text{reg}}\) cells. (b) Quantification of results in (a). Total T cells and T\(_{\text{reg}}\) cells were counted in 5 random fields to give an average for each tumour, and the percent CTLs of total T cells calculated. WT-Ex: \(n=10\); WT-Internal/Ex: \(n=1^{\#}\); ApoE-Sed: \(n=7\); ApoE-Internal/Sed: \(n=3\). Data are shown as individual data points and mean ± SEM. \(p<0.001^{***}\). ApoE-Sed and ApoE-Internal/Sed groups were compared using a two-tailed, unpaired student’s T test. Low sample numbers precluded statistical analysis of WT-Internal/Ex group. \(^{\#}\) The second sample in the WT-Internal/Ex group was temporarily misplaced during staining of all other samples and unable to be stained at a later time point due to time constraints.
In order to determine whether differences in the T cell microenvironment due to exercise were perhaps masked by unequal running distances by individual mice in a cage, we correlated total intratumoral T cells, CTL percentage and T<sub>reg</sub> cell percentage with the measured running distance of individual mice. We found a significant negative correlation between intratumoral T<sub>reg</sub> cell percentage and running distance for WT, but not for ApoE<sup>−/−</sup> mice (Figure 4.6; p<0.05). No correlation was found between total T cell number or CTL percentage and running distance (supplementary Figure 7.2) for either genotype. This suggests that exercise reduces the proportion of T<sub>reg</sub> cells within the tumour in a dose-dependent manner in WT mice. Small numbers of ApoE<sup>−/−</sup> mice available for this analysis preclude drawing conclusions as to the role of hyperlipidaemia.

![Figure 4.6: Individual running distance negatively correlates with T<sub>reg</sub> cell percentage in WT mice. Correlation of individual mouse running distance with intratumoral T<sub>reg</sub> cell percentage. WT: n=7; ApoE: n=4. p<0.05*. Data were analysed by Pearson correlation.](image)

Contrary to expectation, the results in this section suggest that hyperlipidaemia promotes a less immunosuppressive tumour microenvironment. In addition, tumour location may play a role in the intratumoural T cell composition in ApoE<sup>−/−</sup> mice. Furthermore, exercise reduces intratumoral T<sub>reg</sub> cell percentage in a dose-dependent manner in WT mice.

**4.3.2 Analysis of Circulating Inflammatory Biomarkers**

Exercise has been reported to alter levels of circulating inflammatory proteins such as CRP (in patients) and MCP-1 (in tumour-bearing mice) (120, 157). There is clinical interest in finding a prognostic biomarker that is altered by exercise. CRP and MCP-1 have both shown promise in this area, with reports that they are reduced by exercise in breast cancer patients or tumour-bearing mice, respectively (132, 157).
4.3.2.1 Serum CRP Levels are unaltered by Exercise, Hyperlipidaemia, Tumour Location or Tumour Burden

We first investigated the level of CRP in serum from WT and ApoE\(^{-/-}\) mice. There was no difference in serum CRP between tumour-bearing and non-tumour-bearing exercising mice (Figure 4.7a). In addition, we observed no difference between exercising and sedentary groups, nor between mouse genotypes (Figure 4.7b). Furthermore, there was no change in serum CRP levels of mice with internal tumours compared with those with orthotopic tumours (Figure 4.7c).

Figure 4.7: Serum CRP levels are unaltered by exercise, hyperlipidaemia, tumour location or tumour burden.

Serum CRP levels were analysed by ELISA. (a) Analysis of serum CRP levels in exercising tumour-bearing (T) and non-tumour-bearing (NT) mice. WT-T and ApoE-T: n=11; WT-NT and ApoE-NT: n=4. (b) Analysis of serum CRP levels in high exercising (HEx), low exercising (LEx) and sedentary (Sed) WT and ApoE\(^{-/-}\) mice. WT-HEx and ApoE-HEx: n=11; WT-LEx and WT-Sed: n=9; ApoE-LEx: n=14; ApoE-Sed: n=8. (c) Analysis of serum CRP levels in WT and ApoE\(^{-/-}\) mice with orthotopic and internal EO771 tumours. All exercising mice with internal tumours were WT; all sedentary mice with internal tumours were ApoE\(^{-/-}\). Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. WT-Ex: n=20; WT-Internal/Ex: n=2; ApoE-Ex: n=8; ApoE-Internal/Sed: n=7. All Data are shown as individual data points and mean ± SEM.
These results indicate that circulating CRP levels are unaffected by exercise, hyperlipidaemia, tumour location and tumour burden.

4.3.2.2 Exercise Reduces Serum MCP-1 in Mice with Internal EO771 Tumours

Next we investigated the level of serum MCP-1 in WT and ApoE<sup>−/−</sup> mice. A significant increase was detected in tumour-bearing mice compared to non-tumour-bearing mice for both WT and ApoE<sup>−/−</sup> groups (Figure 4.8a; p<0.05). In addition, sedentary mice bearing internal EO771 tumours showed a highly significant increase in serum MCP-1 compared to those with orthotopic tumours (Figure 4.8c; p<0.001). While no difference was observed between exercising and sedentary mice bearing orthotopic EO771 tumours (Figure 4.8b), a strong trend for the reduction of MCP-1 in exercising WT mice compared to sedentary ApoE<sup>−/−</sup> mice bearing internal tumours was noted; however small sample size precluded statistical analysis (Figure 4.8b and c). This result is unlikely to be due to genotype as no differences were observed in serum MCP-1 levels between WT and ApoE<sup>−/−</sup> mice in any other group.
Figure 4.8: Serum MCP-1 is increased in tumour-bearing mice and reduced by exercise in mice bearing internal EO771 tumours.

Serum MCP-1 levels were analysed by ELISA. (a) Analysis of serum MCP-1 levels in exercising tumour-bearing (T) and non-tumour-bearing (NT) mice. WT-T and ApoE-T: n=11; WT-NT and ApoE-NT: n=3. (b) Analysis of serum MCP-1 levels in high exercising (HEx), low exercising (LEX) and sedentary (Sed) WT and ApoE<sup>−/−</sup> mice. WT-HEx and ApoE-HEx: n=11; WT-Lex and WT-Sed: n=9; ApoE-Lex: n=14; ApoE-Sed: n=8. (c) Analysis of serum MCP-1 levels in exercising and sedentary WT and ApoE<sup>−/−</sup> mice with orthotopic and internal EO771 tumours. All exercising mice with internal tumours were WT; all sedentary mice with internal tumours were ApoE<sup>−/−</sup>. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. WT-Ex: n=20; WT-Internal/Ex: n=2; ApoE-Sed: n=8; ApoE-Internal/Sed: n=7. All Data are shown as individual data points and mean ± SEM. p<0.05*; p<0.001*** Data were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison test to detect differences between individual groups.
4.3.3 Analysis of Spleen Weight

The spleen is a secondary immunological organ, and as such can be an indicator of the degree of an immune response by its size.

Figure 4.9: Spleens from sedentary ApoE<sup>−/−</sup> mice bearing internal EO771 tumours are heavier than those from ApoE<sup>−/−</sup> mice bearing orthotopic EO771 tumours.

(a) Analysis of spleen weights from exercising tumour-bearing (T) and non-tumour-bearing (NT) WT and ApoE<sup>−/−</sup> mice. WT-T: n=11; ApoE-T: n=12; WT-NT and ApoE-NT: n=4. (b) Analysis of spleen weights from high exercising (HEx), low exercising (LEX) and sedentary (Sed) WT and ApoE<sup>−/−</sup> mice. WT-HEx and WT-LEX: n=11; WT-Sed: n=10; ApoE-HEx: n=12; ApoE-LEX: n=13; ApoE-Sed: n=7. (c) Analysis of spleen weights from exercising WT and sedentary ApoE<sup>−/−</sup> mice with orthotopic or internal EO771 tumours. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. WT-Ex: n=22; WT-Internal/Ex: n=2; ApoE-Sed and ApoE-Internal/Sed: n=7. All data expressed as mean ± SEM. p<0.01**. Data were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison test to detect differences between individual groups.
No differences in spleen weight were observed between exercising tumour-bearing and non-tumour-bearing mice (Figure 4.9a). In addition, spleen weight was unaltered by exercise or genotype in mice bearing orthotopic EO771 tumours (Figure 4.9b). In contrast, a trend for decreased spleen weight was observed in exercising compared to sedentary mice bearing internal EO771 tumours; however small sample size precluded statistical analysis (Figure 4.9c). This result is unlikely to be due to hyperlipidaemia as no differences were observed in spleen weight between WT and ApoE<sup>-/-</sup> mice in any other group. Furthermore, a significant increase in spleen weight was observed in sedentary ApoE<sup>-/-</sup> mice bearing internal tumours compared to those bearing orthotopic tumours (Figure 4.9c; p<0.01).

Due to obtaining a similar results trend for spleen weight and serum MCP-1 (Figure 4.8, Figure 4.9), we performed Pearson correlation analysis to determine whether there was an association between serum MCP-1 and spleen weight. Serum MCP-1 and spleen weight correlated significantly (Figure 4.10; p<0.001, R=0.6311), indicating that the two events may be linked.

![Figure 4.10: Serum MCP-1 correlates with spleen weight.](image)

Taken together, these results suggest that hyperlipidaemia does not alter the overall degree of the immune response to the tumour. In addition, they suggest that exercise reduces spleen weight in mice bearing internal tumours, but not in those bearing orthotopic tumours. Furthermore, mice bearing internal tumours have enlarged spleens compared to those bearing
orthotopic tumours, and thus may have an abnormal immune response to the tumour. This is supported by the correlation of serum MCP-1 and spleen weight, suggesting that the enlargement of the spleen in mice bearing internal tumours may, at least in part, be due to the increased attraction of monocytes and macrophages, although this requires confirmation.

4.4 Discussion

4.4.1 The Effect of Exercise on Tumour Microenvironmental and Systemic Immunity

Previous preclinical work has indicated that exercise induces favourable changes in the immune microenvironment of the tumour (155, 162, 165, 191). Specifically, it has been reported to cause a shift in macrophage phenotype from M2 to M1 (reviewed in (155)), increase numbers of infiltrating CTLs and reduce numbers of T_{reg} cells (191) and increase mobilisation and cytotoxicity of NK cells (162). Furthermore, there are reports of exercise reducing levels of the inflammatory biomarker CRP in breast cancer survivors (120, 125), and reducing MCP-1 in the serum of tumour-bearing rodents (157).

Results from this chapter only partially support the findings of previously published works. A significant negative correlation between T_{reg} cell percentage and individual running distance was observed in WT mice (Figure 4.6). However, we found no increase in either the number or percentage of CTLs or number of total T cells in exercising compared to non-exercising groups, regardless of genotype (Figure 4.1, Figure 4.2b and c). Similarly, there were no significant differences in T_{reg} cell percentage or number between exercising and sedentary groups, although we did observe a trend for reduced T_{reg} cell percentage in WT HEx mice (Figure 4.4).

Taken together, these results provide a weak trend for a shift towards a more favourable, less immunosuppressive microenvironment within the tumour in high exercising (but not low exercising) mice; however they are far from conclusive. Data from McClellan et al. indicate that Cd8 gene expression is increased and Foxp3 gene expression decreased in mucosal scrapings from a transgenic mouse model for colorectal cancer, the Apc\textsuperscript{Min/+} mouse (191). In this case, differences in results may be due to the method used, as gene expression does not necessarily correlate with protein expression, nor does it give an indication of cell type or location. In addition, the immune microenvironment in the gut is unique in that it tends to be highly immunosuppressive due to the need for tolerance to food and commensal microbiota-derived antigens, and thus these results may not be translatable to other cancer types.
Zhang et al. found a significant reduction in intratumoral T\textsubscript{reg} cells following swimming exercise in a hepatic cancer model (227); however mice were sacrificed and tumours were removed 42 days following tumour cell inoculation, a time period more than twice as long as the tumour-bearing time period for the majority of mice in the current study. The adaptive immune response takes time and provides a delayed response to infection, usually between 4 and 7 days (36). There have also been reports of an adaptive immune response taking up to 2 weeks in response to some pathogens (235). This may be further delayed in the case of cancer, due to the tumour being host-derived (non-foreign) and generating an immunosuppressive microenvironment. Indeed, Huang et al. found that a significant CD8\textsuperscript{+} T cell infiltration was only observed after 35 days of EO771 tumour growth (236). In contrast, the average time for which tumours were allowed to grow in the current study was 16 days. The maximum time for which tumours grew in the current study was 33 days, similar to the time period in the study by Huang et al.; however, only one mouse reached this length of time (and only 4 others had tumours for 28 days or longer). This was due to unusually slow-growing tumours, whereas the tumours in the study by Huang et al. exhibited the usual rapid growth rate and reached approximately 1200 mm\textsuperscript{3} after 35 days. This likely resulted in a different microenvironmental phenotype.

In addition, adaptation of the body and the immune system to exercise takes time, and many preclinical studies investigating the effect of exercise on cancer employ an adaptation period of a number of weeks prior to tumour initiation to allow the exercise response to develop more fully (158, 162). Our study began exercise on the day of tumour cell inoculation in order to more closely mimic the role of exercise as a therapeutic rather than preventative measure. Therefore, it may be that the exercise and tumour-bearing time periods in this study were not long enough for differences in T cell response to the tumour to become clear.

Despite numerous reports of serum or plasma CRP levels being reduced by exercise in breast cancer survivors (120, 124-126, 132, 137), our study found no difference in serum CRP between sedentary and exercising mice bearing EO771 breast tumours (Figure 4.7). This may be due to the fact that CRP is not an acute-phase inflammatory marker in mice in the same way that it is in humans, being only modestly increased during the acute-phase response (237). CRP is, however, used as an indicator of a pro-inflammatory state in some mouse studies (238). In order to include investigation of the effect of exercise on serum CRP in rodent preclinical cancer studies, a transgenic mouse model expressing human CRP could be used. Such models are routinely used in atherosclerosis research (239).
MCP-1 is the principal chemokine responsible for attracting monocytes and immature macrophages to peripheral sites and is produced by a wide variety of cell types including endothelial cells, monocytes/macrophages and adipocytes (154, 240). The role of intratumoral MCP-1 in breast cancer progression is well-documented, with reports indicating a role in macrophage recruitment, pathogenic angiogenesis and metastasis (reviewed in (156)). This results in high intratumoral MCP-1 being associated with poor prognosis and high TAM count (241, 242). To our knowledge, no studies have been conducted investigating the association of serum or plasma MCP-1 with TAM accumulation (241). Nevertheless, the high serum MCP-1 levels found in sedentary mice bearing internal tumours suggest that significant macrophage recruitment may be occurring in these mice. This may also explain the correlation observed between spleen weight and MCP-1. Unfortunately, we were unable to specifically analyse TAMs in tumour sections in this study due to time constraints.

Our results on the effect of exercise on serum MCP-1 partially validate findings from previous studies. MCP-1 is a promising candidate for a prognostic biomarker in cancer that can be regulated by exercise, as it has been reported to be increased in tumour-bearing mice and subsequently decreased by exercise in a transgenic breast cancer model (157). However, only a marginal decrease was observed in exercising mice bearing subcutaneous Lewis lung carcinoma (LLC), although tumour burden significantly increased serum MCP-1 (158). This is concurrent with data from this study, where we observed no difference in serum MCP-1 between sedentary and exercising mice bearing orthotopic EO771 tumours (Figure 4.8b), but found a significant increase in tumour-bearing compared to non-tumour-bearing mice (Figure 4.8a). In contrast, exercising mice bearing internal EO771 tumours displayed a drastic decrease in serum MCP-1 compared to sedentary mice (Figure 4.8c). This aligns with data from Murphy et al in a transgenic breast cancer model (157). The data from the current study and the abovementioned published data together indicate that a decrease in serum MCP-1 following exercise may be dependent on tumour location, type and the model system used. Further investigation is required to establish for which cancer types this decrease is observed, and whether it translates to humans. To our knowledge, no studies have been conducted investigating the effect of exercise on serum or plasma MCP-1 in cancer patients. Taken together, MCP-1 shows promise as a biomarker of cancer that can be regulated by exercise.

The increase in spleen weight observed in sedentary mice bearing internal EO771 tumours is not entirely unexpected, as spleen enlargement has been reported in tumour cell transplantable models of cancer (234). It has been suggested that spleen enlargement in tumour-bearing mice
reflects the body’s immune response to the tumour (243, 244); however this does not translate to an increased T cell infiltrate in the current study, as mice bearing internal tumours have enlarged spleens but no change in total intratumoral T cell number (Figure 4.1, Figure 4.9). More recently, Fang et al. have postulated that splenomegaly in tumour-bearing mice may be due to circulating T cells becoming trapped in the spleen (245). This cannot be ruled out; however, the correlation between spleen weight and serum MCP-1 found in this study suggests that macrophage/monocyte accumulation in the tumour and spleen may also play a role.

Taken together, this study is limited by the short time period in which mice are carrying a tumour and exercising, as exercise-induced effects on the T cell response may need more time to become established. On the other hand, the innate immune response may occur earlier; however this remains conjecture as we were unable to confirm this due to time constraints.

### 4.4.2 The Effect of Hyperlipidaemia on Tumour Microenvironmental and Systemic Immunity

Hyperlipidaemia is known to impair host immunity and, in doing so, can inhibit the anti-tumour response and thereby contribute to tumour growth and progression (228-230, 246). In contrast, we found no differences in CTL percentage, total T cell number, serum CRP or MCP-1 levels, or spleen weight between WT and ApoE−/− mice (Figure 4.2, Figure 4.1, Figure 4.7, Figure 4.8, Figure 4.9). However, we did observe a significant decrease in T_{reg} cell percentage in sedentary ApoE−/− compared to sedentary WT mice, and a similar, if attenuated, trend across both exercising groups (Figure 4.4). Recent work has shown that hyperlipidaemic ApoE−/− mice have impaired functionality of T_{reg} cells, and as such a reduced tolerance, as demonstrated by the increased rejection of cardiac allografts (247). This reduced tolerance appears to be reflected intratumorally by the reduced percentage of T_{reg} cells found in orthotopic EO771 tumours in our study. In addition, our results indicate that exercise may restore tolerance. This would suggest that hyperlipidaemia induces a less immunosuppressive, more favourable tumour microenvironment; however, our data do not support enhanced anti-tumour immunity in hyperlipidaemic mice, as total T cell number and CTL percentage are unchanged.

The young age of mice used in this study, short exercising/tumour-bearing time period and pair housing are all factors that may have affected the results in this chapter. These are discussed fully in Chapter 6.
Taken together, hyperlipidaemia reduces immune tolerance, but further work is required to determine the role this plays in the immune microenvironment of the tumour.
5 The Influence of Exercise and Hyperlipidaemia on Intratumoral Perfusion, Angiogenic Potential and Hypoxia

5.1 Introduction

Evidence from rodent studies indicates that intratumoral perfusion and microvessel density are increased by exercise (159-161, 182). In concurrence with this, intratumoral hypoxia is reduced (159, 182). Together, this results in a normalisation of the tumour microenvironment, causing the tumour to become less aggressive. Whether this remains true in a hyperlipidaemic host is not yet established, particularly as hyperlipidaemia has been associated with impaired angiogenesis (219-221).

The transcription factor HIF-1 is stabilised by hypoxia and contributes to cellular survival and adaptation to hypoxia through the activation of target genes such as GLUT1 (46). This results in a more aggressive tumour phenotype. In addition, the glucose transporter GLUT-1 contributes to tumour growth through an influx of glucose which provides fuel for rapidly proliferating tumour cells (50).

We hypothesise that exercise normalises the tumour microenvironment in both WT and hyperlipidaemic ApoE/− mice through an increase in intratumoral perfusion, a reduction in pathological angiogenesis (angiogenic potential), a decrease in hypoxia and a reduced HIF-1α and GLUT-1 protein level.

5.2 Chapter Aim and Experimental Approach

The primary aim of this chapter was to determine the impact of exercise and hyperlipidaemia on intratumoral oxygenation through analysis of perfusion, angiogenesis, hypoxia, necrosis, HIF-1α protein level and GLUT-1 protein level. A further objective was to compare two different methods of analysis of Hoechst staining.

ApoE/− and WT mice at 6-10 weeks of age were randomised to continuous running (HEx), intermittent running (LEx) or sedentary control following orthotopic inoculation of EO771 tumour cells. Each group contained 10-15 mice. Tumours, organs and serum were harvested when tumours reached a maximum size of 600 mm³. Changes in the vascular/oxic
microenvironment between groups were assessed by analysis of intratumoral perfusion, angiogenic potential, hypoxia, necrosis, HIF-1α expression and GLUT-1 expression.

Intratumoral perfusion was assessed by IV injection of Hoechst 33342 into the lateral tail vein 1 minute prior to euthanasia (section 2.2.2.5), resulting in the immediate distribution of Hoechst 33342 throughout the body’s functional vasculature system and diffusion into the surrounding tissue. Perfusion was quantified using two different imaging methods: (1) Hoechst labelled vessels in frozen tumour sections were quantified by counting the number of perfused vessels in 5 random fields at 10x magnification or (2) by estimation of perfused area using Image J (section 2.2.9.1).

CD31 is an often used marker for vascularity, as it stains endothelial cells (which are predominantly vascular). Angiogenic potential was estimated by immunohistochemical analysis of hotspot areas (high density areas) of CD31⁺ vessels (section 2.2.10). Vessel number at 40x magnification in 5 hotspot areas of tumour sections was counted and averaged.

Pimonidazole is activated via reductase enzymes and binds irreversibly to thiol-containing macromolecules in hypoxic cells, enabling subsequent detection of hypoxia (248). Hypoxia was visualised by IP injection of pimonidazole 90 minutes prior to euthanasia and subsequent immunohistochemical staining of FFPE sections (section 2.2.2.5, section 2.2.10). Scoring was done using a modified H score to quantify the degree of hypoxia by the intensity of staining (0-3) and total hypoxic area by percentage (0-100). Scoring was done by two independent observers blinded to treatment (LB and GD).

Necrosis was estimated as percentage tumour area covered by tissue with typical histological signs of necrosis from H&E stained FFPE sections.

Intratumoral HIF-1α and GLUT-1 expression were determined by Western blot analysis of tumour lysates via densitometric analysis of band intensity (section 2.2.6).

5.3 Results

5.3.1 Analysis of Intratumoral Perfusion

Perfusion was visualised by IV injection of Hoechst 33342. We observed large differences in perfusion between tumours, resulting in highly, moderately and poorly perfused sections (supplementary Figure 7.3).
Perfusion analysis by Hoechst 33342 is typically done by quantification of the number of Hoechst perfused vessels per field of view. However, we observed that some sections contained large numbers of small vessels, while others contained small numbers of large vessels, possibly resulting in a similar perfused area (Figure 5.1). We therefore decided to compare two methods of image analysis: the traditional method of counting Hoechst 33342 perfused vessels and analysis of the percent perfused area (section 2.2.9.1, Figure 5.2). Both methods of analysis were done using Image J software.

![High vessel count](image1.png) ![Low vessel count](image2.png)

**Figure 5.1:** Representative images for tumour sections with high (left) and low (right) vessel count but potentially similar perfused area.
Scale bar represents 200 µm. White outline on the image on the right indicates the edge of the tumour section.

We observed a similar trend in results for each method after analysis of all tumour sections, despite differences in individual sections, for both orthotopic (Figure 5.3) and internal (Figure 5.4) tumours. This suggests that both methods provide an acceptable estimate for the degree of intratumoral perfusion.

Exercise did not significantly alter perfusion in any of the tumours, although we observed a trend for reduced intratumoral perfusion in both WT and ApoE⁻/⁻ mice with exercise (Figure 5.3). In addition, ApoE⁻/⁻ mice tended to have increased intratumoral perfusion compared to WT mice (Figure 5.3). These results suggest that intratumoral perfusion in orthotopic breast tumours may be reduced by exercise and increased by hyperlipidaemia.
Figure 5.2: Representative images for two separate methods of Image J analysis. Scale bar represents 200 µm. (a) Analysis of perfusion by vessel count. Each perfused vessel is indicated by a white *. (b) Analysis of perfusion by vessel area. Perfused area is outlined in yellow.

We observed a trend for intratumoral perfusion of internal compared to orthotopic tumours to be increased in exercising WT mice (Figure 5.4); however, statistical analysis was precluded by small sample size. This trend was not observed in internal and orthotopic tumours from ApoE⁻/⁻ mice. This suggests that intratumoral perfusion may be altered according to tumour location, but that this effect is abolished by hyperlipidaemia.

Figure 5.3: Hyperlipidaemia tends to increase intratumoral perfusion in sedentary mice. Quantification of Hoechst 33342 perfused sections by vessel count (a) or percent perfused area (b) in high exercising (HEx), low exercising (LEx) and sedentary (Sed) WT and ApoE⁻/⁻ mice. WT-HEx: n=10; WT-LEx and ApoE-LEx: n=12; WT-Sed: n=6; ApoE-HEx: n=9; ApoE-Sed: n=8. Data are shown as individual data points and mean ± SEM.

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Figure 5.4: Intratumoral perfusion tends to be increased in internal compared to orthotopic tumours from exercising WT mice.

Quantification of Hoechst 33342 perfused sections by vessel count (a) or percent perfused area (b) in orthotopic and internal tumours from exercising (Ex) or sedentary (Sed) WT or ApoE<sup>−/−</sup> mice. Internal tumours from exercising mice were all from WT animals, while internal tumours from sedentary mice were all from ApoE<sup>−/−</sup> animals. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. WT-Ex: n=22; WT-Internal/Ex: n=2; ApoE-Sed: n=8; ApoE-Internal/Sed: n=5. Data are shown as individual data points and mean ± SEM.

### 5.3.2 Analysis of Microvessel Density

In order to determine whether exercise or hyperlipidaemia affect microvessel density, which is an indication of angiogenic potential, we performed immunohistochemical staining for CD31. Vessel density varied greatly across each tumour section, with the majority of hotspot areas localised near the edge of the tumour. Analysis revealed that vessel density in hotspot areas was unaltered by exercise, as no difference was observed between high exercise, low exercise and sedentary groups, in both WT and ApoE<sup>−/−</sup> groups (Figure 5.5). On the other hand, we noted a significant reduction in the number of CD31<sup>+</sup> vessels in hotspot areas in sedentary ApoE<sup>−/−</sup> compared to sedentary WT mice (Figure 5.5). A similar trend was observed for high and low exercise groups, but this did not reach statistical significance. These results suggest that hyperlipidaemia reduces the angiogenic potential and thus pathological angiogenesis of EO771 breast tumours.
We next analysed CD31 staining in internal compared to orthotopic tumours. There was no difference between internal and orthotopic tumours, either for WT or ApoE⁻/⁻ mice (Figure 5.6). This indicates that tumour location does not affect the angiogenic potential of EO771 tumours.

![Image of CD31 staining](image)

Figure 5.5: Hyperlipidaemia reduces angiogenic potential in sedentary mice.

(a) Representative immunohistochemical staining for CD31 in high exercising (HEx), low exercising (LEx) and sedentary (Sed) WT and ApoE⁻/⁻ mice. Scale bar represents 100 µm (b) Quantification of results in (a). Sections were scored by calculating the average number of CD31⁺ vessels in 5 hotspot areas at 40x magnification. WT-HEx, WT-LEx and ApoE-Sed: n=12; WT-Sed: n=10; ApoE-HEX: n=11; ApoE-LEx: n=13. Data are shown as individual data points and mean ± SEM. p<0.01**. Data were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison test to detect differences between individual groups.
Figure 5.6: Angiogenic potential is unaltered by tumour location.
(a) Representative immunohistochemical staining for CD31 in exercising (Ex) and sedentary (Sed) WT or ApoE−/− mice. Scale bar represents 100 μm. (b) Quantification of results in (a). Sections were scored by calculating the average number of CD31+ vessels in 5 hotspot areas at 40x magnification. WT-Ex: n=24; WT-Internal/Ex: n=2; ApoE-Sed: n=12; ApoE-Internal/Sed: n=6. Data are shown as individual data points and mean ± SEM.
5.3.3 Analysis of Intratumoral Hypoxia, Necrosis and HIF-1α Expression

Intratumoral hypoxia was visualised by IP injection of pimonidazole 90 minutes prior to euthanasia and subsequent immunohistochemical staining for pimonidazole adducts. Hypoxia was unaltered in tumours from exercising compared to sedentary groups in both WT and ApoE–/– mice (Figure 5.7).

Figure 5.7: Hyperlipidaemia reduces hypoxia in orthotopic tumours.
(a) Representative immunohistochemical staining for pimonidazole in high exercising (HEx), low exercising (LEx) and sedentary (Sed) WT and ApoE–/– mice. Scale bar represents 200 µm. (b) Quantification of results in (a). Intensity and percentage of staining were quantified by H score. WT-HEx, WT-LEx and ApoE-Sed: n=12; WT-Sed: n=10; ApoE-HEx: n=11; ApoE-LEx: n=13. Data are shown as individual data points and mean ± SEM. p<0.05*. Data were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison test to detect differences between individual groups.
Figure 5.8: Hypoxia is elevated in internal compared to orthotopic tumours.

(a) Representative immunohistochemical staining for pimonidazole in internal and orthotopic tumours from exercising (Ex) or sedentary (Sed) WT or ApoE<sup>-/-</sup> mice. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. All WT mice with internal tumours were exercising; all ApoE<sup>-/-</sup> mice with internal tumours were sedentary. Scale bar represents 200 µm. (b) Quantification of results in (a). Intensity and percentage of staining were quantified by H score. WT-Ex: n=24; WT-Internal/Ex: n=2; ApoE-Sed: n=12; ApoE-Internal/Sed: n=6. Data are shown as individual data points and mean ± SEM. p<0.01**. Data were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison test to detect differences between individual groups.
In contrast, we observed a decrease in hypoxia in tumours from ApoE\(^{-/-}\) compared to WT mice, which was significant for sedentary and high exercising groups (Figure 5.7; p<0.05). These results indicate that exercise does not affect the level of intratumoral hypoxia; however, hyperlipidaemia reduces hypoxia within orthotopic EO771 breast tumours.

We found a significant increase in intratumoral hypoxia in internal compared to orthotopic tumours from sedentary ApoE\(^{-/-}\) mice (Figure 5.8; p<0.01). A similar trend was observed in internal compared to orthotopic tumours from exercising WT mice; however low numbers in the WT-Internal/Ex group precluded statistical analysis. This indicates that tumour location is an important factor in determining the degree of intratumoral hypoxia.

Figure 5.9: Intratumoral necrosis is unaltered by exercise, hyperlipidaemia or tumour location.

(a) Quantification of necrosis by percent necrotic area in tumours from high exercising (HEx), low exercising (LEx) and sedentary (Sed) WT and ApoE\(^{-/-}\) mice. WT-HEx and WT-LEx: n=12; WT-Sed: n=10; ApoE-HEx and ApoE-Sed: n=11; ApoE-LEx: n=13. (b) Quantification of necrosis by percent necrotic area in internal and orthotopic tumours from exercising (Ex) or sedentary (Sed) WT or ApoE\(^{-/-}\) mice. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. All WT mice with internal tumours were exercising; all ApoE\(^{-/-}\) mice with internal tumours were sedentary. WT-Ex: n=24; WT-Internal/Ex: n=2; ApoE-Sed: n=11; ApoE-Internal/Sed: n=6. Data are shown as individual data points and mean ± SEM.

Analysis of necrotic area within tumours revealed no difference between high exercising, low exercising and sedentary groups, nor between WT and ApoE\(^{-/-}\) mice (Figure 5.9a). In addition, necrosis was unchanged in internal compared to orthotopic tumours (Figure 5.9b). This indicates that the degree of necrosis in EO771 tumours is unchanged by either exercise, hyperlipidaemia or tumour location.
HIF-1α is stabilised by hypoxia and is responsible for cellular adaptation to hypoxia (41). We observed no difference in intratumoral HIF-1α protein levels between exercising and sedentary mice, nor did we observe differences between WT and ApoE−/− mice (Figure 5.10a and c). Furthermore, there was no change in HIF-1α expression on comparison of orthotopic and internal tumours, either from exercising or sedentary mice (Figure 5.10b and d).

GLUT-1 is transcriptionally regulated by HIF-1α, and as such can be used as a measure of HIF-1α transcriptional activity (249). In contrast to our results from the HIF-1α protein level analysis, we observed a trend for increased GLUT-1 expression in ApoE−/− compared to WT mice (Figure 5.11). However, there was no difference in exercising compared to sedentary groups in orthotopic tumours from either WT or ApoE−/− mice (Figure 5.11a and c). In addition, GLUT-1 expression was unchanged in internal compared to orthotopic tumours from exercising
VT or sedentary ApoE−/− mice (Figure 5.11b and d). These results suggest that hyperlipidaemia increases intratumoral GLUT-1 expression.

![Image of Western blots and densitometric quantification graphs]

**Figure 5.11: Hyperlipidaemia tends to increase intratumoral GLUT-1 expression.**

(a) Representative Western blot of GLUT-1 in high exercising (HEx), low exercising (LEx) and sedentary (Sed) WT and ApoE−/− mice. (b) Representative Western blot of GLUT-1 in orthotopic and internal tumours from exercising (Ex) and sedentary (Sed) WT and ApoE−/− mice. All internal tumours from exercising mice were WT; all internal tumours from sedentary mice were ApoE−/−. Tumours from exercising mice were pooled from original HEx or LEx groupings to form Ex groupings. β-actin was used as a loading control. (c) Densitometric quantification of results in (a). WT-HEx: n=12; WT-LEx and ApoE-HEx: n=11; WT-Sed and ApoE-Sed: n=10; ApoE-LEx: n=13. (d) Densitometric quantification of results in (b). WT-Ex: n=23; WT-Internal/Ex: n=2; ApoE-Sed: n=10; ApoE-Internal/Sed: n=4. Data are shown as individual data points and mean ± SEM.

### 5.4 Discussion

Exercise has been reported to increase intratumoral perfusion and reduce hypoxia (159, 182). However, whether this holds true in the presence of comorbidities such as hyperlipidaemia remains to be established, particularly as hyperlipidaemia has been shown to inhibit angiogenesis (219-221).
5.4.1 The Effect of Exercise on Intratumoral Perfusion, Hypoxia and Angiogenic Potential

In contrast to published data by the Dewhirst group (159-161), we did not observe an increase in intratumoral perfusion in exercising compared to sedentary mice (Figure 5.3). Instead, we noted a trend for exercise to reduce intratumoral perfusion. This may be due to tumour location. A recent study has shown that blood flow in rats bearing (subcutaneous) ectopic prostate tumours was reduced compared to those bearing orthotopic prostate tumours (187). Importantly, they also observed that exercise reduced blood flow to the ectopic tumours, while increasing blood flow to orthotopic tumours. In addition, blood flow to the skin and subcutaneous adipose tissue was reduced during exercise (187). The tumour model used in the current study, while orthotopic, is nevertheless localised subcutaneously in the mammary fat pad of the mouse. This may explain the observed trend for exercise to decrease rather than increase intratumoral perfusion. This is supported by the trend for perfusion to be increased in internal compared to orthotopic tumours from exercising mice (Figure 5.4).

The trend for decreased intratumoral perfusion in exercising mice would suggest that hypoxia is increased; however, we observed no change in the degree of intratumoral hypoxia in exercising compared to sedentary mice (Figure 5.7). Data for both hypoxia and perfusion in this study varied widely between individual animals. Thus, it may be that the observed trend in perfusion is too slight for detectable differences in hypoxia to develop, particularly as the H score used to quantify hypoxia is a semi-quantitative, subjective measure, making it difficult to detect small differences. In addition, it may be that vessels are filled with deoxygenated blood, as has been reported to occur in solid tumours (250).

No difference was observed between exercising and sedentary mice in terms of angiogenic potential or intratumoral HIF-1α expression (Figure 5.5, Figure 5.10). This is again in contrast to studies by the Dewhirst group, in which they found a significant increase in microvessel density (159, 160) and intratumoral HIF-1α expression (160, 161) in tumours from WT mice. Both studies by Jones et al. (using human breast tumour xenografts in athymic mice and orthotopic prostate tumours in WT mice) had much longer tumour-bearing and thus exercising periods (44 days and 53 days, respectively, compared to a mean of 16 days in the current study) (160, 161). In addition, EO771 tumours in the study by Betof. et al. were allowed to reach approximately 900 mm³ in the exercise group and 1200 mm³ in the sedentary group, at which point a difference in tumour growth rate had been established (159). Therefore, it may be that
the time period in our study was not long enough for exercise-dependent effects to become apparent.

5.4.2 The Effect of Hyperlipidaemia on Intratumoral Perfusion, Hypoxia and Angiogenic Potential

We found that tumours from ApoE\(^{-/-}\) mice had reduced microvessel density, reduced hypoxia and a trend for increased perfusion compared to tumours from WT mice (Figure 5.3, Figure 5.5, Figure 5.7). In addition, they tended to have increased GLUT-1 protein expression compared to tumours from WT mice (Figure 5.11).

The significant reduction in microvessel density in sedentary ApoE\(^{-/-}\) compared to sedentary WT mice, as well as a similar trend in both high and low exercising groups (Figure 5.5), is in contrast to results from Pelton et al. and Kimura et al. in studies using high fat diets to induce hyperlipidaemia (85, 251). However, Pelton et al. utilised an immunodeficient model of orthotopic human breast cancer, and Kimura et al. used the ectopic LLC model. In addition, tumour size in the study by Kimura et al. reached 2500 mm\(^3\), a size far exceeding appropriate ethical and physiologically relevant limits. Therefore, results from both studies may not reflect physiological processes and should be interpreted with caution. The results from the current study are in accord with preclinical results in rodents indicating that angiogenesis is inhibited by hyperlipidaemia (219-221), as well as an epidemiological study in patients suggesting that angiogenesis in breast carcinoma is impaired by hypercholesterolaemia, resulting in a reduced risk of metastasis (252). Taken together, this suggests that hyperlipidaemia reduces microvessel density and thus pathological angiogenesis.

Intratumoral hypoxia was significantly reduced in ApoE\(^{-/-}\) (high exercising and sedentary) compared to WT mice, and a similar trend was observed for the low exercising mice (Figure 5.7). In concurrence with the observation that intratumoral perfusion tended to be increased in sedentary ApoE\(^{-/-}\) compared to WT mice (Figure 5.3), this suggests that hyperlipidaemia contributes to a more normalised tumour microenvironment. At first glance, it may seem that the trend for increased perfusion is contradictory to the decreased angiogenic potential observed in ApoE\(^{-/-}\) mice; however it is important to note that CD31 staining does not give an indication as to the functionality of the vessel. In addition, quantification of hotspot areas of CD31\(^{+}\) vessels does not provide a measure of vessel density throughout the entire tumour. Therefore, it is possible that tumours from hyperlipidaemic ApoE\(^{-/-}\) mice are better perfused and thus less
hypoxic due to a more even distribution of functional vessels, despite reduced angiogenic potential.

We identified a trend for increased GLUT-1 expression in tumours from ApoE−/− compared to WT mice (Figure 5.11). ApoE deficiency impairs cellular lipid uptake from the bloodstream, thereby potentially depriving cells of an important energy source. It may be that the observed increase in GLUT-1 expression is due to cells compensating for the failure to take up sufficient lipids by the increased uptake of glucose as an alternative energy source.

5.4.3 The Effect of Tumour Location on Intratumoral Hypoxia

We observed a significant increase in hypoxia in internal compared to orthotopic tumours from sedentary ApoE−/− mice (Figure 5.8). A similar trend was observed for internal compared to orthotopic tumours from exercising WT mice. In addition, similar results were obtained by our group upon comparison of the degree of hypoxia in internal and orthotopic EO771 tumours from much older (26-52 weeks) WT and ApoE−/− mice (207). This is likely due to the highly aggressive nature of the internal tumours, and potentially tumour cells in internal tumours outgrowing the blood supply more quickly than in orthotopic tumours. In support of this, when a cut-off of 0.54% pHH3 positive cells is applied (according to Gerring et al. in human breast cancer (224)), no internal tumours fall into the poorly proliferative category, whereas at least one orthotopic tumour for both WT and ApoE−/− mice does (Figure 3.11).

The young age of mice used in this study, short exercising/tumour-bearing time period and pair housing are all factors that may have affected the results in this chapter. These are discussed fully in Chapter 6.

Counterintuitively, the results from this chapter suggest that hyperlipidaemia normalises the tumour microenvironment through an increase in intratumoral perfusion and a reduction in both angiogenic potential and hypoxia. In addition, exercise-mediated effects may require a longer study period to become apparent.
6 Discussion

The current study is the first to investigate the effect of exercise on breast tumour growth and the breast tumour microenvironment in conjunction with hyperlipidaemia. Preclinical research in exercise oncology has been plagued by inconsistent effects of exercise on tumour growth, likely due to the disparity in model systems used, while hyperlipidaemia has been shown to increase breast tumour growth rate and proliferation in a number of preclinical studies (82, 84, 85).

The two main aims of this study were (1) to develop a relevant mouse model that appropriately mimics the clinical situation of increased exercise following diagnosis and (2) to determine the effect of exercise and hyperlipidaemia on the breast tumour microenvironment. In order to achieve this, WT C57BL/6 and hyperlipidaemic ApoE−/− mice inoculated with orthotopic EO771 breast tumour cells were randomised to continuous running, intermittent running or sedentary control directly after implant in order to obtain graded exercise levels.

Results from this study suggest that hyperlipidaemia induces a normalisation of the tumour microenvironment through an increase in intratumoral perfusion, a reduction in hypoxia and a reduction in T_{reg} cell percentage (Figure 4.4, Figure 5.3, Figure 5.7). In contrast, exercise-mediated effects remain subtle, potentially due to the short exercising and tumour-bearing time period or to unequal activity levels between mouse pairs overshadowing individual effects.

6.1 Mouse Model Development

The current study provides novel insights into the development of an appropriate mouse model of exercise and hyperlipidaemia for use in cancer research. This is the first study to use graded exercise groups to attempt to narrow down the optimal exercise dose required for a response and determine dose effects, and the first to use ApoE−/− mice on a normal chow diet as a model for hyperlipidaemia.

We encountered several issues with the model system used in the current study. Our aim was to mimic the clinically relevant scenario of exercise being recommended upon cancer diagnosis and so mice were randomised to running or sedentary control on the day of tumour cell inoculation. However, in combination with the very rapid growth of EO771 tumours, this resulted in an exercise period of only 2-3 weeks in the vast majority of cases. This may not be
long enough for strong effects on the tumour to be observed. We postulate that the lack of significant effects due to exercise are partially attributable to this.

In addition, a major limitation of the current study is the inability to quantify running distance and thus exercise dose for individual mice. We decided to house mice in pairs in order to reduce stress caused by individual housing (222, 223). However, it is likely that both mice in a cage do not run equal distances, but rather that one mouse dominates, potentially confounding our results. Indeed, tumour growth data for the few mice for which we have individual running data suggest that exercise may result in an increased tumour lag phase (Figure 3.9a). This may provide a further explanation as to why we observed only subtle differences between exercising and sedentary mice.

The age of mice used in this study may be an important factor in the development of changes in the tumour due to hyperlipidaemia. Our mice were 6-10 weeks of age, a standard age for mice used in preclinical cancer studies (160, 166). However the full ApoE<sup>−/−</sup> phenotype may not be expressed at this age (although subclinical atherosclerotic lesions may be present (253)). A study conducted by our group using orthotopic EO771 tumours in sedentary 26-52 week old ApoE<sup>−/−</sup> mice found a significant increase in proliferation of tumours from ApoE<sup>−/−</sup> compared to WT mice (207). This suggests that in older mice, hyperlipidaemia may be more established, resulting in greater microenvironmental changes.

An important consideration for the interpretation of the results in this study is that hyperlipidaemia is induced by knockout of A<sub>poe</sub>, thereby impairing cellular lipid uptake from the bloodstream. It is important to note that lipid uptake in ApoE<sup>−/−</sup> mice is therefore also inhibited in the EO771 tumour cells transplanted into mice in the current study, as ApoE is a circulating protein and its absence means that lipids cannot be taken up by receptor-mediated endocytosis initiated by the binding of ApoE. The majority of proposed mechanisms for the effect of hyperlipidaemia on breast cancer growth rely on the uptake of cholesterol from the bloodstream (Figure 1.3). When ApoE is deficient, this uptake is impaired, which may result in tumour cells being exposed to less rather than more cholesterol (and triglycerides), potentially skewing the results of this study.

To our knowledge, only one other study has used ApoE<sup>−/−</sup> mice to investigate the effect of hyperlipidaemia on breast cancer growth (82). In contrast to results from the current study, they observed a significant increase in tumour growth rate. However, the investigators additionally kept mice on a high fat, high cholesterol diet, resulting in serum cholesterol levels more than
10 fold above the physiological level (>2000 mg/dL). This huge excess in blood cholesterol likely resulted in off-target effects and other comorbidities. Therefore, it would be important to directly compare tumour growth in ApoE⁻/⁻ mice and HFHC diet fed WT mice to determine whether the method of induction of hyperlipidaemia plays a role in tumour development and growth. In addition, serum cholesterol and triglycerides should be measured to confirm the degree of hyperlipidaemia (further discussed in section 6.3).

Mice tend to run more than humans, with reported distances in voluntary models ranging from approximately 2-21 km/day (160-163, 165). To our knowledge, there is no established way of estimating the equivalent running distance in humans. This precludes direct comparison of mouse and human activity levels. However, as the current study (and other mouse exercise studies) compares levels of activity between mice and does not directly relate this to humans, this difference is accounted for.

6.2 Tumour Microenvironmental Effects of Exercise, Hyperlipidaemia and Tumour Location

Our data suggest that hypoxia promotes an immunosuppressive microenvironment within the tumour, as indicated by the high proportion of T_{reg} cells and low proportion of CTLs found in highly hypoxic internal tumours (Figure 4.3, Figure 4.5, Figure 5.8). This is supported by published work showing that intratumoral hypoxia decreases tumour cell susceptibility to CTL-mediated killing (254, 255) and increases T_{reg} cell attraction, induction and immunosuppressive function ((256-258), reviewed in(259)). In addition, hypoxia promotes an immunosuppressive phenotype in myeloid cells (reviewed in (259)).

We observed a number of differences in the microenvironment of tumours from ApoE⁻/⁻ compared to WT mice. We found a reduction in microvessel density (representative of angiogenic potential) in ApoE⁻/⁻ compared WT mice; this was significant for sedentary groups (Figure 5.5). In addition, the percentage of intratumoral T_{reg} cells and the degree of hypoxia were reduced in ApoE⁻/⁻ compared to WT mice (Figure 4.4, Figure 5.7). T_{reg} cells have been shown to induce angiogenesis, both in a tumoral and physiological context (258, 260). Therefore, the observed reduction in angiogenic potential in ApoE⁻/⁻ mice may be attributable to the decreased percentage of T_{reg} cells, which in turn may be due to the reduction in hypoxia. Likewise, the reduction in hypoxia may be due to a trend for increased perfusion in ApoE⁻/⁻ compared to WT mice (Figure 5.3). Taken together, hyperlipidaemia appears to promote a more
normalised tumour microenvironment through increased perfusion, reduced hypoxia, a reduced proportion of $T_{reg}$ cells and a decrease in angiogenic potential, at least in young female mice.

Exercise tended to reduce intratumoral perfusion in both WT and ApoE$^{-/-}$ mice (Figure 5.3). This suggests a reduction in functional vessels. This may result in reduced extravasation of T cells from the bloodstream into the tumour, reflected in a trend for a reduced total T cell number and CTL number in high exercising compared to sedentary WT and ApoE$^{-/-}$ mice (Figure 4.1, Figure 4.3c). However, this is purely conjecture and requires confirmation through future work.

To our knowledge, this is the first study to investigate exercise or hyperlipidaemia-induced changes in the immune and vascular/oxic microenvironment of the same tumour. Taken together, our data suggest an important link between these two microenvironmental components which may provide important insights into the regulation of the tumour microenvironmental phenotype by exercise and hyperlipidaemia.

### 6.3 Future Directions

As discussed in section 6.1, our development of a mouse model of exercise encountered a number of limitations. Future studies should ensure that exercise duration is long enough to ensure that exercise-mediated effects become apparent. This could be approached in two ways. Firstly, if using a model of aggressive tumour growth such as the EO771 model used in the current study, exercise could begin prior to tumour cell inoculation. However, in order to model changes induced by exercise post-diagnosis, a less aggressive, slower growing tumour model (such as transgene or carcinogen-induced tumours) should be selected in order to allow exercise to begin at tumour induction/detection.

The drawback of housing mice in pairs is that running distance per individual mouse cannot be quantified, and assuming that both mice in a cage run equal amounts may confound results. However, individual housing is not an ideal solution due to stress caused to the animal (222, 223), which may also confound results. Instead, an estimate as to the degree of exercise undertaken by individual mice could, for example, be determined by analysis of cytochrome c oxidase (COX) activity (261) or protein expression (262, 263) in skeletal muscle. This would enable grouping of data according to individual exercise dose. Indeed, we have collected muscle tissue from some animals in the current study and will determine COX activity and/or protein expression in the future.
The hyperlipidaemic phenotype observed in ApoE−/− mice may be enhanced with age (discussed in section 6.1). Therefore, further studies using ApoE−/− mice as a model for hyperlipidaemia should use older animals. This has the added advantage of mimicking the clinical situation, where most breast cancer patients are over 60 years old.

Serum cholesterol and triglycerides should be measured (for example by ELISA) to confirm the degree of hyperlipidaemia caused by ApoE knockout. This is planned to be done in the future via Canterbury Health Laboratories using serum samples from the current study.

In the current study, immunofluorescence was used to analyse the abundance of T cell subsets within the tumour. While providing important information as to the distribution of T cells throughout the tumour, this method is semi-quantitative rather than quantitative. A more accurate measure of the relative proportion and absolute numbers of T cell subsets would be fluorescence activated cell sorting (FACS). This method could be used to analyse intratumoral and splenic T cells (following tissue dissociation), as well as T cells in the bloodstream. In addition, this method could be applied to the quantification of other immune cells such as macrophages or neutrophils.

Furthermore, simple staining of T cell subsets and cell number does not provide an indication as to the functionality of these cells. T cell exhaustion is well-characterised as a dysfunctionality of effector T cells and frequently occurs in intratumoral T cells (264). Therefore, it would be of interest to conduct functional assays (such as cytotoxicity or suppression assays) on T cell subsets isolated from tumours from sedentary and exercising WT and ApoE−/− mice in order to determine whether exercise and/or hyperlipidaemia alter T cell functionality.

In order to further delineate the association between hypoxia and T cell phenotype, visualisation of T cell subsets together with pimonidazole and/or Hoechst 33342 would have been ideal to do in the current study. However, attempts to visualise pimonidazole by fluorescence resulted in high background due to the anti-pimonidazole antibody being mouse-derived. This was unable to be resolved in the time-frame of the current study, but future studies should continue optimisation work to reduce background fluorescence. In addition, the T cell immunofluorescence protocol was optimised for sample fixation with acetone, which washed out Hoechst 33342. Future attempts to visualise both T cells and Hoechst 33342 in the same section should test different fixation solutions (e.g. formalin).

The trend for an increased number of intratumoral T cells as well as a trend for increased perfusion in sedentary ApoE−/− mice raises the intriguing possibility of a connection between
intratumoral perfusion and T cell infiltration by extravasation. In order to determine whether the two observations are linked, immunofluorescent analysis of tumour sections could be performed using Hoechst 33342 as an indicator of perfusion and CD3 as a T cell marker (taking into account the technical difficulties outlined in the paragraph above). Localisation of T cells with respect to perfused vessels may provide an initial indication as to whether increased perfusion results in increased T cell infiltration by extravasation.

This study focussed on adaptive immunity by assessment of T cell infiltrate, but the elevated serum levels of MCP-1 found in sedentary mice bearing internal tumours and subsequent reduction in exercising mice suggest that macrophage recruitment in these mice may be altered by exercise. Current literature indicates that macrophage polarisation is altered by physical activity, from a pro-tumour M2 to an anti-tumour M1 phenotype; however, the question whether macrophage recruitment to the tumour is altered remains unanswered (reviewed in (155)). Thus, future work should include analysis of the number of intratumoral macrophages in both orthotopic and internal tumours, either by FACS, immunofluorescence or immunohistochemistry.

In our study, we estimated angiogenic potential by the quantification of CD31+ vessels in hotspot areas of vessel density. However, other measures of angiogenic potential (such as VEGF expression) could be applied in future studies in order to obtain a more accurate estimate.

6.4 Conclusion

The current study provides novel insights into the selection of an appropriate mouse model for research in exercise oncology and into changes in the breast tumour microenvironment caused by exercise and/or hyperlipidaemia. We found that while exercise reduces body weight or prevents weight gain, no difference was seen in tumour growth rate or tumour cell proliferation in exercising compared to sedentary mice or in WT compared to ApoE−/− mice. Contrary to expectation, hyperlipidaemia appeared to generate a more normalised tumour microenvironment through reduced intratumoral T_{reg} cell percentage, reduced microvessel density and reduced hypoxia. Exercise-mediated effects remained subtle, although we did observe a vast decrease in serum MCP-1 in exercising compared to sedentary mice bearing internal tumours and a significant negative correlation of intratumoral T_{reg} cell percentage with individual mouse running distance.
We identified a number of issues during the development of an exercise model of breast cancer in this study. Future studies should ensure that the time period of the study is sufficiently long for exercise-mediated effects to become apparent, and that mice can be grouped according to individual exercise dose.

Normalisation of the tumour microenvironment through improved vessel patency resulting in improved perfusion, oxygenation and subsequent alleviation of the immunosuppressive tumour microenvironment provides an attractive goal for anti-tumour strategies (265). We hypothesised that this normalisation could be achieved through exercise in both WT and hyperlipidaemic ApoE−/− mice; however we observed limited changes in the tumour microenvironment attributable to exercise. In contrast, hyperlipidaemia appeared to normalise the tumour microenvironment through an increase in intratumoral perfusion, a reduction in hypoxia and a reduction in Treg cell percentage. However, whether this is due to hyperlipidaemia itself or the impaired cellular lipid uptake from the bloodstream caused by ApoE deficiency remains to be established. Further studies are required to fully elucidate the effect of exercise and hyperlipidaemia on the breast tumour microenvironment.
7 Appendix

7.1 Supplementary Material for Chapter 3

Figure 7.1: Average distance run per 24h does not correlate with lag phase of tumour growth. Correlation of individual mouse running distance with lag phase data for tumour growth (time to reach 100 mm³). n=13. p=0.7, R=0.1176. Data were analysed by Pearson correlation.
7.2 Supplementary Material for Chapter 4

Figure 7.2: T cell number and CTL percentage do not correlate with individual running distance. Correlation of individual running distance with intratumoral T cell number and CTL percentage in WT (a, c) and ApoE−/− (b, d) mice. WT: n=7; ApoE: n=4. Data were analysed by Pearson correlation.
Figure 7.3: Representative images of highly, moderately and poorly perfused tumour sections. Scale bar represents 200 µm.
Immunohistochemical pimonidazole staining is quantified by a modified H score. The H score is given by multiplication of the staining intensity (assigned a value from 1-3 to represent high, moderate and low intensity) with the percent stained area (0-100). This gives a value between 0 and 300. Scale bar represents 200 µm.

8 References


Byon CH, Hardy RW, Ponnazhagan S, Welch DR, McDonald JM, Chen Y. Free fatty acids enhance breast cancer cell migration through plasminogen activator inhibitor-1 and SMAD4. Laboratory investigation; a journal of technical methods and pathology. 2009;89(11):1221-8.


188. McClellan JL, Davis JM, Steiner JL, Enos RT, Jung SH, Carson JA, Pena MM, Carnevale KA, Berger FG, Murphy EA. Linking tumor-associated macrophages, inflammation,


198. Mason G, Würbel H. What can be learnt from wheel-running by wild mice, and how can we identify when wheel-running is pathological? Proceedings of the Royal Society of London B: Biological Sciences. 2016;283(1824).


