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Investigating lifestyle and aspects of immunity among healthy women, patients undergoing neoadjuvant therapy for breast cancer and survivors of the disease

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Investigating lifestyle and aspects of immunity among healthy women, patients undergoing neoadjuvant therapy for breast cancer and survivors of the disease

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ABSTRACT

Breast cancer is the most common cancer in the UK (CRUK, 2017). T-lymphocytes destroy cancerous cells through recognition of tumour-associated antigens. Unhealthy lifestyles can negatively influence immune function and detrimentally affect risk, treatment and survival of breast cancer. This thesis assesses the relationship between lifestyle, psychological factors, health and T-lymphocyte function in healthy women, breast cancer patients and survivors.

86% of healthy women (n=50) possessed tumour-associated antigen specific T-lymphocytes, which, given links between immune function and cancer, may be protective. Tumour-associated antigens mammaglobin-A (MamA) and carcinoembryonic antigen (CEA) elicited the highest proportion of T-lymphocyte IFN-γ secretion (52% of women) and may prove useful targets for immunotherapeutic treatments.

Cytomegalovirus (CMV) seropositive healthy women (n=22/50), had higher numbers of tumour-associated antigen specific T-lymphocytes; MamA (p=0.009), CEA (p=0.042), Cl6 (p=0.033), CycB1 (p=0.050), ERB ICD (p=0.005) and SUR (p=0.033) versus seronegative counterparts. CMV seropositive tumour-associated antigen specific possessing individuals had less healthy lifestyles or psychological profiles ($p<0.05$), perhaps due to increased prior exposure to antigens. It may be an anti-cancer T-lymphocyte response helps individuals remain free from cancer.

Breast cancer patients receiving neoadjuvant chemotherapy (n=6) demonstrated reductions in predicted $\dot{V}O_2max$ post-chemotherapy. Hb (haemaglobin) levels were higher pre- versus mid- (p=0.046) and post-chemotherapy (p=0.043). These detrimental changes should be targeted with future interventions. A higher number of tumour-associated antigen specific T-lymphocytes were observed in a patient with a pathological clinical response (pCr), demonstrating a potential marker of prognosis.

In breast cancer survivors, 8-weeks of remotely monitored (n=15) or partly supervised (n=15) exercise saw no change in $\dot{V}O_2max$ (p=0.707 and 0.215) or body fat (p=0.685 and 0.365). Remotely monitored was deemed non-inferior to partly supervised exercise for body fat changes but not non-inferior in regards $\dot{V}O_2max$, systolic blood pressure or 6-minute walk time. A partly supervised intervention is preferred to improve health following breast cancer treatment.

ABBREVIATIONS

3-PUFA	n-3 Polyunsaturated fatty acids
A	Adenine
ACM	All-cause mortality
ACSM	America College of Sports Medicine
AI	Aromatase inhibitor
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ATP	Adenosinetriphosphate
AU	Arbitrary unit
β	Beta
Bcl	B-cell lymphoma
bFGF	Basic fibroblast growth factor
BIA	Bioelectrical impedance
BMD	Bone mineral density
BMI	Body mass index
BMR	Basal metabolic rate
bpm	Beats per minute
BRCA	Breast Cancer
BZLF1	BamHIZ leftward reading frame 1
C	Cytosine
CC	Chemokine
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen-related adhesion molecule
CI	Confidence interval
Cl6	Claudin-6
CM	Central memory
cm	Centimetre(s)
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CR	Complete response
CRP	C-reactive protein
CT	Computerised tomography
CTLA	Cytotoxic T-lymphocyte associated
CycB1	Cyclin-B1
Δ	Delta

DASS-21	Depression, anxiety and stress questionnaire
DCIS	Ductal carcinoma in situ
DEXA	Dual energy x-ray absorptiometry
DIT	Diet induced thermogenesis
dl	Decilitre
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
ϵ	Epsilon
EBV	Epstein-Barr virus
EBNA1	Epstein-Barr nucleic antigen 1
EDTA	Ethylenediaminetetraacid
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme linked immunosorbent spot
EM	Effector memory
EMRA	Effector memory terminally differentiated
EORTC QLQ-C30	European organization for research and treatment of cancer quality of life questionnaire
ER	Oestrogen receptor
ErbB	Receptor tyrosine-protein kinase
ERB ECD	Receptor tyrosine-protein kinase ErbB-2 extracellular domain
ERB ICD	Receptor tyrosine-protein kinase ErbB-2 intracellular domain
ESR	Erythrocyte sedimentation rate
ESSI	ENRICHD Social Support Instrument
FBS	Foetal bovine serum
FEC	Fluorouracil, epirubicin and cyclophosphamide
FEC-T	5-fluorouracil, epirubicin, cyclophosphamide and docetaxel
FEC-TH	5-fluorouracil, epirubicin, cyclophosphamide, docetaxel, trastuzumab and pertuzumab
FISH	Fluorescence in situ hybridisation
fTh	Follicular T helper
G	Glutamine
g	Gram(s)
gE	Glycoprotein-E
GLUT	Glucose transporter type
$G_{(2)}$-M	Growth to mitosis phase
GP	General Practitioner

H	Hour(s)
Hb	Haemaglobin
HER2	Human epidermal growth factor receptor 2
Hg	Mercury
HHV	Human Herpes virus
HLA	Human leukocyte antigen
HR	Heart rate
HR	Hazard ratio
HR_{max}	Maximum heart rate
HPV	Human Papillomavirus
IAP	Inhibitor of apoptosis protein
IARC	International agency for research on cancer
ICSH	International council for standardisation in haematology
IE	Immediate early
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IGF	Insulin like growth factor
IL	Interleukin
iNKT	Invariant Natural Killer T cells
IPAQ	International physical activity questionnaire
IU	International unit(s)
κ	Kappa
kB	Kilobyte(s)
kDa	Kilodalton(s)
kg	Kilogram(s)
kcal	Kilocalorie(s)
kph	Kilometers per hour
L	Litre(s)
LCIS	Lobular carcinoma in situ
M	Metastases stages
m	Metre(s)
MAGE-3	Melanoma associated antigen 3
MamA	Mammaglobin-A
MCF	Michigan Cancer Foundation
MDA-MB	MD Anderson metastasis breast cancer
MET	Metabolic equivalent

mg	Milligram(s)
MHC	Major histocompatibility complex
Min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
MMP11	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MMTV	Mouse mammary tumour virus
MP1	Matrix protein 1
MUC1	Mucin-1
N	Node stages
NA	Naive
NEFA	Non-esterified fatty acids
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram(s)
NHS	National Health Service
NK	Natural Killer
NKCA	Natural Killer cytotoxic activity
NKT-like	Natural-Killer-T-like
NLR	Neutrophil lymphocyte ratio
nm	Nanometre
NMSC	Non-melanoma skin cancer
NP	Nucleocapsid protein
O_2	Oxygen
OR	Odds ratio
p	Probability
PACES	Physical activity enjoyment scale
PAL	Physical activity level
PAMPS	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pCR	Complete pathological response
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PET	Positron emission tomography
PLR	Platelet lymphocyte ratio

pp65	phosphoprotein 65
pPR	Partial pathological response
PR	Partial response
PR	Progesterone receptor
PRRs	Pathogen recognition receptors
PVDF	Polyvinylidene difluoride
OR	Odds ratio
RAG2	Recombination activating 2
RANTES	Regulated on activation, normal T-cell expressed and secreted
Rb	Retinoblastoma
RBC	Red blood cells
RER	Respiratory exchange ratio
RMR	Resting metabolic rate
RNA	Ribonucleic acid
RPE	Rating of perceived exertion
RPMI	Roswell Park Memorial Institute medium
ROS	Reactive oxygen species
RR	Risk ratio
RUH	Royal United Hospital
SD	Stable disease
SD(PPS)	Standard deviation of the change within the partly supervised group
SD(RM)	Standard deviation of the change within the remotely monitored group
SFU	Spot forming units
SHBG	Sex hormone-binding globulin
S-phase	Synthase phase
SPSS	Statistical Package for Social Sciences
Sur	Survivin
T	Tumour stages
TAG	Triglycerides
TARP	TCRgamma alternate reading frame protein
TCR	T-cell receptor
T	Thymine
Th	T helper
TLR	Toll-like receptor
TNM	Tumour, nodal and metastasis

TNF-α	Tumour necrosis factor-alpha
TNTC	Too numerous to count
UK	United Kingdom
URTI	Upper respiratory tract infection
VEGF	Vascular endothelial growth factor
$\dot{V}O_2$	Oxygen consumption
VZV	Varicella zoster virus
W:H	Waist to hip ratio
WHEL	Women's healthy eating and living study
WHO	World Health Organisation
WINS	Women's intervention nutrition study
X	Chi
γδ	Gamma-delta
μ	Mu
μL	Microliter(s)
μm	Micrometer(s)
λ	Lamda

CHAPTER ONE: Introduction

1.1 PERSPECTIVES AND OVERVIEW

Cancer is a disease of uncontrolled cell growth, resulting in a tumour mass developing that subsequently affects normal body functions. Cancer is the second leading cause of mortality worldwide (WHO, 2018). Amongst 66 million inhabitants in the United Kingdom there were around 367,000 new cancer cases each year from 2015-2017 (CRUK, 2017). Breast cancer is the most common cancer in the UK, accounting for almost a sixth (15%) of all cases in males and females combined (CRUK, 2017). Previously, almost four in ten (36.8%) cancer cases in women in the UK were attributable to known risk factors such as obesity, smoking and physical inactivity (Brown *et al.*, 2018). Therefore, replacing features of an unhealthy lifestyle with those of a healthy lifestyle might reduce the incidence of certain cancers. One example is breast cancer whereby being physically active can reduce the risk of developing this disease by nearly 25% (Friedenreich and Orenstein, 2002).

Epidemiological studies continue to show associations between unhealthy lifestyles and breast cancer incidence (Bhaskaran *et al.*, 2014) however the mechanisms underlying this relationship remain unclear and speculative. For example, exercise is thought to reduce the risk of breast cancer by lowering lifetime exposure to oestrogen or exercise-induced effects on the endocrine system (Irwin *et al.*, 2008b). In addition, in obese and overweight individuals, higher circulating levels of insulin, insulin-like growth factors and inflammatory mediators, have been associated with breast cancer occurrence (De Pergola and Silvestris, 2013). This seems plausible considering the known effects of insulin on cell growth processes, and because it is thought that inflammation itself increases the risk of cells undergoing a malignant transformation (Coussens and Werb, 2002). Finally, some diets considered as being 'healthy' (higher fruit, vegetable and fibre intake and reduced red meat intake) have been linked to a lower incidence of breast cancer (Dunn *et al.*, 2002a). In the case of diet, it might be that individuals who eat a diet rich in fruit and vegetables exhibit greater antioxidant defences, which provide protection from reactive oxygen species (ROS) that can damage DNA (deoxyribonucleic acid) leading to mutations and cancer (Reuter *et al.*, 2010). Many features of a healthy lifestyle have also been linked to better immune function, which is critical in both preventing and eliminating cancer. Indeed, it has been speculated that exercise might stimulate aspects of immune function to provide better protection against cancer (Irwin *et al.*, 2008b). The present work aims to test this, filling the knowledge gap within the current literature.

The immune system protects against infectious agents such as bacteria and viruses, but also targets cancerous cells (Hanahan and Weinberg, 2011). While decreased exposure to cancer-promoting substances (i.e., the mechanism by which some dietary antioxidants are thought to make cancer less likely to occur), reduces the chance of a cell adopting cancer-like characteristics, some cells become cancerous spontaneously, and if this occurs, it is the immune system that is the final defence. Within the immune system, specialised cells recognise and destroy cells that show signs of cancer before a tumour can develop (Jakobisiak *et al.*, 2003). There are several lines of evidence to support the anti-cancer properties of the immune system. For example, there is a lower incidence of cancer in individuals who exhibit a higher than normal killing ability of certain immune cells found in peripheral blood (e.g., Natural Killer (NK) cells) (Mandal and Viswanathan, 2015). In addition, there is a considerably greater risk of cancer, including breast cancer, for organ transplant recipients prescribed immunosuppressive medication (Vesely *et al.*, 2011; Buell *et al.*, 2005). Indeed, it is known that several different types of immune cells contribute to the anti-cancer effects of chemotherapy and radiotherapy (Ghiringhelli *et al.*, 2009; Casares *et al.*, 2005b; Mattarollo *et al.*, 2011a). Furthermore, in patients diagnosed with breast cancer, low numbers of certain immune cells (T-lymphocytes) in breast tumours have been linked with poor effectiveness of chemotherapy (DeNardo *et al.*, 2011). Finally, the most recent advances in cancer therapy such as immunotherapy and monoclonal antibody therapy, provide supra-physiological stimulation to some cells of the immune system (e.g., T-lymphocytes), to improve their ability to detect and eliminate cancer cells (Finn, 2008). Despite the immune system becoming a focus for mainstream cancer researchers, the relationship between cancer, lifestyle and immune function, until now, has been neglected in the literature.

1.2 OVERALL AIM AND RESEARCH QUESTIONS

The aim of this thesis is to examine, in healthy people and in breast cancer patients, whether relationships exist between lifestyle variables (e.g., cardiorespiratory fitness, body mass index (BMI), percentage body fat, habitual diet) and the ability of specialised cells of the immune system (cytotoxic T-lymphocytes) to recognise proteins expressed on the cell surface of tumour cells (tumour-associated antigens). The outcome will be a greater understanding of potential mechanisms underlying the protective effects of a healthy lifestyle on the risk of developing cancer, and whether leading a healthy lifestyle prior to, and during cancer therapy, improves clinical outcomes in patients, providing a potential utility to optimise treatment. This thesis will also investigate different ways in which to

improve health in breast cancer survivors, following treatment through exercise interventions.

1.3 THE IMMUNE SYSTEM

The immune system protects the body by selectively eliminating pathogens that cause sickness by recognising specific molecules (antigens) that trigger an immune response to eliminate toxins or other harmful substances and removing damaged, altered or dying cells. The major effectors of the immune system are white blood cells (leukocytes) which originate in bone marrow and differentiate into other cells with specialised functions. Leukocytes consist of granulocytes (60-70% of circulating leukocytes), monocytes (5-15%) and lymphocytes (20-25%) and each of these sub-types differentiate further into more specialised cells. The immune system is traditionally split into two compartments; the innate and acquired, however much overlap and cooperation exists between the two.

1.3.1 Innate Immunity

The innate immune system provides a first line of defence by recognising and removing pathogens such as viruses, bacteria, fungi or even cancer cells through non-specific, fast acting effectors, providing a similar response each time. Cells of the innate immune system comprise of granulocytes (neutrophils ~90%, eosinophils ~2%, basophils ~5%, mast cells ~3%), lymphocytes (NK cells ~5-15%) and myeloid cells, including monocytes (which are referred to as macrophages when they are tissue resident) and other predominantly tissue resident cells, such as dendritic cells. Many granulocytes are rapidly recruited to the site of infection by a process known as chemotaxis, and go on to destroy these cells by various processes (Stuart and Ezekowitz, 2005). Natural killer (NK) cells detect and eliminate virus infected cells or tumour cells through recognition of the down regulation of key cell surface molecules (major histocompatibility complex (MHC) molecules), releasing the cytotoxic enzyme perforin, to activate apoptosis, disrupting the cell membrane causing the infected cell to lyse, by release of proteases.

Monocytes circulate in the bloodstream before migrating to tissues where they differentiate into macrophages (and sometimes dendritic cells dependent on the immediate microenvironment). Upon pathogen recognition, macrophages secrete a series of chemokines (small soluble proteins) and cytokines (proteins released by cells) that attract other cells from blood to the site of inflammation (Bachmann *et al.*, 2006) and facilitate the movement of plasma proteins to the site of infection through increased vessel permeability

(Moser and Leo, 2010). Macrophages are also involved in antigen presentation, an essential process to trigger the acquired immune response. However, the 'master' professional antigen presenting cells are the dendritic cells, which initiate acquired immune responses by recognising pathogen-derived molecules or endogenous signals released by damaged or dying cells (Joffre *et al.*, 2009). Dendritic cells exist in an immature state, present in high numbers in tissues in direct contact with the external environment (e.g., skin and mucosal tissue) where they act as sentinels awaiting pathogen invasion or tissue damage. Upon antigen ingestion, dendritic cells become activated, mature, and degrade protein antigens while traveling to lymph nodes where they activate naïve T-lymphocytes – cells of the acquired immune system (Moll, 2003; Moser and Leo, 2010). Pathogen recognition comes about by pathogen recognition receptors (PRRs) present on the cell surface of immune cells recognising pathogen-associated molecular patterns (PAMPS) in the extracellular environment.

1.3.2 Acquired Immunity

The acquired immune system has three key properties; the ability to distinguish self from non-self, (tolerance), the ability to form immunological 'memory' and the ability to exhibit specificity so that it can differentiate between proteins that are identical except for a single amino acid. The acquired immune system consists of two main lymphocyte sub-populations; T-lymphocytes (60-75% of all lymphocytes) and B-lymphocytes (5-15% of lymphocytes). T-lymphocytes elicit cell-mediated immunity whereby they eliminate target cells infected with viruses, bacteria, or those that have turned cancerous, whereas B-lymphocytes elicit humoral immunity, whereby soluble mediators (antibodies) bring about the immune response. During their differentiation, both T- and B-lymphocytes undergo a process by which the genes encoding the structure of their cell-surface antigen receptors randomly shuffle to produce an almost infinite number of receptors with the ability to recognise almost any possible sequence of amino acids (Murre, 2007). Thus, a primary immune response may arise from a single antigen-specific cell which will become activated and proliferate rapidly to produce daughter cells also specific for the antigen. Memory cells are subsequently generated by clonal expansion and differentiation to enable a rapid and effective response on re-exposure and are the basis of the immunological memory characteristic of acquired immunity (Fabbri *et al.*, 2003).

T-lymphocytes recognise specific sequences of amino acids, typically between 8-15 amino acids long presented to them by complex cellular machinery[1]. Antigen presenting cells phagocytose antigens (e.g., cell debris or pathogens) in peripheral tissues and migrate to the lymph nodes. During this transition, these cells degrade the ingested proteins into short peptide sequences by means of the proteasome and assemble them onto MHC molecules in the endoplasmic reticulum. There are two forms of MHC molecules. MHC-class I molecules (which cytotoxic T-lymphocytes recognise) and MHC-class II molecules (which another form of T-lymphocytes recognise; helper cells). Professional antigen presenting cells express both forms of MHC molecules, but all other cells only express MHC-class I. Thus, MHC-class I molecules on normal cells allow them to signal to CD8+ T lymphocytes that the cell is infected or transformed. During the immune response, while the dendritic cell travels to the lymph nodes, it also moves the peptide-MHC complexes to the cell surface to present the processed antigen to a naïve T-lymphocyte that has not encountered the antigen that its antigen receptor – the T cell receptor (TCR) – is specific for. During this interaction, the naïve CD8+ T-lymphocyte becomes 'licenced' to kill, and divides and differentiates into hundreds or thousands of so-called effector-memory CD8+ cytotoxic T-lymphocytes. This differentiation process provides the T-lymphocytes with potent killing capacity and stimulates them to patrol the body, searching for cells expressing the peptide that their TCRs are specific for. Indeed, as part of this process, some of these expanded clones of antigen-specific T-lymphocytes become long-lived memory cells, ensuring the immune system responds more rapidly and effectively to a pathogen that has been encountered previously (Gourley *et al.*, 2004).

1.3.2.1 T-lymphocytes

T-lymphocytes enable the acquired immune system to target intracellular pathogens or cells that have undergone a malignant transformation by recognition of peptides expressed in the context of MHC molecules. There are two main populations of T-lymphocytes: T-helper (CD4+) and T-cytotoxic (CD8+) lymphocytes. A number of other minor T-lymphocyte populations exist, such as Natural-killer-T-like (NKT-like) cells and gamma-delta (γδ) T-lymphocytes, which together account for <10-20% of the broader population of cells. NKT-like cells express receptors normally found on NK cells, but as with conventional T-

[1] This topic is covered in depth in Chapter 2, General Methods because it is relevant to explaining the laboratory techniques used in this thesis. For reasons of flow and conciseness, only an outline is presented here.

lymphocytes, these cells recognise peptides presented by MHC molecules. γδ T-lymphocytes are unique in their ability to respond to multiple stimuli via their TCR, or other receptors, such as toll-like receptors, in a manner similar to innate immune cells. For reasons of conciseness and scope, only the broader populations of T-lymphocytes will be discussed in this thesis.

1.3.2.2 T-lymphocyte sub-populations

CD4+ T-helper lymphocytes comprise 60-70% of all T-lymphocytes and recognise antigens 14-20 amino acids long presented by MHC-class II molecules expressed on professional antigen presenting cells (Loureiro and Ploegh, 2006). Once activated, T-helper lymphocytes control immune responses by secreting cytokines to attract other T-lymphocytes influencing their differentiation (Mosmann *et al.*, 1986). CD4+ lymphocytes can differentiate into six sub-populations (Type 1 (Th1), Type 2 (Th2), follicular T-helper (fTh), Th17, Th9 and regulatory cells) differing in secretion and subsequent cell activation (Alberts, 2008; Finkelman *et al.*, 2004; Breitfeld *et al.*, 2000; Harrington *et al.*, 2005; Luckheeram *et al.*, 2012).

Another sub-population of T-lymphocytes are CD25+, Foxp3 and CTLA-4 expressing T-regulatory cells that make up 10% of all T-lymphocytes. CTLA-4 is an inhibitory molecule which binds to the costimulatory molecule CD80 on dendritic cells in the immune synapse, whilst Foxp3 is a member of the forkhead transcription factor family which suppresses IL-2 and T-lymphocyte cytokines (Kim, 2009). T-regulatory cells secrete the cytokine IL-10 to provide a negative feedback loop to 'turn off' the immune response, thus playing both a beneficial and negative role by blocking unsuitable immune reactions directed to self-antigens (Kim *et al.*, 2007) but sometimes aberrantly inhibit the development of protective immune responses against non-self-antigens (Oldenhove *et al.*, 2003).

CD8+ cytotoxic T-lymphocytes comprise 30-40% of T-lymphocytes which are essential for killing virus infected cells and cancer cells (Loureiro and Ploegh, 2006). CD8+ cytotoxic T-lymphocytes respond to pathogens by engagement of their TCR with peptides 8-9 amino acids in length presented in the context of MHC-class I molecules on normal cells. Upon recognition, these cells produce a number of soluble factors expressing anti-pathogenic effects inducing death of infected or damaged cells (Cerottini *et al.*, 1970). CD8+ cytotoxic T-lymphocytes have two strategies to eliminate target cells. First, release of a pore-forming protein (perforin) from secretory vesicles by local exocytosis which forms transmembrane channels allowing the intracellular delivery of proteases (granzymes) into the cytoplasm of

the target cells. Granzymes initiate an apoptotic response through the activation of a caspase cascade, a group of proteins that lead to rapid cell death (Chowdhury and Lieberman, 2008). The second killing mechanism is elicited by interaction between the so-called Fas-ligand protein on the T-lymphocyte and the Fas receptor protein on the target cell which recruits protocaspase-8-molecules again leading to a caspase cascade (Nagata, 1999).

1.4 MODULATORS TO IMMUNE FUNCTION

1.4.1 Exercise, physical activity and immune function

Links between exercise and immune function were first established in 1893 by studies examining leukocytosis (an elevated number of white blood cells), showing that exercise results in an increased number of leukocytes in peripheral blood (Clark *et al.*, 1989). These findings were confirmed by studies undertaken in 1901 and 1902 reporting an acute leukocytosis in individuals who had completed marathon running race events (Cabot *et al.*, 1901; Larrabee, 1902). During the 1970s it was questioned whether exercise-induced changes to immune function were responsible for the high frequency of infections reported by athletes following endurance events (Roberts, 1986; Kujala *et al.*, 1988) and thus potentially impacting upon training and performance in athletic settings (Daniels *et al.*, 1985). By the 1990s it was assumed by coaches, scientists, and the general public that participants of marathons and ultra-marathon events exhibit an increased risk of upper respiratory tract infections (Nieman *et al.*, 1990). This led to the development of the J-shaped curve: a hypothesis proposing that the volume and intensity of exercise training undertaken by individuals is related to the risk of infections (Nieman, 1994). This model suggests that moderate volumes and intensities of exercise training protect against infections, whereas low volumes (i.e., being sedentary) but perhaps more predominantly, high volumes and intensities of training, increase the risk of infection. Exercise-induced impairments to immune function were blamed, partly due to the observation that following the acute increase in some immune cells post-exercise, other cells (in particular lymphocytes) fall to approximately half of normal levels for up to 24 hours. This period was coined the ‘open window theory’ of increased susceptibility to infections following acute exercise.

By the year 2000 however, some doubt was cast over this idea. Only seven of the twenty eight studies reporting an increased incidence of infections in athletes met the criteria of research excellence in terms of originality, significance and rigor (Shephard, 2010). The

majority of this research focused on self-reported illness symptoms and did not provide laboratory measurements to confirm infections (e.g., microbiology) or did not corroborate self-reports with clinical diagnoses by medical personnel. These results were subject to positive bias whereby those athletes experiencing symptoms would be more likely to return questionnaire responses. Even if there was an increased incidence of infections among participants of endurance races or international competitions, this is more than likely a result of increased exposure to pathogens, due to foreign travel, interaction with hundreds or thousands of people in relatively confined places, and poor hygiene. For example, air travel has been linked with and can predict infection or illness in athletes and can disrupt other physiological and psychological factors such as sleep, dehydration and stress which in turn have a detrimental effect on immune function (Choudhry *et al.*, 2006; Svendsen *et al.*, 2016; Schwellnus *et al.*, 2012).

The most recent thinking is that it is actually just a subset of athletes who are more prone to illness who are responsible for reporting the high incidence of infections around the time of competition or increased training loads. These individuals appear to exhibit inappropriate immune responses to novel antigens, whereby upon stimulation of their immune cells, rather than there being a robust pro-inflammatory response (e.g., characterised by secretion of IFN-γ, TNF-α and IL-2), elicit an even stronger anti-inflammatory response (e.g., IL-10) (Gleeson *et al.*, 2012). Indeed, the advancements in laboratory methodology and the number of research papers published over the past twenty years now clearly indicate that exercise 'stimulates' aspects of immune function rather than causing 'suppression' (Campbell and Turner, 2018).

As mentioned, during exercise, or immediately afterwards, the composition of blood shifts whereby neutrophils, monocytes and lymphocytes all undergo an increase in number (Kruger and Mooren, 2014; Simpson *et al.*, 2009; Shek *et al.*, 1995). It is generally accepted that immediate leukocytosis is due to demargination of leukocytes from the endothelium likely by increased shear stress and catecholamine-induced down-regulation of adhesion molecule expression (Timmons and Cieslak, 2008; Benschop *et al.*, 1993; Kappel *et al.*, 1991), whilst delayed leukocytosis may be due to the action of cortisol (Gleeson *et al.*, 2013; Dimitrov *et al.*, 2010). The extent to which cells mobilise operates via activation of the β_2-adrenergic receptors on lymphocytes, (the highest expression levels are seen on CD8+ T-lymphocytes and NK cells), affecting adhesion properties (Dimitrov *et al.*, 2009), whereby increased expression of receptors leads to higher sensitivity to mobilisation. This means a large influx of NK-cells and CD8+ T-lymphocytes and a smaller influx of CD4+ T-lymphocytes and B-lymphocytes (Shek *et al.*, 1995; Gannon *et al.*, 2001). Particularly,

effector memory CD8+ cytotoxic T-lymphocytes and the highly cytotoxic 'CD56dim' NK cells, are the largest contributors to the acute and transient lymphocytosis that occurs in peripheral blood during intensive exercise (Gleeson *et al.*, 1995). The short-term increase in blood leukocytes reflects a mobilisation of cells into the blood from various body compartments as the blood is a critical compartment through which immune cells must pass in order to maintain their normal surveillance pathways and to rapidly reach sites of immune activation.

In humans, stress induced changes in immune cell distribution are accompanied by enhancement of immune function in organs to which immune cells traffic during stress (Edwards et al 2007, Rosenberger et al 2009). Naïve CD8+ T-lymphocytes, along with the regulatory so-called 'CD56bright' NK cells, traffic from the bloodstream to the spleen or lymph nodes, where they are likely to be activated by antigen presenting cells. However, effector memory CD8+ T-lymphocytes that are present in the blood under resting conditions, traffic to tissues such as the skin or the mucosal/ epithelial lining of the gut where they are likely to encounter antigens to which they have been previously exposed (Dhabhar *et al.*, 2012). This ensures a greater number of antigen-experienced lymphocytes at sites of potential immune challenge, even before the challenge is administered (Engler *et al.*, 2004). This exercise-mediated mobilisation of immune cells towards tissues (Shephard and Shek, 1999; Fridman *et al.*, 2012) might represent an immune focused defence mechanism against cancer growth (Pedersen *et al.*, 2016) whereby each bout of exercise and subsequent immune mobilisation surveys the body for cancer cells. The majority of exercise responsive leukocytes have high cytotoxic effector properties demonstrating a protective evolutionary component whereby these immune cells are deployed in preparation for immune challenges that may be imposed (Dhabhar, 2009).

Exercise appears to stimulate most aspects of immune function, and not just the number of cells in the bloodstream. For example, it has been shown that acute bouts of exercise result in immune-enhancement in the context of vaccination, whereby a larger secondary response demonstrates heightened immune function (Edwards *et al.*, 2007). Responses to vaccination are a measure of integrated immune function, elicited by antigen exposure and measured by antibody levels and/or cell-mediated response (Edwards and Booy, 2013). Acute and chronic exercise results in increased antibody responses to vaccination in older adults (Woods *et al.*, 2009b). Younger adults appear to show a smaller effect of exercise on vaccine responses, likely due to the normally robust immune systems in young healthy adults versus weaker immune function in older adults, demonstrating exercise has an ability

to enhance cellular and humoral immune responses to antigen exposure (Silberman *et al.*, 2003; Dhabhar and Viswanathan, 2005).

1-2h post-exercise, the composition of blood changes once more when compared with rest and exercise, whereby some cells fall below baseline levels (lymphocytopenia and monocytopenia when lymphocyte and monocytes numbers fall respectively), whereas other cells exhibit an even larger increase than during exercise (e.g., neutrophilia whereby neutrophil numbers increase) (Kruger and Mooren, 2014; Simpson *et al.*, 2009; Shek *et al.*, 1995). Those cells previously rapidly mobilised such as CD8+ T-lymphocytes and NK cells exhibit a larger lymphocytopenia post-exercise (Lund *et al.*, 2011). The CD8+ T-lymphocyte subsets also differ in the magnitude of their response to exercise, with EM (effector memory) and EMRA (effector memory terminally differentiated) showing a greater ingress into peripheral blood during exercise and a greater lymphocytopenia post exercise compared to NA (naïve) and CM (central memory) (Shek *et al.*, 1995; Campbell *et al.*, 2009). This mobilisation leads to distinct functional and cellular characteristics. The subsequent short term decrease in blood leukocyte number represents the trafficking of cells out of the blood to target organs, sites of immune activation, including potentially cancerous cells, demonstrating a fundamental survival response essential for effective immune surveillance (Dhabhar and McEwen, 1997; Dhabhar and Viswanathan, 2005).

Animal studies have shown that exercise training may have a beneficial effect on the immune system. After 4 weeks of voluntary running in mice, natural cytotoxicity and T-lymphocyte proliferation increased, reducing stress induced antibody formation (Moraska and Fleshner, 2001). Moderate intensity exercise training (5 times a week for 6 months) has been suggested to upregulate Th cell mediated immune functions and reduce risk of infection and autoimmune disease in individuals over 60 years old (Shimizu *et al.*, 2008). Regular moderate intensity endurance exercise has shown to increase lymphocyte proliferation, and IL-2 production in ageing rats (Nasrullah and Mazzeo, 1992). Positive effects on immunosurveillance and host protection elicited from moderate exercise training as demonstrated by improved response to vaccine in physically active individuals (Kohut *et al.*, 2004; Kohut *et al.*, 2002; Woods *et al.*, 2009b; Smith *et al.*, 2004; de Araújo *et al.*, 2015; Grant *et al.*, 2008) is likely due to the cumulative effect of positive changes that occur during each individual exercise bout. Interestingly, the positive effects of moderate exercise training have been proven to improve immune function to a greater extent in individuals who are less fit or sedentary at baseline (Brolinson and Elliott, 2007; Klentrou *et al.*, 2002) in relation to influenza and self-reported URTI (upper respiratory tract infection) symptoms

such as runny, stuffy nose, sore throat, coughing, sneezing, coloured discharge, or in mice whose immune system is compromised (Rogers *et al.*, 2008).

1.4.2 Body composition and immune function

Obesity is a disorder of energy imbalance whereby energy intake is greater than energy expenditure and is linked to a wide variety of health problems such as cardiovascular disease and hypertension (high blood pressure) (Samartín and Chandra, 2001). Obese individuals often present with characteristics associated with metabolic syndrome such as insulin resistance and elevated markers of systemic inflammation. Evidence suggests that excess adiposity generated with obesity negatively impacts immune function and host defence as visceral adipose tissue (the hormonally active component body fat), is immunologically dynamic, playing home to resident immune cells with specialised functions. Adipose resident cells include CD4+ (Winer *et al.*, 2016), CD8+, T-reg (Feuerer *et al.*, 2009), invariant natural killer T-cells (iNKT) (Lynch *et al.*, 2009), and B-lymphocytes (Winer *et al.*, 2011). Epidemiological data suggests obese humans are at greater risk for nosocomial (hospital induced) infections, alongside an increased susceptibility to poor wound healing following surgery when compared to lean counterparts (Marti *et al.*, 2001). Furthermore, recent findings demonstrate the impact obesity has on immunity and pathogen defence in terms of disruption of lymphoid tissue, alterations in leukocyte development and phenotypes and the coordination of the innate and adaptive immune responses (Andersen *et al.*, 2016). Murine models of obesity have shown a decrease in all T- and B-lymphocyte subsets (Kimura *et al.*, 1998) demonstrating lower lymphocyte responsiveness to mitogens in obese versus lean animal models and impairments in NK cell activity and dendritic processing and presentation (Karlsson and Beck, 2010; Smith *et al.*, 2009; Kimura *et al.*, 1998). Both high fat diet fed and ob/ob obese mice (mutant mice that eat excessively due to mutations in the gene responsible for the production of leptin (the hormone responsible for appetite regulation and fat storage)), experienced increased mortality in response to staphylococcus aureus induced sepsis (Strandberg, 2009).

The diminished immune function, in obese individuals has been confirmed in humans, whereby a lower proliferative capacity of lymphocytes in response to mitogen activation, suppression in phagocytic function, and oxidative burst activity of monocytes has been seen in obese individuals as determined by body mass index (BMI) (Marcos *et al.*, 2003; Marti *et al.*, 2001; Henson *et al.*, 1999). Circulating mononuclear cells in obese individuals have been shown to exhibit a pro-inflammatory state (raised NF-κB , IL-6, and TNF-α) compared with people of a healthy BMI (Ghanim *et al.*, 2004), perhaps affecting immune competency.

Interestingly, obesity has been shown to enhance thymic aging and reduce T-lymphocyte diversity by increasing apoptosis of developing T-lymphocytes and the age-related reduction of recently generated T-lymphocytes (Yang *et al.*, 2009). Obesity has thus been shown to decrease the number of NA and EM T-lymphocytes in the periphery, thus accelerating age-related thymic involution and immunosurveillance (Yang *et al.*, 2009). Furthermore, evidence has suggested obese individuals do not respond to vaccines (a reduced B-lymphocyte antibody response has been observed) of tetanus (Eliakim *et al.*, 2006), hepatitis B (Bandaru *et al.*, 2013) and influenza (Ocak and Eskiocak, 2008) when compared with lean individuals (Marti *et al.*, 2001; Sheridan *et al.*, 2012). However, research has often defined obesity through the use of BMI, which has several limitations such as an inability to determine the relative contributions of fat and muscle to overall body composition or assess regional body fat distribution which is often related to overall health (Kok *et al.*, 2004).

The mechanisms behind reduced immune function in obese individuals surrounds the relationship between adipose tissue metabolism and immunocompetent cell functions (Trim *et al.*, 2018). Excess nutrient intake leads to adipose tissue expansion, promoting activation of a chronic proinflammatory state (Guilherme *et al.*, 2008). Adipose resident macrophages often switch from M2 to M1 phenotypes, whereby M1 are more inflammatory, partially explaining the obesity related inflammatory state (Weisberg *et al.*, 2003; Lumeng *et al.*, 2007; Ferrante Jr, 2013; Kraakman *et al.*, 2014). This obesity related chronic, low grade inflammation (Hotamisligil, 2006; Bastard *et al.*, 2006) in turn increases circulating concentrations of inflammatory markers (Dalmas *et al.*, 2011; Odegaard and Chawla, 2011) such as TNF-α, IL-6, IL-1β and C-reactive protein (CRP) (Hotamisligil *et al.*, 1993; Kern *et al.*, 1995; Bullo *et al.*, 2003) and chemokines (e.g. CCR2) (Kanda *et al.*, 2006). This causes leukocyte infiltration and an inflamed adipose phenotype, further expanding the inflammatory environment (Guilherme *et al.*, 2008; Bremer and Jialal, 2013; Altintas *et al.*, 2011). Furthermore, compared to resting T-lymphocytes, which have low energy needs, T-lymphocyte activation requires more energy, triggering increases in insulin receptor expression and glucose transporter type 1 mediated glucose uptake. This may help explain why the presence of insulin resistance is more common in obese populations, as obesity may suppress insulin signalling on top of generating insufficient T-lymphocyte activation in response to pathogens (McIver *et al.*, 2008). Finally, obesity can lead to immune system dysfunction partly because of adipose deposition in primary lymphoid tissues (Sheridan *et al.*, 2012; Kanneganti and Dixit, 2012), which alters leukocyte populations and distribution of inflammatory phenotypes (Kanneganti and Dixit, 2012; Yang *et al.*, 2009; Ghanim *et al.*, 2004; Sheridan *et al.*, 2012).

1.4.3 Nutrition and immune function

The concept of immunonutrition refers to the ability of certain nutrients to modulate immune function through energy metabolism and protein synthesis (Grimble, 1997; Moreira *et al.*, 2007). Both undernutrition and excess of some nutrients can impair the immune system in terms of host protection (Chandra, 2002; Calder and Kew, 2002).

With inadequate nutrition, particularly insufficient protein intake, the immune system is deprived of the nutrients required to generate an effective immune response (Marcos *et al.*, 2003). It is well accepted that inadequate intake of protein impairs host immunity with particularly detrimental effects on T-lymphocytes, resulting in an increased incidence of infections in animal models and immunocompromised hosts (Chandra, 1997; Scrimshaw and SanGiovanni, 1997; Calder and Kew, 2002). In humans, protein-energy malnutrition has been found to depress the number of mature, fully differentiated T-lymphocytes and the in vitro proliferative response to mitogens, (Daly *et al.*, 1990). Additionally, a lack of protein intake causes the T-lymphocyte CD4+/CD8+ ratio to markedly decrease and phagocytic cell function, cytokine production and complement formation to be impaired (Gleeson *et al.*, 2004). Protein deficiency impairs immunity as immune defences are dependent on the production of proteins for rapid cell replication and producing immunoglobulins, acute phase proteins and cytokines. Several studies have demonstrated that undernutrition is associated with immunosuppression and can lead to both increased susceptibility to infection and all-cause mortality (ACM) (Alwarawrah *et al.*, 2018; Bourke *et al.*, 2016; Rahman and Adjeroh, 2015).

Research suggests that intake of polyunsaturated fatty acids has a negative impact on immune function. Several studies have reported lower T-lymphocyte proliferation, decreased cytotoxic T-lymphocyte activity, and inhibition of cell mediated immune responses after feeding a high fat diet rich in polyunsaturated fatty acids found in foods such as walnuts and fish (Miles and Calder, 1998). The mechanism behind this is predicted to surround the link between polyunsaturated fatty acids and inhibition of human antigen presenting cells (Hughes and Pinder, 2000). There is little evidence to suggest that dietary intake of sugars and/or fats influence immune response in individuals however, their contribution to energy intake may prove crucial in terms of regulating adiposity and inflammation.

It is believed that antioxidant nutrients play a pivotal role in preserving the adequate function of immune cells (De la Fuente, 2002). A lack of vitamins A, C and E impair immune function

and decrease the body's resistance to infection (Scrimshaw and SanGiovanni, 1997; Calder and Kew, 2002; Calder and Jackson, 2000). These vitamins play a pivotal role in maintaining the antioxidant/oxidant balance in immune cells and in protecting them from oxidative stress whilst preserving their adequate function (Marcos *et al.*, 2003; Grimble, 1997; De la Fuente, 2002; Chandra, 2002).

One vitamin that has received recent attention in terms of influence on immune function is vitamin D. It has been identified that vitamin D metabolising enzymes (1-α-hydroxylase) and vitamin D receptors are present on a variety of innate and adaptive immune cells (Battault *et al.*, 2013; Prietl *et al.*, 2013) which can subsequently synthesise the active vitamin D metabolite. This suggests that vitamin D may modulate the immune system, suggesting insufficient vitamin D levels may lead to dysregulation of immune responses as shown by increased susceptibility to infection in 19,000 individuals with a vitamin D deficiency (Ginde *et al.*, 2009). This finding has been mirrored and rates of influenza (Cannell *et al.*, 2006; Urashima *et al.*, 2010), bacterial vaginosis (Bodnar *et al.*, 2009), HIV (Villamor, 2006) and respiratory tract infection (Laaksi *et al.*, 2007) are elevated in those who have less than optimal vitamin D levels. However, randomised-controlled evidence is still somewhat lacking. Mechanisms surrounding the protective effect of vitamin D on immune function are multifactorial. Mechanisms include the role vitamin D plays in innate antimicrobial response, decreased production of inflammatory cytokines and increased productions of anti-inflammatory cytokines (Aranow, 2011).

Several minerals, including zinc and iron are also known to exert modulatory effects on immune functions (Cherayil, 2010; Dardenne, 2002). Studies have associated iron deficiencies with increased morbidity from infectious disease (Sherman, 1992). However, an excess of these minerals can also impair immune function and increase susceptibility to infection (Sherman, 1992).

1.4.4 Anti-viral serostatus and immune function

Positive viral serostatus for numerous virus' has been known to alter overall immune status. One virus that has been known to do so is Epstein Barr Virus (EBV). EBV is a gamma-herpes virus with a 172-kb DNA genome which infects more than 90% of the world population. The virus utilises resting B-lymphocyte biology to infect, persist and replicate within human cells (Thorley-Lawson, 2001; Thorley-Lawson and Gross, 2004; Chen *et al.*, 2016). The infection is controlled by T-lymphocytes causing an increase in EBV specific memory T-lymphocytes (up to 50% of all T-lymphocytes, (Chen *et al.*, 2011)), which, if the

infection occurs in adolescence or adulthood, causes clinical symptoms of infectious mononucleosis. Recent studies have also indicated that EBV positivity may regulate CD8+ NKT-like development which may contribute to disease development (He et al 201) as NKT cells are involved in regulating cell mediated immunity to various infectious organisms; cancer, allergy and autoimmune disease (Chen *et al.*, 2011).

Another virus known for producing alterations in immune function is the human cytomegalovirus (CMV). CMV is a member of the beta herpesvirus with a ~236-kB genome expressing up to ~750 protein encoding open reading frames (Stern-Ginossar *et al.*, 2012; Balázs *et al.*, 2017). Infection with CMV has three distinct phases: Firstly, a systemic replication phase in peripheral tissues that activates the innate immune system and NK cells, priming a diverse antibody and T-lymphocyte effector memory response. Secondly, a tissue located persistent phase that continues for months to years and continues to shape the innate and adaptive immune systems and finally, multisite latency with restricted viral gene expression that promotes immune inflation during subsequent exposure (Karrer *et al.*, 2003; Seckert *et al.*, 2012; Jackson *et al.*, 2011). Despite inducing a robust and diverse innate and adaptive immune response, CMV successfully progresses through the three phases and establishes a persistent latent infection that is never cleared by the host (McGeoch *et al.*, 2006). This leaves a permanent mark on the immune system in the form of phenotypically distinct T-lymphocyte and NK cell subsets at high frequencies (Souquette *et al.*, 2017) which can sporadically reactivate leading to further antigenic stimulation and secondary immune responses. This drives further immune cell differentiation and further increases the frequency of CMV specific T-lymphocytes, a concept termed ‘memory inflation’ (O’Hara *et al.*, 2012).

Evidence of CMV negatively affecting immune function is demonstrated by increased inflammation and acceleration of immunosenescence (Nikolich-Žugich and van Lier, 2017; Kaczorowski *et al.*, 2017). CMV seropositivity and the persistence of the virus as a chronic antigenic stressor is associated with many of the same T-lymphocyte changes that are biomarkers of immune ageing and mortality such as the expansions of memory phenotypes (Looney *et al.*, 1999). In humans the percentages of CMV specific T-lymphocytes occupying the memory compartment is highly variable (on average 10% but can be up to 50% of the total memory T-lymphocyte compartment). Such high numbers of effector type like memory CD8+ cells may subsequently cause naive CD8+ cells to be diminished, potentially reducing the response to novel antigens (Jackson *et al.*, 2017). Little evidence exists surrounding the impact of VZV or Flu on overall immune function.

1.5 CANCER BIOLOGY

1.5.1 Fundamental principles of cancer

Cancer is a disease of dysregulated signalling brought about by mutations to DNA – the code for all proteins within the body. DNA is tightly coiled around proteins called histones forming the structural units of chromosomes in cells. DNA is a double helix consisting of two polynucleotide chains composed of four types of nucleotide subunits; adenine (A), cytosine (C), guanine (G) or thymine (T). When DNA is replicated, each strand represents a template encoding the sequence of amino acids that form proteins with specific functions (Alberts, 2008). Cancer results from cumulative mutations to DNA that can cause a cell to out-grow, out-divide, and out-live its neighbours. Mutations in DNA can lead to specific genes being permanently switched on (e.g. HER2 or the RAS family of genes) or off (e.g. BRCA1, BRCA2, and p53 or TP53), which in turn can lead to proteins being permanently expressed (or not expressed), which can affect signalling pathways controlling proliferation, cell death (apoptosis) and DNA repair processes. Therefore, cancer can develop due to both an up regulation of growth, or, down regulation of cell death. Mutations can comprise of the substitution, insertion, deletion, inversion or translation of any of the four nucleotide bases. When the mutation occurs in the promoter region of a key gene (e.g., a gene concerned with repair of DNA such as BRCA1, BRCA2 or p53, control of proliferation e.g. HER2 or RAS or apoptosis e.g. BRCA1, BRCA2, p53 and TP53) a cellular transformation can occur. In some cases, these mutations will be insignificant, eliciting no effects. In other cases, spontaneous cell death may occur. In most cases, these mutations will be repaired, but sometimes a malignant transformation does occur whereby key regulatory processes (cell growth and death) are affected. The genetic damage can result in a cancer cell that starts to grow rapidly or avoid cell death, to form tumours causing tissue failure unless the cancerous, abnormal cells are removed or destroyed or a cancer that is resistant to cell death and thus continues to divide (Schneider, Dennehy & Carter, 2003).

1.5.2 Risk of developing cancer

Age is considered to be a risk factor for cancer due to the ever increasing number of cell divisions taking place across the life course (Hanahan and Weinberg, 2000; Niccoli and Partridge, 2012). In addition, lifestyle factors such as lack of exercise, eating a poor diet, consuming alcohol, and tobacco use, have all previously been associated with an increased risk of cancer (Parkin, 2011; Brown *et al.*, 2018). Thus, the concept of whether cancer occurs by a random process of ‘chance’ and ‘bad luck’ or alternatively, whether this process

is due to modifiable factors, has been a topic of debate. Studies have focussed on intrinsic or extrinsic risk factors for cancer. Intrinsic factors include those that result in mutation due to random errors in DNA replication, whereas extrinsic or modifiable factors are environmental factors that affect mutagenesis (e.g., ultraviolet radiation, ionising radiation, exposure to carcinogenic chemicals, and lifestyle variables, such as lack of exercise).

Previous epidemiological analysis of risk factor exposure and distributions among the total number of cancer cases in the UK in 2010, provided evidence that approximately 43% of the 338,623 cancer cases were caused by external environmental exposures, increasing the chance of a random mistake in DNA replication happening (Parkin, 2011). This figure includes; tobacco (19.4%), diet (consumption of meat, fruit and vegetables, fibre and salt) (9.2%), overweight/obesity (5.5%), alcohol consumption (4%), occupation (3.7%), ultraviolet radiation (3.5%), infections (3.1%), ionizing and solar radiation (1.8%), inadequate exercise (1%), reproductive factors (0.9%) and post-menopausal hormones (0.5%) (Parkin, 2011). Thus, in theory, just over one third of all cancer cases could be prevented by modifying aspects of the environment and lifestyle. In 2015, a landmark paper suggested one third of cancer cases are caused by inherited predispositions and/or external environmental factors (e.g., lack of exercise). This work proposed that the remaining two-thirds of cancer cases are caused by 'bad luck', whereby random mutations were associated with the lifetime number of stem cell divisions within tissues. This paper concluded that the majority of changes to DNA occur by chance during DNA replication, rather than as a result of modifiable factors. However many cancers such as breast cancer were not included in this analysis, and the conclusions were based on mathematical models of stem cell division over a lifetime (Tomasetti and Vogelstein, 2015). In contrast, another landmark paper published in 2016 suggested almost the opposite; that more than two-thirds of cancers are caused by external environmental factors, and less than one third of cancers were due to 'bad luck'. These conclusions were made from analysis of epidemiological studies reporting the relationship of cancer risk versus total number of tissue cell divisions and cancer risk versus intrinsic stem-cell mutation errors (Wu *et al.*, 2016).

On balance, it is likely that the figure for the number of cancers caused by modifiable risk factors is somewhere between the estimates by Tomasetti and Vogelstein (2015) and Wu et al (2016), however, estimating is a complicated task, given that some of these factors include exposure to radiation from rocks and cancer risk from infections that are very difficult to avoid (e.g., EBV).

1.5.3 Established characteristics of cancer cells

Tumour development can be viewed as having three stages; tumour initiation, tumour promotion and tumour progression. After tumour initiation, has taken place, brought about by mutations to DNA, the tumour promotion stage takes over, characterised by clonal expansion of tumour cells with hyperproliferation, apoptosis, tissue remodelling and inflammation. Finally, during tumour progression, pre-neoplastic cells develop into invasive tumours through further clonal expansion, usually associated with alterations in gene expression and additional genetic damage due to progressive genomic instability (Rogers *et al.*, 2008; Pitot, 1989). In order for a tumour to develop and evolve through these stages, it is thought that a number of properties must be adopted by cancer cells in order to evolve, multiply and spread. These properties have been categorised into six cellular alterations that drive the transformation of normal cells into malignant cells, and it is thought that a cell must adopt all six of these 'hallmarks' to survive successfully as a cancer cell (Hanahan and Weinberg, 2000) (Figure 1.).

Firstly, cancer cells must gain an ability to grow autonomously – a process that is normally tightly regulated by growth factors and growth factor receptors. Some cancer cells become hyper-sensitive to ambient levels of growth factors, often by overexpressing growth factor receptors (e.g., Human epidermal growth factor receptor-2 (HER2)). Other cancer cells exhibit deregulated cell growth by synthesising their own growth factors (e.g., platelet derived human growth factor or tumour growth factor alpha). Secondly, cancer cells become insensitive to anti-growth signals (e.g., soluble growth inhibitors and immobilised inhibitors) driving their proliferation further. Thirdly, cancer cells obtain the ability to evade apoptosis; a precisely choreographed series of steps leading to death, whereby cellular membranes are disrupted, cytoplasmic nuclear skeletons are broken down, chromosomes are degraded, the nucleus is fragmented, and the shrivelled cell is engulfed. Normally apoptotic sensors (e.g., the extracellular sensor CD95 and the intracellular sensor *p*53) detect DNA damage and, in response, destroy the cell. Yet, mutation and subsequent inactivation of the *p*53 tumour suppressor gene is apparent in more than 50% of human cancers (Harris, 1996), preventing repair. Fourth, cancer cells develop an ability to divide infinitely (i.e., they avoid the Hayflick limit of 40-70 divisions). Telomeres – a sequence of TTAGG nucleotides repeated several thousand times in human cells – form the ends of chromosomes, and progressively shorten each time a cell divides because DNA polymerase is unable to copy the entire length of the DNA sequence. Thus, telomeres act as molecular clocks: critically short telomeres stimulate a state of replicative senescence, preventing protein-encoding DNA being miscopied. In 85-95% of all cancer cells, the enzyme telomerase, which

synthesises and elongates telomeres, is up-regulated allowing the cells limitless replicative potential (Shay and Wright, 2000). Fifth, cancer cells turn on an angiogenic switch, stimulating a growth of blood vessels. All cells need to be within 100 μm of a capillary for an oxygen and nutrient supply and for removal of waste products. Thus, cancer cells produce factors that expand blood vessels; inhibiting anti-angiogenic factors such as endostatin, angiostatin, and thrombospondin and increasing the release of angiogenic factors such as vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF). Without overcoming this hallmark, cancer cells are unable to grow more than 1-2 mm in size. Sixth, cancer cells develop an ability to invade tissues and break away from the original tumour, surviving independently. Pioneer cells leave the tumour mass, breaking into the lymphatic system, undergoing local invasion, disrupting tissues and their function sometimes moving through the circulation to other tissues where there is less competition for nutrients and space. These new tumour masses are called metastases and are the primary cause of death in cancer (Hanahan and Weinberg, 2000).

1.5.4 Emerging characteristics of cancer cells

The six established hallmarks of cancer cells are thought to be acquired by means of two enabling characteristics; the development of genomic instability and the inflammatory environment surrounding premalignant cells, in part driven by cells of the immune system. In addition, two other hallmarks have been developed: a major reprogramming of cellular energy metabolism and evasion by cancer cells from attack and elimination by immune cells (Hanahan and Weinberg, 2011) (Figure 1.).

Amongst cancer cells there is an on-going process of selection or ‘survival of the fittest’. For cancer cells to have a selective advantage over other clones, enabling their outgrowth and dominance in a local tissue, they must have genome instability so that mutations take place. A mutation needs to lead to a defect in the DNA-maintenance machinery involved in detecting and repairing DNA damage and inactivating mutagenic molecules. This accelerates the rate at which the premalignant cells gain favourable genotypes. Cancer cells become more sensitive to mutagenic agents to increase the rate of mutation to permit tumorigenesis (Negrini *et al.*, 2010). Tumour-promoting inflammation can influence the tumour environment making it proinflammatory by providing bioactive molecules, growth factors and survival factors that limit cell death and promote proliferation, enzymes that modify the extracellular matrix to encourage metastasis, and stimulating inflammatory cells to release ROS that can further damage the DNA.

In order for cancer cells to develop, cellular energetics and metabolism must become deregulated. Normal cells rely primarily on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes. Uncontrolled division in cancer cells requires energy whereby glucose is broken down even when oxygen is low. To sustain oxygen delivery, the cancer cell acquires genetic mutations that functionally alter receptor initiated signalling pathways (Vander Heiden *et al.*, 2009; Liberti and Locasale, 2016); a phenomenon termed 'The Warburg Effect'. This leads to a reduction in adenosine triphosphate (ATP) and an up-regulation of GLUT1 (glucose transporter type 1), a transporter protein that imports glucose to the cancer cell (Warburg, 1956) resulting in the uptake of nutrients that meet or exceed the bioenergetics demands of cell growth and proliferation (Vander Heiden *et al.*, 2009). Some cells, such as those in the middle of the tumour, maintain anaerobic glycolysis, especially if oxygen is scarce. This process appears to activate oncogenes which increases proliferation and prevents cell death. Indeed, this idea of excessive glucose use by cancer cells is exploited by a common clinical imaging technique; positron emission tomography (PET) scanning, which uses a radiotracer called fluorodeoxyglucose, which accumulates in fast-dividing cells rapidly taking up glucose, making it possible to identify tumours (NHS UK, 2016).

Finally, and perhaps most importantly, cancer cells attempt to avoid immune destruction. Cells of the immune system undertake constant surveillance of cells and contribute to tumour eradication (Kim *et al.*, 2007). The process of immunoediting, a refinement of the cancer immunosurveillance theory, explains how the immune system is responsible for both eliminating tumours but also promoting tumour development (Dunn *et al.*, 2002a). Cancer immunoediting has three processes: elimination, equilibrium and escape. During the elimination phase, invasive growth of cancer cells causes minor disruptions in the surrounding tissues, stimulating inflammatory signals leading to the recruitment of innate immune cells and tumour cell killing (Coughlin *et al.*, 1998; Qin and Blankenstein, 2000). Tumour cell debris formed as a result of this cell death is ingested by local dendritic cells which home to the draining lymph nodes, inducing tumour specific CD8+ T-lymphocyte cytotoxicity (Pardoll, 2002; Ferlazzo *et al.*, 2002; Shankaran *et al.*, 2001b). The host immune system and surviving tumour cells then enter into dynamic equilibrium whereby the immune system applies selection pressure on the tumour cells that is enough to contain, but not fully eliminate a tumour. Surviving tumour cell variants arise carrying different mutations with increased resistance to immune attack through a lack of costimulatory molecules or danger signals (Abken *et al.*, 2002), secretion of immunosuppressive mediators or blocking molecules, decreased death receptor expression, release of suppressive cytokines or a lack

of tumour-associated antigen expression and down-regulation of MHC molecules, (Garrido *et al.*, 1997). The subsequent increased resistance provides developing tumours with a mechanism to escape immunological detection and elimination which then expand in an uncontrolled manner resulting in clinically malignant disease (Dunn *et al.*, 2004).

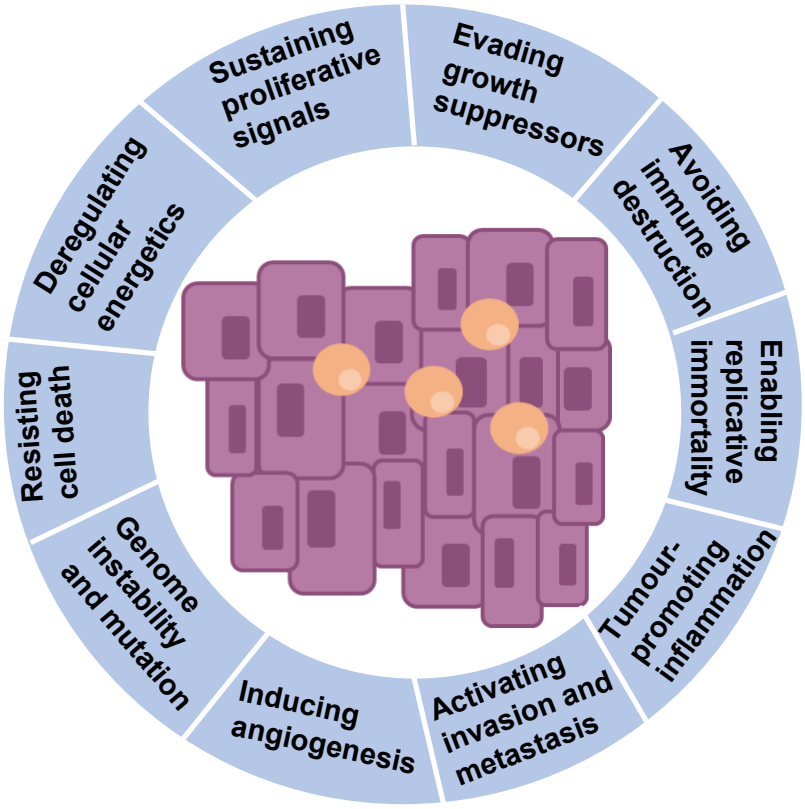

Figure 1. Biological Hallmarks of Cancer adapted from Hanahan and Weinberg, 2017

1.6 NATURAL DEFENCES AGAINST CANCER

Natural defences against cancer operate at all stages of the carcinogenesis process; initiation, promotion and progression (Hursting *et al.*, 1999). If one defence fails, another takes its place in a hierarchical fashion. These natural defence mechanisms against cancer could be classified into either non-immune or immune-based mechanisms (Jakobisiak *et al.*, 2003).

1.6.1 Non-immune mechanisms

Oxidative stress, is defined as a disturbance in the balance between the production of very reactive molecules and antioxidant defenses (Betteridge, 2000) which can cause damage and alterations in DNA. ROS and free radicals (molecules with one or more unpaired

electrons) are produced through a number of processes, for example mitochondrial electron transport, auto-oxidation of molecules (e.g., thiol molecules, quinones), enzymes (e.g., xanthine oxidase, aldehyde oxidase) and microsomal oxidations (Halliwell, 1999). In addition, carcinogens present in our diet, ultraviolet and ionising radiation can all damage DNA or stimulate cell proliferation, increasing the probability of spontaneous errors during DNA replication. However, a number of antioxidant mechanisms exist, which stabilise free radicals, including activation of enzymes typically found within cells (e.g., superoxide dismutase and catalase) but also extracellular molecules (e.g., plasma uric acid, ascorbic acid, alpha-tocopherol, and albumin), which have antioxidant and therefore anti-carcinogenic effects (Pigeolet *et al.*, 1990). Anti-carcinogenic substances in the diet include; vitamins A, C, E, B6, B12, folic acid, phenols, flavones, phytosterols, selenium, uric acid, glutathione and coumarins which can be found in grapes, berries, dark green vegetables and nuts, which can inhibit the formation of, and trap ROS, preventing their contribution to carcinogenesis.

As cancer is a disease caused by mutations in several DNA repair mechanisms, including 0^6-methyl-guanine-DNA methyltransferase base-excision repair, nucleotide-excision repair, homologous recombination and non-homologous recombination (Wood, 1996). In most cases the DNA is successfully repaired by one of the above processes, however, even if a mutation is missed, it is unlikely to result in a malignant transformation as it is thought that multiple genetic mutations are required (Hahn and Weinberg, 2002). Proto-oncogenes and tumour suppressor genes (e.g. *p*53) are crucial in regulating cell growth involved in signalling pathways, cell proliferation, differentiation and programmed cell death, all promoting proliferation. Tumour suppressor genes continuously participate in the defence against cancer; inhibiting proliferation, inducing cell senescence, cell death and differentiation, repairing DNA, inhibiting angiogenesis, interfering with metastasis and influencing immune surveillance. However, even if the proto-oncogenes and tumour suppressor genes become mutated, growth is limited by the Hayflick limit and the requirement for angiogenesis, which can only be evaded by chance mutations in critical genes.

If a cancer cell manages to overcome the previous defences then the tumour cells must also overcome natural protection against metastases. Metastases consists of; penetration of basement membrane, detachment of cells from the primary tumour, movement of cells into the blood or lymph (intravasation), survival of cells in circulation, arrest and interaction of cells with endothelial cells, movement of cells across the blood vessel wall (extravasation) and finally migration of cells into the tissue and growth at the new site which displays the

appropriate chemokines and receptors (Hanahan and Weinberg, 2000). These processes require adhesion molecules which either inhibit or facilitate progression (Carmeliet and Jain, 2000) as they regulate the adhesion of tumour cells to each other or to adjacent non-tumour cells, preceding their intra and extravasation during the metastatic process. Two protease systems also have an important role against metastasis and tumour invasion; urokinase-type plasminogen activators which actively degrade all components of the extracellular matrix (Kähäri and Saarialho-Kere, 1999) and gelatinases which facilitate the development of the immune response against cancer and enable the migration of dendritic cells which might carry tumour-associated antigens from skin to regional lymph nodes (Ratzinger *et al.*, 2002). It is estimated that only 0.01% of circulating tumour cells will form metastatic colonies (Liotta *et al.*, 1991).

1.6.2 Immune mechanisms

One of the last defences against cancer is the activity of the innate and adaptive immune systems (Jakobisiak *et al.*, 2003). The innate immune system is involved in protection by secreting a number of cytokines with direct and indirect anti-tumour activity. NK cells provide rapid detection and elimination of potentially dangerous cells, immediately finding their targets and killing them, primarily by recognition of downregulated MHC class I molecules (Leiden *et al.*, 1989; Pena *et al.*, 1990). Macrophages and neutrophils are capable of phagocytosis of tumour cells and secretion of growth inhibitory cytokines during the advanced stages of tumour progression (Jakobisiak *et al.*, 2003).

Cytokines produced by monocytes/macrophages and T-lymphocytes participate in every aspect of immune responses including the defence against cancer. IFN-γ regulates tumour growth through either direct or indirect mechanisms. IFN-γ exerts cytotoxic effects towards tumour cells, induces expression of chemokines that inhibit angiogenesis, up-regulates the expression of MHC class I and II molecules, activates macrophages and causes other immune cells to clone and differentiate to effector cells to remove the infectious agent (Harao *et al.*, 2015; Blankenstein and Qin, 2003). TNF-α possesses anti-tumour effects attributed to both the direct cytotoxic effects on tumour cells and angiogenesis, lipid metabolism and pro-coagulant properties. Initially transformed cells may contain too little antigen to initiate a response, known as immune ignorance, yet, with time there is a gradual increase in the number of tumour cells and thus antigen (Igney and Krammer, 2002; Chouaib *et al.*, 1997)

1.7 CANCER IMMUNE-SURVEILLANCE

For the last 100 years, there has been debate as to whether the immune system can detect and eliminate cancer cells. In the 1890s, William B. Coley observed that some cancer patients experienced spontaneous remission of their tumours when they contracted acute infections (Coley, 1991). Soon after, in 1909, Paul Ehrlich proposed that the immune system was able to recognise and protect against tumours (Ehrlich, 1909). This idea was pursued by Medawar and colleagues, who clarified the role of cellular components of the immune system in recognising and mediating transplant rejection whereby immune responses characterized by lymphocyte infiltration of genetically dissimilar grafts (but not of autografts) were responsible for rejection (Medawar, 1946, 1944). Furthermore, subsequent work with mouse models showed that immune cells could detect the presence of transformed tissue either by recognising specific structures on the tumour cell surface or be 'immunised' against tumours, establishing the notion of tumour specific antigens (Old and Boyse, 1964; Klein, 1966). In 1957, the theory of 'immune surveillance' was suggested by Lewis Thomas and Frank McFarlane Burnet, suggesting that T-lymphocytes patrol the body to identify and eliminate malignancies through recognition of specific tumour antigens (Thomas, 1982; Burnet, 1970), provoking an effective immune response, leading to regression of the tumour (Burnet, 1970).

This theory of immune surveillance was considered controversial. A series of experiments examining the CBA/H strain of so-called athymic nude mice (which lack hair, but also do not have a thymus, and do not develop T-lymphocytes) showed that these mice did not form carcinogen induced tumours (e.g., tumours caused by the chemical carcinogen methylcholanthrene) either earlier or more frequently than their immunocompetent controls (Stutman, 1974). In hindsight there were several experimental design problems that the investigators could not have known at the time, specifically, that nude mice are not totally immunocompromised. It is now known that nude mice have high levels of NK cells and other innate immune cells (Dunn *et al.*, 2004). In addition, the CBA/H strain of mice are more susceptible than wild-type mice to carcinogen-induced tumour formation (Heidelberger, 1975) and the tumour monitoring periods of 3–7 months in these experiments were too short to detect spontaneous tumour formation in controls. Despite these concerns over the robustness of some studies in the 1970s, the concept of cancer immune surveillance became unpopular and lost attention in the scientific literature.

Later studies provided some more support for the theory of cancer immune surveillance (Herberman and Holden, 1978). The experiments previously undertaken by Stutman and

colleagues were repeated. Immunodeficient mice with a genetically targeted disruption of the recombination-activating gene-2 (RAG2) that is expressed only in lymphocytes and essential for their function, developed tumours earlier than wild type mice. It was also shown in these mice that the immune cells were essential to suppress the development of chemically induced tumours (Shankaran *et al.*, 2001b). Indeed, rather than just a presence or absence of cells, it became clear that loss of certain effector functions was most important in the susceptibility to cancer (Dunn *et al.*, 2002a). For example, mice genetically modified so that their T-lymphocytes could not produce IFN-γ, or so that other cells did not express the IFN-γ receptor, exhibited an increase in chemically induced carcinogenesis and spontaneous tumour development (Dighe *et al.*, 1994; Kaplan *et al.*, 1998). In addition, mice deficient for perforin, a key killing mechanism of T-lymphocytes and NK cells, were 1000-fold more susceptible to transplanted lymphomas compared with immunocompetent mice (Smyth *et al.*, 2000).

Evidence of cancer immune surveillance is not just restricted to animal models. Human studies have shown that transplant recipients on immunosuppressive medication have a heightened risk of virus-associated cancers (Boshoff and Weiss, 2002; Adami *et al.*, 2003). In addition, in immunocompromised patients, such as individuals with acquired immune deficiency syndrome (AIDS) susceptibility to solid cancers compared to the wider population is increased (Chaturvedi *et al.*, 2007; Kirk *et al.*, 2007). Finally, patients with higher levels of tumour infiltrating CD8+ T-lymphocytes have been shown to have prolonged survival (Clark *et al.*, 1989; Clemente *et al.*, 1996). Immune surveillance is now a well-established concept with tumour cells expressing tumour-associated antigens (oncofetal, oncoviral, overexpressed, cancer-testis, lineage-restricted, mutated, post-translationally altered or idiotypic (explained further in Methods, 2.6.1.2) (Decker, 2003)) that T-lymphocytes recognise (van der Bruggen *et al.*, 1991; Jäger *et al.*, 2000), leading to the subsequent elimination of cancer cells (Swann and Smyth, 2007). These tumour-associated antigens are also targeted with recent cancer therapies such as monoclonal antibody therapy and adoptive immunotherapy (Wolff *et al.*, 2013; Cheever *et al.*, 2009). The ideal tumour-associated antigens possess therapeutic functions, immunogenicity (elicits an immune response), oncogenicity and specificity (Cheever *et al.*, 2009).

1.8 BREAST CANCER

Breast cancer is the most common female malignancy (excluding non-melanoma skin cancer) in most countries worldwide (Ajithkumar, 2011) and is the most common cause of death among women aged 40-50 years, accounting for about a fifth of all deaths in this age

group (Dixon and Montgomery, 2008). In the UK in 2015-2017, each year, on average, there were 55,176 new cases of invasive breast cancer diagnosed and 11,399 deaths from the disease (CRUK, 2017). Around 27% of these breast cancer cases were deemed preventable. There are many types of breast cancer; ductal carcinoma in situ (DCIS), lobular carcinoma in situ (LCIS) (early, non-invasive types), invasive lobular, invasive ductal carcinoma, and rarer diagnoses such as inflammatory breast cancer, medullary breast cancer, mucinous breast cancer, metaplastic breast cancer and papillary breast cancer. The stage and grade of a tumour can be quantified and tumours can be typed according to the presence of steroid receptors; the oestrogen receptor, progesterone receptor and HER2. If a tumour does not express any of the three receptors, this is classified as are 'triple negative' breast cancer.

1.8.1 Breast cancer risk

1.8.1.1 Breast cancer risk: traditional factors

Besides age, important risk factors for breast cancer include; family history, variables which reflect exposure to oestrogens (e.g., early menarche, late menopause), high energy intake, high intake of fat, obesity and weight gain, and age. In the UK between 2011 and 2013, on average almost half of female breast cancer cases were diagnosed in females over 65 years old. Incidence rates generally rise from ages 30-50 then further again from age 65, dropping and plateauing after 70 years (Abdelmagid *et al.*, 2016).

Familial breast cancer risk depends on the relative affected (first or second degree), the age at which the relative developed cancer, and the number of relatives affected. The risk of developing breast cancer almost doubles if a first-degree relative (mother, sister or daughter) has had breast cancer and triples if two first-degree relatives have developed breast cancer, compared to individuals with no family history of breast cancer (Singletary, 2003). Around 5 to 10% of all breast cancer cases are explained by the inheritance of mutations in one of the two major breast cancer susceptibility genes (Claus *et al.*, 1996). The genes *BRCA1* and *BRCA2* encode proteins that are implicated in DNA repair and tumour suppression, and account for around 75% of hereditary breast cancer cases (Benson *et al.*, 2009). The mutated version of the genes impairs defences against tumours, increasing the lifetime risk of breast cancer to around 55%.

Women who start menstruating early in life or go through the menopause later than average have an increased risk of developing breast cancer, likely due to increased oestrogen

exposure. For every year younger at menarche, breast cancer risk increases by 5% and for every year older at menopause risk increases by 3% (Brinton *et al.*, 1988). For example, it has been shown that in women who began menstruating at the age of 15 or later had a 23% lower risk of breast cancer than those with menarche before age 12 (Brinton *et al.*, 1988). At the other end of the reproductive period, women who reached artificial menopause (through removal of ovaries) before age 40 had a 45% reduced risk compared to women who did not reach menopause until age 50 (Brinton *et al.*, 1988).

Nulliparity, or giving birth to a first child later in life, increases lifetime risk of breast cancer. For example, women giving birth to their first child over the age of 30 are twice as likely to develop breast cancer as women giving birth to their first child before the age of 20 years. This risk is higher for women who give birth to their first child after the age of 35 years. It is thought that such a delay in becoming pregnant increases exposure to oestrogen, which is thought to promote tumour survival and is a factor that increases the risk of developing breast cancer (Eliassen *et al.*, 2006; Pike *et al.*, 1979). It is also thought that the beneficial effects of early full-term pregnancy could be due to high concentrations of progesterone and/or prolactin (enables women to produce milk) protecting breast cells from oestrogen in the long term (Benson *et al.*, 2009).

1.8.1.2 Breast cancer risk: exercise and physical activity

Evidence suggests that exercise can exert preventative or therapeutic effects at all stages of carcinogenesis (Bigley *et al.*, 2014). Breast cancer is the most widely studied cancer in the context of exercise, physical activity, and interventions along the framework of the cancer continuum; prevention, detection, treatment preparation, treatment effectiveness, recovery and rehabilitation, disease relapse, palliation and survival (Courneya, 2014)[2].

There is consistent and substantial evidence that physical activity reduces risk of breast cancer by 25% when comparing the most active populations to least active populations in both pre- and post-menopausal women (Leitzmann *et al.*, 2015a). This seems to follow a dose-response pattern with breast cancer risk reduced when comparing insufficiently active

[2] For simplicity in this thesis, the stages of *prevention* and *detection* from the cancer continuum are grouped into 'cancer risk'. The stages of *treatment preparation/coping* and *treatment effectiveness/coping* from the cancer continuum have been grouped into 'cancer treatment'. Finally, the stages of *recovery/rehabilitation, disease prevention/health promotion, palliation and survival* from the cancer continuum have been grouped into 'following cancer treatment'

women (undertaking less than 600 MET (metabolic equivalent) minutes per week of total physical activity), slightly active women (600-3999 MET minutes per week), moderately active women (4000-7999 MET minutes per week) and highly active women (>8000 MET minutes per week) by 3%, 6% and 14% respectively (Jung *et al.*, 2016a). Risk reductions surrounding physical activity and breast cancer range from 20-80% (Monninkhof *et al.*, 2007) depending on exercise intensity and duration (Monninkhof *et al.*, 2007). Overall assessment of the dose-response data suggests that a total of 30 to 60 minutes per day of moderate to vigorous physical activity is required for the reduction of breast cancer risk (around 25 MET.h week^{-1}) (IARC 2002).

Both lifetime recreational activity and recent leisure time physical activity have been associated with a reduced risk (odds ratio (OR) = 0.70; 95% CI = 0.56-0.88) of breast cancer (Verloop *et al.*, 2000). Greater leisure time activity has been associated with a 37% reduction in relative risk of breast cancer even after adjustments for age and body mass, with risk reduction greatest in pre-menopausal women below 45 years (Thune *et al.*, 1997). A decreased risk of both pre- and post-menopausal breast cancer has been shown by comparing women who were active both in adolescence (16% reduction) and adulthood (32% reduction) (Matthews *et al.*, 2001). Evidence of breast cancer primary prevention studies suggest that increasing activity levels later in life may also reduce risk of breast cancer (Newman *et al.*, 1997).

Direct evidence of an anti-cancer effect of exercise has recently been proven to be a result of exercise-induced immune cell infiltration into tumours in mice. Tumour-bearing mice randomised to a condition of voluntary wheel running versus inactivity, showed that exercise promoted NK cell infiltration into tumours, which correlated inversely to tumour burden, and correlated positively with survival. Further, depletion of NK cells enhanced tumour growth and blunted the beneficial effects of exercise on survival (Pedersen *et al.*, 2016). It must now be subsequently investigated whether the mobilisation of CD8+ lymphocytes with exercise enhances T-lymphocyte cytotoxic activity towards cancer.

1.8.1.3 Breast cancer risk: body composition

Obesity is an important but under recognised contributor as a preventable cause of breast cancer in pre- and post- menopausal women (McDonnell *et al.*, 2014; Renehan *et al.*, 2008; Ligibel *et al.*, 2008). Women with a BMI greater than 28kg.m^2 had a 23% increased risk of breast cancer compared to women with a BMI lower than 22kg.m^2 (Rohan *et al.*, 2013). BMI appears to be a more prevalent risk factor for breast cancer in post- than in pre-menopausal

women (Dixon and Montgomery, 2008; Matthews *et al.*, 2007; Fortner *et al.*, 2016). Whilst the relationship between obesity and breast cancer risk has long been recognised, central rather than general obesity, may be in more important in predisposing individuals to the development of breast cancer (Stoll, 1994; Bruning *et al.*, 1992). Dual-energy x-ray absorptiometry (DEXA) measurements of body fat have also shown strong positive associations with breast cancer risk. The multi-variable adjusted HR for the upper quintile vs lowest quintile of fat mass of the trunk ranged from 2.05 (95% CI 1.50-2.79) (Rohan *et al.*, 2013). Furthermore, more basic anthropometric measures of obesity (BMI 1.97, 1.45-2.68), waist circumference (1.97, 1.46-2.65) and waist to hip ratio (W:H) (1.91, 1.41-2.58) were also positively associated with breast cancer risk. Interestingly, DEXA derived body fat variables were only positively associated with risk of ER positive breast cancer not ER negative. The association between obesity and breast cancer risk seems to follow a dose-response relationship. A 12% increased risk for breast cancer reported among overweight postmenopausal women was increased to 25% in those women who were obese (as defined by BMI) (Eliassen *et al.*, 2006).

In adults, exercise may lower breast cancer risk by regulating weight, thus decreasing hormone and growth-factor levels (Velasquez-Manoff, 2015). Mechanisms surrounding this association are likely to include higher levels of insulin, insulin-like growth factors and sex hormones that occur with higher BMI (Del Giudice *et al.*, 1998; Suga *et al.*, 2001). Obesity is linked to elevated levels of oestrogen (Hankinson *et al.*, 1995; Cleary and Grossmann, 2009) and the strong association between obesity and ER positive breast cancer risk compared to ER negative breast cancer demonstrates the importance of oestrogenic milieu of obesity (Munsell *et al.*, 2014; Suzuki *et al.*, 2009). Increased oestrogen in postmenopausal women due to the conversion of androstenedione to oestrone by adipose tissue and subsequent long-term increase in plasma oestradiol from adipose tissue in this group explains why body composition may have a stronger relationship in post- versus pre-menopausal women (Martin and Weber, 2000; Cleary and Grossmann, 2009).

Obesity-associated inflammation is also strongly correlated to breast cancer risk and progression. The cytokine production in excess adipose tissues creates a chronic inflammatory microenvironment which favours tumour development. Chronic inflammatory components such as CRP and IL-6 are important constituents in the carcinogenic process and tumour microenvironment, where elevated levels can promote tumour-cell proliferation and inhibition of apoptosis, consequently leading to transformation of a normal cell to tumour cells which can survive and proliferate (Reuter *et al.*, 2010; Landskron *et al.*, 2014). This has been shown to increase the risk of breast cancer (Asegaonkar *et al.*, 2015;

Esquivel-Velázquez *et al.*, 2015) through associations with intracellular transcription factors that are involved in each step of carcinogenesis including apoptosis, migration, cell proliferation, inflammation, angiogenesis and metastasis (Custódio *et al.*, 2016). Recent work suggests that NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) mediates tumor progression through proinflammatory cytokines such as IL-6, IL-8 CCL2, CCL5 and VEGF (Esquivel-Velázquez *et al.*, 2015) which not only drives obesity mediated inflammation but also stimulates antiapoptotic genes and breast cancer proliferation, invasion, angiogenesis, and metastasis (Prasad *et al.*, 2010).

Thankfully, the increase in breast cancer risk with increasing obesity does seem reversible and prospective cohort studies show that weight loss is associated with a reduction in the risk of postmenopausal breast cancer (Eliassen *et al.*, 2006).

1.8.1.4 Breast cancer risk: nutrition

Poor dietary habits characterised by the high intake of refined starches, sugar, alcohol, and both saturated and trans-saturated fats, are known to increase inflammation and therefore linked with increased risk of breast cancer by impacting both cancer initiation and progression (Kotepui, 2016).

Alcohol consumption can elevate the level of oestrogen related hormones in the blood and affect oestrogen metabolism in the liver. A meta-analysis of 53 epidemiological studies indicated that an intake of 35-44g alcohol per day can increase risk of breast cancer by 32%, with a 7.1% increase in the relative risk (RR) for each additional 10g per day (Bagnardi *et al.*, 2015; Jung *et al.*, 2016a). However, it is unknown whether this is down to the alcohol content itself or to the culmination of calories within alcohol (approximately 7kcal per gram). Other epidemiological studies have shown women who drank more than 5 glasses of alcoholic beverages a week had 20% increased risk of breast cancer compared with women who self-reported that they had never consumed alcohol (Mahabir *et al.*, 2004).

3-PUFA (polyunsaturated fatty acids) intake has been found to be inversely associated with breast cancer risk (Abdelmagid *et al.*, 2016) due to the growth inhibitory pro-apoptotic and anti-angiogenic effects of n-3 fatty acids (Abdelmagid *et al.*, 2016). Carbohydrates can affect glucose and circulating insulin levels (Belardi *et al.*, 2013) which might affect breast cancer risk through stimulation of insulin receptors in breast tissues and via the increased bioactivity of IGF-1 which in turn may stimulate cell proliferation. However, omega 3

polyunsaturated fatty acids have anti-inflammatory properties and are thought to be critical in breast cancer prevention (Abdelmagid *et al.*, 2016).

A high fat diet may increase breast cancer risk through an increase in endogenous hyperinsulinaemia (Dawson *et al.*, 2013). Epidemiological evidence suggest that lower levels of insulin may be important for cancer prevention. In 9,778 individuals over 20 years of age, cancer death was significantly higher in individuals with hyperinsulinaemia (fasting insulin level >10IU/ml) (HR 1.89, 95% CI 1.07-3.34) (Tsujimoto *et al.*, 2017). Other research compared women who developed breast cancer and women who did not and reported a significant positive association between endogenous insulin levels and breast cancer risk (HR for the highest compared with the lowest quartile 1.46, 95% CI 1.00–2.13) (Gunter *et al.*, 2009) with evidence showing that this relationship was still apparent for lean women, indicating this mechanism is independent from obesity (Kabat *et al.*, 2009). In mice models using mice with reduced insulin gene dosage and therefore a reduced endogenous insulin production without impairing glucose homeostasis, pancreatic intraepithelial neoplasia was reduced by ~50% (Duvillié *et al.*, 1997; Mehran *et al.*, 2012), with fasting insulin levels correlating with tumour area and weight (Zhang *et al.*, 2019). The pro-cancerous effects of hyperinsulinaemia may be promoted by inflammatory adipokines and proinflammatory cytokines such as IL-6 and TGF-α.

Other dietary products can reduce the risk of breast cancer. For example, eating products containing antioxidants or vitamin D, have been shown to reduce RR of breast cancer (Saxe *et al.*, 1999; Bauer *et al.*, 2013). Fruit and vegetable intake is also associated with a reduced risk of cancer and reduced all-cause mortality (ACM) (Aune *et al.*, 2012) which may be explained by the antioxidant vitamins present within them which protect DNA from oxidative damage. It is believed that vitamin C for example, reduces breast cancer risk due to the antioxidant capacity and ability to inhibit cell proliferation, maintaining DNA methylation and hormonal metabolism (Emaus *et al.*, 2015; Griffiths *et al.*, 2016; Eliassen *et al.*, 2012).

1.8.1.5 Breast cancer risk: ageing

Research suggests that the transformation of a malignant cell requires age-associated changes in the cellular microenvironment or tissue, such as increased inflammation or decreased immune function to support proliferation and dissemination of transformed cells (Naylor *et al.*, 2013; DeGregori, 2013).The incidence of breast cancer increases with age, doubling every 10 years until the menopause, at which point the rate of increase slows dramatically (Dixon, 2012). The relationship between age and cancer risk may be due to

the accumulation of genetic and epigenetic mutations (Niccoli and Partridge, 2012; Finkel *et al.*, 2007). The midlife point (defined as 45-64 years of age) is a period of life when the prevalence and accumulation of multiple cancer risk factors is high (e.g. oestrogen exposure) (Ory *et al.*, 2014). Although, a risk factor of 5.8 was predicted for individuals over 65 years old compared to individuals under 65 years of age (whereby a risk factor of 1 indicates no extra risk and a risk factor of 10 indicates a 10 fold increase risk) (Races, 2002). Unlike most cancer types the relationship between incidence and age is not linear for breast cancer, as other risk factors prove increasingly important, such as, age of first menstrual cycle, age of menopause and age of first full term pregnancy (Pike *et al.*, 1983). This suggests breast tissue ageing and hormone level, rather than ageing alone, maybe closely related to breast cancer risk and incidence.

1.8.1.6 Breast cancer risk: viral serostatus

Several cancers in humans (e.g. cervical cancer, liver cancer, and adult T-cell leukaemia) are known to be caused by viruses and the detection of viral DNA, mRNA (messenger ribonucleic acid), and/or antigens in tumour tissues. However, the area of viruses and breast cancer risk, despite ongoing research, is poorly understood compared to genetic, environmental and behavioural risk factors. The possibility that viruses may have a role in breast cancer aetiology was initiated in 1936 by John Bittner and colleagues who observed that mouse milk contained an unknown factor, which caused mammary tumours in their pups when they grew to adulthood (Bittner, 1936). This unknown factor was later identified as breast cancer promoting mouse mammary tumour virus (MMTV) (Hennighausen, 1999). Thanks to more sensitive detection methods, links between infectious agents and breast cancer are becoming clearer. In normal rodent cells, CMV (infectious virus or virus DNA) induced mutations in genes that are critical for malignant transformation (Geder *et al.*, 1976; Geder *et al.*, 1977; Nelson *et al.*, 1984; Boldogh *et al.*, 1994; Shen *et al.*, 1997; Doniger *et al.*, 1999). These findings led to the speculation that CMV contributes to oncogenesis by a 'hit-and-run' mechanism (Nelson *et al.*, 1984; Boldogh *et al.*, 1994; Shen *et al.*, 1997; Doniger *et al.*, 1999). This seemed unlikely as it supposed that virus nucleic acids are not retained in transformed cells and there was no conclusive evidence for the transformation of normal cells after CMV infection in humans.

Links between EBV, CMV and breast cancer are inconsistent (Bonnet *et al.*, 1999; Magrath and Bhatia, 1999; Xue *et al.*, 2003). It has previously been hypothesised that some breast cancers might be predominantly caused by late exposure (in adulthood rather than in childhood) to common virus' (Richardson, 1997; Savu *et al.*, 2008; Hjalgrim *et al.*, 2000).

Case-controlled evidence suggests a relationship between increased breast cancer risk with increasing age at onset of self-reported infectious mononucleosis (caused by EBV), suggesting that a delayed exposure to the virus is the mechanism behind increased risk (Yasui *et al.*, 2001; Bonnet *et al.*, 1999). This is supported by evidence concerning countries with a low incidence of breast cancer (e.g. Asia), whereby exposure to CMV and EBV occurs in childhood whereas in countries (Western) with higher incidences of breast cancer, exposure usually occurs later in life (Richardson, 1997; Richardson *et al.*, 2004). Age-adjusted odds ratios of breast cancer in women who reported a history of infectious mononucleosis relative to women who did not, increased from 0.55 (95% confidence interval (CI), 0.05–6.17)) for women who had onset at 0–9 years to 2.67 (CI, 1.04–6.89) for women with onset at >25 years (p=0.016). However, the self-reported nature of data collection in this study is prone to bias. The likely mechanisms surrounding breast cancer risk and delayed primary infection appear to be associated with a prolonged stress on the immune system with increased production of proinflammatory cytokines (IL-6 and TNF-α) which stimulate aromatase function converting androstenedione to oestrone in adipose tissues (Hornef *et al.*, 1995; Foss *et al.*, 1994; Andersson, 1996; Macdiarmid *et al.*, 1994; Zhao *et al.*, 1996; Simpson *et al.*, 1997; Singh *et al.*, 1997; Purohit *et al.*, 1999). Interestingly, aromatase function is the major source of endogenous oestrogen in the postmenopausal period, when breast cancer risk is already increased (Simpson *et al.*, 1997).

Furthermore, higher mean CMV IgG (immunoglobulin G) levels have previously been found in women with breast cancer, suggestive of more recent infection with CMV. This suggests that late exposure to CMV is a risk factor for breast cancer (Richardson *et al.*, 2004), whereby mean CMV IgG levels were higher in cases than controls, with an adjusted OR per unit increase in CMV IgG of 1.46 and 95% CI 1.06–2.03. This agrees with the hypothesis that late exposure to CMV is a risk factor for breast cancer. However, this association did not exist for EBV. To investigate whether CMV IgG levels were elevated before diagnosis of breast cancer, a nested case-control study with two serum samples taken at least four years before diagnosis and controls were tested for CMV IgG (Cox *et al.*, 2010). The risk of breast cancer, adjusted for parity, was greater per unit difference in CMV IgG between samples: OR 1.7 (95% CI 1.1–2.5). In an analysis restricted to parous cases and age-matched parous controls, the OR for seroconversion between samples in the same individual, adjusted for parity and age at first birth, was 9.7 (95% CI 1.2–77.3). This association between CMV and breast cancer may be due to CMV being an ubiquitous virus that is shed in breast milk, saliva, urine, cervical secretions, and semen, therefore persistently infecting epithelial cells (Sissons and Carmichael, 2002). As breast milk is one

of the major routes for CMV transmission it may also be a major site of latent CMV infection. CMV infection is found in the breast of healthy and normal tissues but at a higher rate in the epithelium of breast carcinoma (97%) (Harkins *et al.*, 2010). No evidence of an association between EBV IgG levels and breast cancer were seen but research is inconsistent (Xue *et al.*, 2003; Cox *et al.*, 2010; Mant and Cason, 2004) partly because of the extremely low EBV viral loads, the latency of EBV with minimal protein expression and inconsistent methodologies (Bonnet *et al.*, 1999; Fina *et al.*, 2001; Khan *et al.*, 2011a; Khan *et al.*, 2011b).

1.8.2 Breast cancer treatment

1.8.2.1 An overview of breast cancer treatment

After a breast cancer diagnosis, the exact treatment regimen depends upon both the disease stage, (which is determined by tumour size, the number and location of lymph nodes involved, the presence or absence of distant metastatic disease (Mantovani *et al.*, 2008), menopausal status and pathological features such as hormone receptor status and tumour grade (based on how abnormal the tumour cells and the tumour tissue look under a microscope, indicating how quickly a tumour is likely to grow and spread). Traditional therapies for breast cancer include surgery, radiotherapy, chemotherapy, hormone therapies and biological therapies (also referred to as immunotherapy – predominantly monoclonal antibody therapy). Breast cancer surgery can include the removal of the primary tumour with a circumferential margin (1 mm - 1 cm) of normal tissue; wide local excision (a surgical procedure removing a small area of cancerous cells with a margin of normal tissue), segmental mastectomy or quadrantectomy (partial mastectomy) which can take place prior to or after any treatment.

Radiotherapy is a treatment consisting of energy deposition in tissues causing ionisation resulting in the production of free radicals and ROS that damage DNA causing single or double strand breaks and stimulating cell death. Radiotherapy is usually administered post-operatively and is recommended for patients who have undergone surgery and are at high risk of reoccurrence (Mantovani *et al.*, 2008). Radiation therapy is typically delivered over a 5-8-week period in small repeated doses to maximise the killing of cancer cells and minimise the damage to normal cells.

Chemotherapy refers to a wide group of cytotoxic drugs that interfere with cell division and DNA synthesis. The discovery of chemotherapeutic drugs stems from World War II whereby

individuals poisoned by mustard gas, a deadly chemical, exhibited a low number of immune cells in their blood. Goodman and Gilman hypothesised that if mustard gas could destroy normal white blood cells it could also destroy cancerous cells which proved to be the case in a lymphoma patient in 1946 (Christakis, 2011). Similar but more effective agents (called alkylating agents) were then developed that killed rapidly growing cancer cells by damaging their DNA. In addition, Farber and Diamond (1948) continued their work on anti-folates which cause a deficiency of folic acid that is instrumental in folate-dependent enzymes and their role in cellular production and building processes (Goodman *et al.*, 1946; Farber and Diamond, 1948). Common chemotherapy regimens for breast cancer include a variety of drugs; anti-metabolites, alkylating agents, anthracyclines, taxanes, monoclonal antibodies and oestrogen receptor modulators. Anti-metabolites, alkylating agents, anthracyclines and taxanes halt cell replication and ultimately lead to cell death in rapidly dividing cells.

Chemotherapy can cause several side effects including fatigue, anorexia, nausea, anaemia, neutropenia, thrombocytopenia (low blood platelet counts), peripheral neuropathies, ataxia (co-ordination, balance and speech disorders) and cardiotoxicity. Chemotherapy can be administered with adjuvant or neoadjuvant regimens. Adjuvant chemotherapy is used post-operatively to eradicate the micro-metastases that remain and can cause relapse if not destroyed. Neoadjuvant chemotherapy (chemotherapy before surgery for operable breast cancer) aims to downsize large tumours to reduce the need for mastectomy or to make locally advanced breast cancers operable (Dixon and Montgomery, 2008). The use of neoadjuvant chemotherapy was first introduced in the 1970s and led to the improvements in the number of patients able to undergo breast conserving surgery (Mantovani *et al.*, 2008). Neoadjuvant chemotherapy achieves clinical regression of tumours in 70-80% of patients with 10-20% achieving complete pathological response (disappearance of the tumour from the breast and axillary nodes) (pCR).

Around 20-25% of breast cancers overexpress HER2 and can be treated using the drug Trastuzumab (Wolff *et al.*, 2013). Trastuzumab is a humanised monoclonal antibody that uses the immune system to target the extracellular domain of the HER2 protein, hence clinically used in combination with chemotherapy in patients that are HER2 positive and have a tumour >1 cm. Trastuzumab binds to the HER2 receptor, preventing Epidermal Growth Factor binding and also preventing non-specific downstream signalling and, due to overexpression, subsequently prevents proliferation. It also promotes antibody-dependent cell-mediated cytotoxicity whereby monoclonal antibodies target tumour-associated antigens leading to cell cycle arrest, reduction in angiogenesis and inhibition of extracellular domain cleavage. Treatment with Trastuzumab has been associated with increased tumour

infiltration of NK cells (Arnould *et al.*, 2006; Gennari *et al.*, 2004) and T-lymphocyte subsets (Varchetta *et al.*, 2007). Adding Trastuzumab to chemotherapy has previously increased pCR rates in patients (Joensuu *et al.*, 2009; Perez *et al.*, 2011; Irwin *et al.*, 2008b).

Hormonal therapy is used in ER positive and PR positive breast cancer patients (around 70% of all patients), following chemotherapy to block oestrogen binding to receptors on breast cancer cells which would normally stimulate proliferation. Drugs, such as Tamoxifen, compete for the oestrogen receptor to prevent oestrogen stimulating the cells to divide and grow.

1.8.2.2 Breast cancer treatment: the effect of exercise and physical activity

Physical activity has been increasingly examined as an intervention alongside conventional treatments to combat the physiological and psychological effects of treatment by improving the overall health of patients or by managing specific symptoms that may cause treatment delays (Friedenreich and Orenstein, 2002). In breast cancer patients, the aims of exercise during treatment are to regain and improve physical function, aerobic capacity, strength and flexibility, body image, quality of life, body composition and cardiorespiratory, endocrine, neurological, muscular, cognitive and psychological outcomes. Other outcomes are to prevent long-term and late effects of cancer, enhance ability to withstand treatments and to promote treatment success by mitigating adverse symptoms associated with treatment e.g. fatigue and nausea (Schmitz *et al.*, 2010).

Although adequate rest is often encouraged at the time of treatment, insufficient activity over time leads to loss of physical conditioning and muscular strength. Women diagnosed with breast cancer are estimated to decrease their physical activity levels by 11%, (by 2 hours per week), according to a self-report study (Irwin *et al.*, 2003). This decline in physical activity has been linked with weight gain (Demark-Wahnefried *et al.*, 2001) and poorer survival (Holmes *et al.*, 2005). It has been shown that $\dot{V}O_2$ peak, a measure of cardiorespiratory fitness, is lower in patients with breast cancer receiving cytotoxic chemotherapy than for healthy, sedentary women by 34, 30, 25 and 17% at ages 40, 50, 60 and 70 years respectively (Betof *et al.*, 2013). In line with these significant declines in physical function there is a negative effect on bodily pain, social functioning and emotional health (Kroenke *et al.*, 2004) with 50% of women with breast cancer encountering depression, anxiety or both upon a year following diagnosis (Burgess *et al.*, 2005). Exercise during cancer treatment has been shown to help individuals cope with their disease with physical activity having modest positive effects on not only aerobic fitness, physical

functioning and muscular strength, but also on fatigue, and some aspects of quality of life (Blanchard *et al.*, 2008). Although exercise is evidently beneficial and safe during treatment it must be remembered that cancer variables such as type, stage and treatment may influence a person's ability and willingness to exercise (Courneya, 2014).

1.8.2.3 Breast cancer treatment: the effect of body composition

Body fat gain is apparent in breast cancer patients undergoing a variety of treatments including chemotherapy, radiotherapy and surgery, with menopausal status affecting the extent of weight gain (>5% gain in body fat seen in was 56% of premenopausal women and 42% of postmenopausal women n=131) (Liu *et al.*, 2017; Ali *et al.*, 2014). Previous studies have reported 34% to 96% of women with early-stage breast cancer gain 0.9–7 kg compared to their pre-treatment weight after treatment of either mastectomy, breast radiation or chemotherapy (Caan *et al.*, 2006; Caan *et al.*, 2012a; Fitzmaurice *et al.*, 2015; Sheean *et al.*, 2012). However, weight gain is more prevalent in women undergoing chemotherapy (Demark-Wahnefried *et al.*, 1997a). It must be considered that weight gain has often been measured using BMI which fails to decipher accurately between lean and fat mass (Fitzmaurice *et al.*, 2015; Muraro *et al.*, 2011; Freedman *et al.*, 2004; Demark-Wahnefried *et al.*, 1997a; Demark-Wahnefried *et al.*, 2001; Ingram and Brown, 2004; Cheng *et al.*, 2010). One study using computerised tomography (CT) scans to assess body composition (a more robust measure of lean and fat mass), showed that the majority of women undergoing adjuvant treatment gained body fat, particularly visceral fat, irrespective of the direction of weight change (Cheney *et al.*, 1997).

Nodal status, alongside the duration and intensity of treatment has also been shown to affect the extent of weight gain during treatment of breast cancer. Women of normal weight at the time of diagnosis are more likely to increase body fat and weight, compared to those who are overweight or obese at the time of diagnosis (Goodwin *et al.*, 1988; Nissen *et al.*, 2011). One consideration is whether reported increases in body fat pre- versus post-treatment are due to treatment or the general effect of ageing as increases in body fat and decreases in lean mass occur in healthy women with the ageing process, regardless of disease or treatment (Wang *et al.*, 1994).

Potential mechanisms for weight gain during or after breast cancer treatment involve changes in metabolism, physical activity (Demark-Wahnefried *et al.*, 1997a; Winningham *et al.*, 1994), and dietary intake (Champ *et al.*, 2012; Boltong *et al.*, 2012). An increase in body fat, rather than lean mass, causes higher amounts of circulating oestrogens, IL-6, TNF-α,

CRP and leptin (Champ *et al.*, 2012). Differences observed in pre- versus post-menopausal women may be explained by different sources of oestrogen production for pre- menopausal women (the ovaries are the main source) compared to post-menopausal women (adipose tissue is the main source) (Cleary and Grossmann, 2009) and lower levels of serum sex hormone binding globulin (SHBG) in post-menopausal women where SHBG binds to oestradiol and therefore increase levels of circulating oestrogen which may promote tumour growth (Champ *et al.*, 2012).

1.8.2.4 Breast cancer treatment: the effect of nutrition

Nutritional needs and wants can change during breast cancer treatment due to changes in taste and smell, and disturbances of the gastrointestinal tract, affecting how the body digests and absorbs food (Ollenschlaeger *et al.*, 1989; Nitenberg and Raynard, 2000; Schattner and Shike, 2006). However, research regarding dietary intake during breast cancer treatment is inconclusive with studies showing no changes (Del Rio *et al.*, 2002) increases (Grindel *et al.*, 1989) or decreases in energy intake during chemotherapy (Demark-Wahnefried *et al.*, 1997a; Custódio *et al.*, 2016).

An observational study of 1,901 early-stage breast cancer patients indicated that women following a diet with high intakes of fruits, vegetables, whole grains, and poultry had a statistically significant decreased risk of overall death (HR for highest quartile, 0.57; 95% CI, 0.36 to 0.90) and death from non-breast cancer causes (HR for highest quartile, 0.35; 95% CI, 0.17 to 0.73) (Kwan *et al.*, 2009a). The Women's Intervention Nutrition Study (WINS) study, a randomised multi-centre clinical trial investigating reduced fat intake (20% of total diet) during breast cancer treatment in 2,437 postmenopausal women with early stage breast cancer, suggested that a low-fat diet leads to a reduction in risk for disease recurrence, with a greater reduction in risk observed in oestrogen negative breast cancer (Chlebowski *et al.*, 1986; Hoy *et al.*, 2009). The HR of relapse events in the women on the low-fat diet compared with the control group was 0.76 (95% CI, 0.60 to 0.98). One consideration is that weight loss was not the aim of the WINS trial, but the intervention group did reduce body weight, which may be the primary explanation for these findings, rather than the specific nutritional content of the diet. A review of observational cohort studies from 1985 to 2002 showed 5/8 studies concluded vegetables and fruit intake was positively related to survival (Rock and Demark-Wahnefried, 2002) from breast cancer, demonstrating that evidence is not yet conclusive. Elevations in endogenous insulin levels may promote aggressive and metastatic cancers with poorer prognosis (Goodwin *et al.*, 2002; Goodwin *et al.*, 2009a). Mice models have shown that aggressive growth in mammary tumours were

apparent in hyperinsulinaemia type 2 diabetic muscle lysine to arginine mice with a reduction in tumour burden observed when hyperinsulinaemia was reduced (LeRoith, 2010; Novosyadlyy *et al.*, 2010; Fierz *et al.*, 2010). The American Cancer Society who recommend a diet low in fat and refined carbohydrates and high in vegetables and fruits to improve prognosis in breast cancer patients (Doyle *et al.*, 2006).

In terms of supplements, most oncologists advise against taking higher doses of supplements with antioxidant activity during chemotherapy or radiotherapy as antioxidants could repair cellular oxidative damage to cancer cells that contributes to the effectiveness of these treatments (Labriola and Livingston, 1999).

1.8.2.5 Breast cancer treatment: viral serostatus

Some evidence supports the hypothesis that particular viruses may play a role in the progression of breast cancer (Brower, 2004), however evidence is controversial. It has been shown that CMV positive breast tissue is detected in the majority of breast cancer patients. Polymerase chain reaction (PCR) analysis has further shown CMV in breast tumour and normal tissue, with CMV genetic material found in a higher proportion of tumour tissue than normal tissue (Harkins *et al.*, 2010; Tsai *et al.*, 2005; Tsai *et al.*, 2007), suggesting that the virus may be implicated in breast cancer tumorigenesis. However, evidence also exists showing no correlation between CMV or EBV and breast cancer (Antonsson *et al.*, 2012; Utrera-Barillas *et al.*, 2013). Differences in results are likely due to variation in tissue handling and sample size, PCR primer designing and sites of tissue sampling (Lazzeroni and Serrano, 2012).

Whilst CMV is not typically regarded as an oncogenic virus, the term oncomodulation has been proposed to describe the increased malignancy associated with CMV- infected tumour cells and the virus' role in changing the tumour microenvironment and genome as well as initiation and promotion of tumour cells (Soroceanu and Cobbs, 2011; Michaelis *et al.*, 2009b; Cinatl Jr *et al.*, 2004; Cinatl *et al.*, 2004; Baryawno *et al.*, 2011). It has been postulated that tumour cells provide a genetic environment, characterised by disturbances in intracellular signalling pathways, transcription factors, and tumour suppressor proteins, that enables CMV to exert its oncomodulatory potential (Michaelis *et al.*, 2009b). CMV is known to increase malignancy through various mechanisms (Figure 2.), specified in the hallmarks of cancer (Hanahan and Weinberg, 2000). CMV influences the cancer cell cycle which is dysregulated by CMV immediate early proteins IE1 (immediate early) and IE2, promoting entry into S phase, as well as the activity of the UL97 protein which

phosphorylates and inactivates tumour suppressor Rb alongside accumulation of p53 gene, leading to p53- and p21-dependent inhibition of cell cycle progression (Sanchez and Spector, 2008; Murphy *et al.*, 2000; Poma *et al.*, 1996; Pajovic *et al.*, 1997; Hagemeier *et al.*, 1994; Hume *et al.*, 2008; Speir *et al.*, 1995; Song and Stinski, 2005). This is supported by evidence that has shown an abundance of IE1 from breast biopsies from humans with breast cancer (Taher *et al.*, 2013). In turn, both IE1 and IE2 proteins can prevent cancer cell apoptosis by affecting death receptor signalling pathways such as the TNF-mediated death receptor-signalling pathway (Zhu *et al.*, 1995). This demonstrates that the effects of CMV on the cell cycle and cell proliferation may depend both on the context of the internal cellular environment and on the properties of virus regulatory proteins. Resistance to apoptosis has also been evidenced as a hallmark for cancer (Hanahan and Weinberg, 2000). It has previously been shown that CMV protects fibroblasts from apoptosis, through the effects of IE proteins and p53-dependent and non-dependent mechanisms (Zhu *et al.*, 1995). It has subsequently been shown that CMV can protect tumour cells from apoptosis by the induction of certain cellular proteins (Cinatl Jr *et al.*, 2004; Cinatl *et al.*, 1998).

In order to survive and progress cancer cells must induce angiogenesis (Wojtukiewicz *et al.*, 2001; Goon *et al.*, 2006). In some cancers such as leukaemia and glioma, CMV has promoted expression of tumour angiogenesis promoter IL-8 (Murayama *et al.*, 1997; Murayama *et al.*, 2000). In addition, ectopic expression of IE1 induces telomerase mRNA and enhances telomerase activation in normal human diploid fibroblasts in vitro and purified CMV virions have reported to induce chromosomal breaks in primary human foreskin fibroblasts (Stråât *et al.*, 2009; Fortunato *et al.*, 2000).

CMV influences cancer cell immunogenicity. CMV is highly adept at manipulating the host immune system to avoid recognition (Scalzo *et al.*, 2007; Michaelis *et al.*, 2009a; Powers *et al.*, 2008). As previously mentioned, the ability of cancer cells to evade recognition by the immune system is essential for growth and progression (Drake *et al.*, 2006; Nazarenko *et al.*, 2008). The cmvIL-10 protein is a homolog of human IL-10 encoded by the UL111A gene product of CMV. cmvIL-10 binds to the cellular IL-10 receptor (IL-10R) and displays many of the immune suppressive functions of human IL-10 (Slobedman *et al.*, 2009; Spencer *et al.*, 2002). Elevated levels of IL-10 are frequently detected in the serum of breast cancer patients and correlate with poor prognosis (Llanes-Fernández *et al.*, 2006; Nicolini *et al.*, 2006; Asadullah *et al.*, 2003; Najm and Althwani, 2011), suggesting that IL-10 may contribute to immune suppression and protect tumour cells from CD8+ T lymphocytes by down-regulation of class I and class II MHC. This has been demonstrated in murine models whereby CMV-encoded class I homolog m144 protected lymphoma cells from NK cell lysis

resulting in increased tumour growth and decreased survival (Cretney *et al.*, 1999). It has also been demonstrated that cmvIL-10 can enhance other properties associated with tumour cells, such as enhancing movement toward other growth factors (Oseguera and Spencer, 2014). This suggests that infection with CMV may enhance the invasive potential of human breast cancer cells and stimulate metastases.

CMV which infects macrophages, induces an atypical M1/M2 phenotype, representative of the tumour-associated macrophage phenotype, in turn associated with the release of cytokines involved in poor breast cancer prognosis (Herbein and Kumar, 2014). Poor prognosis is particularly evident in inflammatory breast cancer, a highly metastatic, aggressive, and fatal form of breast cancer (Utrera-Barillas *et al.*, 2013), however this diagnosis is uncommon (estimated 1% to 5% of all breast cancers). It has been found that the number of metastatic lymph nodes in patients with breast carcinoma tissues containing CMV genotypes is significantly higher in inflammatory breast cancer patients than other breast cancer patients with a less aggressive form of the disease. This suggests that CMV infection may augment invasion and motility of breast carcinoma cells (Mohamed *et al.*, 2014). CMV may be a potential therapeutic target for patients with CMV-infected tumours (Cinatl Jr *et al.*, 1996; Cinatl *et al.*, 1998; Cinatl Jr *et al.*, 2004). One approved drug is sorafenib, which inhibits the replication of CMV in several cell types in vitro, inhibiting cell death and inducing apoptosis in several breast cancer cell lines including MCF-7 (Michigan Cancer Foundation 7) and MDA- MB-231 (MD Anderson metastatic breast cancer) (Michaelis *et al.*, 2009b; Fumarola *et al.*, 2013; Cinatl *et al.*, 1998). In a phase II clinical trial involving patients with HER2 negative breast cancer, combination of sorafenib and capecitabine has been reported to improved progression free survival in locally advanced, or metastatic breast cancer patients (Baselga *et al.*, 2012). Collectively, evidence suggests that CMV may be involved in the progression of breast cancer, however, in order to obtain conclusive results, clinical findings need to be analysed on large cohorts and in vitro findings need further validation.

EBV is another virus that has potential implications for breast cancer treatment. EBV-positive breast cancer has shown a tendency to be present in tumours with a more aggressive phenotype, e.g., oestrogen negative and with high histological grade (Utrera-Barillas *et al.*, 2013; Bonnet *et al.*, 1999; Ko *et al.*, 2003; Mazouni *et al.*, 2011). The replicative form of EBV, as investigated using anti-ZEBRA titres, has previously correlated with poorer disease outcomes, whereas positive clinical outcomes were found to have a measurable EBV DNA load, together with a high frequency of IFN-γ and TNF-α producing PBMCs (peripheral blood mononuclear cells), indicating the benefit of Th1-type polarized

immune responses (Marrão *et al.*, 2014). Furthermore, EBV infection may also confer resistance to some treatment therapies such as paclitaxel (Taxol) with the infection of EBV in breast carcinoma cells provoking overexpression of a multidrug resistance gene (Arbach *et al.*, 2006). Results are contradictory and other authors demonstrated that EBV plays no relevant role in breast cancer prognosis (McCall *et al.*, 2001). In mouse models, presence of more than one virus (EBV, HPV (human papillomavirus), and MMTV) were detected in 72% of breast tumour tissue and in 13% of breast-milk samples without breast cancer (Glenn *et al.*, 2012). More accurate techniques and methodologies need to be established to help confirm whether EBV is associated with breast cancer.

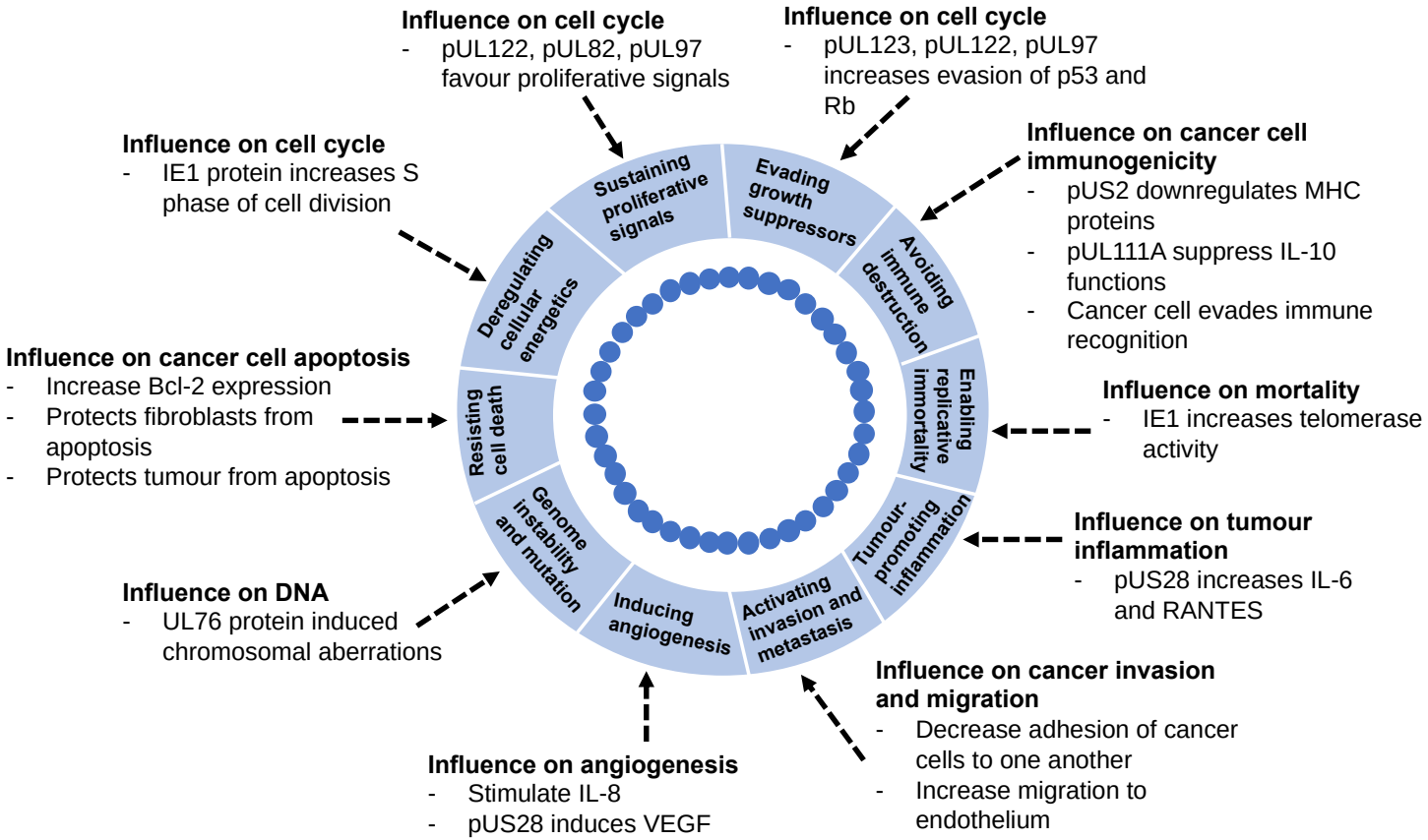

Figure 2. Influence of CMV on the Hallmarks of Cancer (Hanahan and Weinberg, 2011)

IL; interleukin. IE; immediate early/ Rb; retinoblastoma. MHC; major histocompatibility complex. RANTES; Regulated on Activation, Normal T Cell Expressed and Secreted. VEGF; vascular endothelial growth factor. DNA; deoxyribose nucleic acid. Bcl; B-cell lymphoma. S phase; synthase phase. CMV; cytomegalovirus.

1.8.2.6 Breast cancer treatment: the effect of ageing

Worldwide, breast cancer is the most common cancer in women younger than 40 years old (Fredholm *et al.*, 2009).Whilst age is an important risk factor for breast cancer, patient age at diagnosis is also related to breast cancer survival (McPherson *et al.*, 2000). Numerous studies have shown different breast cancer outcomes and treatment options based on patient age at diagnosis. On the whole, younger women tend to have more aggressive tumours that are more likely to recur both locoregionally and distantly, and older women more commonly have less aggressive disease (Fredholm *et al.*, 2009; Klauber-DeMore, 2006; Nixon *et al.*, 1994; Kurtz *et al.*, 1990; Albain *et al.*, 1994; Leborgne *et al.*, 1995). As the incidence is generally low in younger ages, perhaps reduced breast cancer screening in this population may be responsible for the larger masses and more advanced disease (Dobi *et al.*, 2011; Alieldin *et al.*, 2014; Cluze *et al.*, 2009; Kataoka *et al.*, 2014). Middle-age breast cancer patients are more likely to be associated with increased survival. After adjusting for potential confounding factors, middle-age at breast cancer diagnosis was shown to be an independent predictor of favourable outcomes in terms of overall survival, (HR, 0.92; 95%CI, 0.87–0.98; p = 0.007) (Chen *et al.*, 2016). After middle-age, ~60 years, prognosis of breast cancer becomes poorer. Women who were under 40 years old and over 80 years old at diagnosis had a statistically significant higher 10-year mortality rate compared to women aged 40 to 49 years, (RR: 1.40; 95% CI: 1.04 to 1.88 and RR: 1.80; 95% CI: 1.45 to 2.25 respectively for ages under 40 years and over 80 years), but this association only remained in women over 80 years old after adjustment for confounding factors such as stage of diagnosis (Brandt *et al.*, 2015), (likely due to 10-year survival rate being lower in this group given their age). Not all evidence is in agreement and some studies have reported elderly women experience poorer outcomes than younger patients (Schonberg *et al.*, 2010; Yancik *et al.*, 2001). This may be due to inconsistent thresholds for age ranges and small sample sizes.

1.8.3 Following breast cancer treatment

1.8.3.1 Exercise and physical activity following treatment

Breast cancer has a risk of recurrence even 20-30 years after initial diagnosis (Dixon and Montgomery, 2008). Less than one-third of breast cancer survivors participate in the levels of physical activity recommended by government agencies (Irwin *et al.*, 2004) compared to 58% of women aged 16 or over in the UK (Scholes and Neave, 2013). This is despite the American Cancer Society (Kushi *et al.*, 2012a; Campbell *et al.*, 2019b) recommending

physical activity as a strategy to help cancer survivors manage post-treatment symptoms, improve quality of life and possibly extend survival. Exercise following treatment has been reported to improve cardiorespiratory and cardiovascular function, body composition, immune function, strength, flexibility, body image, self-esteem, mood, reduces the number and severity of side effects, stress, depression and anxiety - all of which contribute to improvements in quality of life (Schmitz *et al.*, 2010). A 2010 systematic review of controlled intervention trials from a variety of all cancer patients (Speck *et al.*, 2010) concluded that exercise following treatment has overall positive effects on functional and wellbeing outcomes.

Exercise capacity is an established strong predictor of mortality in numerous clinical studies (Erikssen *et al.*, 1998; Paterson *et al.*, 1999) and exercise and/or physical activity has been described as a modifiable risk factor for recurring breast cancer (Chlebowski, 2013). A review of intervention and self-report studies summarised that participation in regular exercise after diagnosis is associated with 34% fewer deaths from breast cancer and 41% fewer deaths from ACM (Dieli-Conwright and Orozco, 2015). $\dot{V}O_2$ peak increases with increasing exercise or physical activity levels and may be an independent predictor of survival. Previously median survival after breast cancer was 16 months for patients reporting a $\dot{V}O_2$ peak <1.09 L.min^{-1} versus 36 months for those with a $\dot{V}O_2$ peak >1.09 L.min^{-1} (Betof *et al.*, 2013). The effect of exercise has been observed to be stronger for women who are overweight or obese, with ER positive and HER2 positive breast cancer (Dieli-Conwright and Orozco, 2015). Exercise has been proven to improve quality of life and physical functioning, manage chronic and late appearing effects of treatment (fatigue, lymphedema, fat gain, bone loss), reduce the likelihood of cancer recurring and reduce the likelihood of developing other chronic diseases (for which they may be at increased risk (e.g., osteoporosis, heart disease and diabetes)) (Rohan *et al.*, 1995; Brower, 2004). Indeed, women undertaking 2.5 hours of moderate intensity activity each week for two years after breast cancer diagnosis had a 67% lower overall risk of ACM risk than inactive women (Irwin *et al.*, 2008b). Women who had decreased their physical activity after diagnosis had four times greater risk of dying than women who had been consistently inactive (i.e. before and two years after diagnosis) (Irwin *et al.*, 2008b) and the five-year survival for women exercising for >9 MET.h week^{-1} was 97% versus 93% for those exercising <3 MET.h week^{-1}. Ten-year survival for the same exercise categories was 92% and 86% respectively. A lot of these studies used self-reported methods for assessing exercise levels which may be open to bias and inaccuracy of results.

1.8.3.2 Body composition following treatment

Weight gain is common for many breast cancer survivors and consequently associated with adverse health consequences. This has been seen in large cohorts (n=3993) whereby 6 years after diagnosis each 5kg gain was associated with a 13% increase in breast cancer death and a 12% increase in ACM (Nichols *et al.*, 2009). Weight gain can continue for a period of time following treatment. Previous literature has shown that whilst no significant change in mean body weight was seen during treatment, one year after treatment, 14/18 pre-menopausal women receiving adjuvant chemotherapy, had gained an average of 3.8kg (Demark-Wahnefried *et al.*, 1997a). This weight gain may also be progressive. A longitudinal study confirmed in 185 women diagnosed with stage I-III breast cancer, the mean weight change across all women was 1.5kg one year after diagnosis, 2.7kg after two years and 2.8kg after three years (Makari-Judson *et al.*, 2007) whilst another study reported at the third year from diagnosis, 68% of women with stage I-III breast cancer had gained an average of 3.8kg (Irwin *et al.*, 2005b). This amount of weight gain is larger than typical weight gain in women from the general population over this time period. Furthermore, unfavourable changes to a sarcopenic phenotype (increase in fat and decreases in lean tissue) (Cheney *et al.*, 1997; Caan *et al.*, 2006; Kutynec *et al.*, 1999) can increase the risk for comorbidities and affect breast cancer specific disease-free survival (Carmichael, 2006).

Mechanisms surrounding increased risk of breast cancer reoccurrence with weight gain are similar to mechanisms involved in the increased risk of disease in healthy women, such as increased adipose tissue derived circulating oestrogens, which are known to play a role in breast cancer initiation and promotion of breast cancer by stimulating cell division, and increasing the likelihood of DNA mutations. Increases in circulating insulin, insulin-like growth factor and leptin, with increased fat mass may also promote cell proliferation by downregulating concentrations of sex hormone binding globulin, thereby increasing oestradiol (Stephenson and Rose, 2003).

Breast cancer survivors are also reported to have lower BMD which increases the risk of fractures and osteoporosis (Broeckel *et al.*, 2000; Chen *et al.*, 2005). This is likely due to the effects of treatments such as chemotherapy and standard hormone suppressant therapy known as aromatase inhibitors (AIs) (Greep *et al.*, 2003), which can affect the number and size of bone cells (Friedlaender *et al.*, 1984) whilst decreasing the amount of oestrogen produced in the body (Pfeilschifter and Diel, 2000).

1.8.3.3 Nutrition following treatment

Dietary intake and its contribution to a healthy lifestyle plays a role in breast cancer survivorship as cancer, alongside cancer treatments, can change the metabolic and physiological demands of the patient and therefore alter the patient's nutritional needs (Vandebroek and Schrijvers, 2008). Cancer survivors receive a wide range of advice regarding foods they should eat, foods they should avoid, and what types of supplements they should take. However, advice is inconsistent and rarely supported by robust evidence and more research needs to take place to decipher the optimal diet for breast cancer survivors. Observational studies suggest that diet and food choices may affect cancer recurrence, and overall survival. Findings demonstrate high intake of fruits and vegetables, alongside wholegrains, poultry and fish, are associated with reduced mortality when compared to a diet with higher intakes of refined grains, processed and red meats and high fat products (Kushi *et al.*, 2012a; Holmes *et al.*, 2005; Caan *et al.*, 2006; Pekmezi and Demark-Wahnefried, 2011; Norman *et al.*, 2007; Rock *et al.*, 2012). Furthermore, a cross-sectional study in 100 breast cancer survivors demonstrated those with better nutritional status had fewer clinical symptoms (Mohammadi *et al.*, 2013). Some evidence has demonstrated a 43% reduction in overall mortality in breast cancer survivors whose diet consisted of a high intake of vegetables and whole grains (Kwan *et al.*, 2009b). The WINS trial of 2437 post-menopausal breast cancer patients saw a lower risk of relapse free survival (borderline statistically significant), especially in those who had previously had an ER positive breast cancer, in women who had a low-fat diet (fat contributed to less than 15% of total energy intake) (Chlebowski, 2013). Interestingly, these women also lost weight over the intervention thus it may be the weight loss, rather than the reduction in fat intake that is responsible for the lower risk of relapse free survival. Conversely, the WHEL study in 3088 pre- and post-menopausal breast cancer survivors, demonstrated that a diet rich in fruit and vegetables and low in fat is only beneficial to survival when accompanied by physical activity (Gandini *et al.*, 2000; Pierce *et al.*, 2007). It must also be noted that any extreme in nutrient intake, including inadequate energy intakes, may be negatively associated with breast cancer survival (Brown *et al.*, 2003; Goodwin *et al.*, 2003). To note, breast cancer survivors are often at increased risk of cardiovascular disease therefore dietary recommendations are often in line with reducing the risk of cardiovascular events (McGuire, 2011).

Dietary supplement use is of interest to breast cancer survivors, yet evidence suggests that supplements are unlikely to improve survival after breast cancer. In fact, vitamin, mineral or multivitamin use is increased in women following a breast cancer diagnosis (range of 57-

87% women) (Velicer and Ulrich, 2008) compared to the general population (Radimer *et al.*, 2004). However, observational studies demonstrated that any dietary supplement or multivitamin use was not associated with breast cancer recurrences or breast cancer-specific mortality (Saquib *et al.*, 2011; Kwan *et al.*, 2011). Whilst in 12,019 breast cancer survivors, vitamin E intake was associated with a decreased risk of disease reoccurrence, vitamin C intake associated with a decreased risk of mortality, and vitamin D intake associated with a decreased risk of disease reoccurrence in ER positive cancers only (Poole *et al.*, 2013). Furthermore, evidence has suggested that breast cancer survivors have high rates of vitamin D insufficiency, thus supplementation is necessary in this population (Boynton *et al.*, 2007). Other research suggests omega 3 fatty acids may have specific cancer specific benefits (Gogos *et al.*, 1998) however research is inconsistent, and benefits may rather be seen in lowering the risk of cardiovascular disease (McGuire, 2011). There is a need for larger scale, high quality research trials to fully assess the benefit of various nutritional supplements for breast cancer survivors.

Perhaps most importantly, given that obesity is more established with worsened outcomes in terms of mortality after breast cancer, is that women have a diet that manages weight, rather than focussing on the specific nutrient content of the diet (McDonald *et al.*, 2011).

1.9 MECHANISMS UNDERLYING RELATIONSHIPS BETWEEN EXERCISE AND BREAST CANCER

Exercise may reduce risk of developing breast cancer by dampening the processes involved in the promotion and progression of carcinogenesis (Rogers et al., 2008). These mechanisms either prevent mutations happening or directly target the cancer cells after a mutation has occurred. Several plausible biological mechanisms exist for the association between physical activity and cancer mainly involving; modulation of hormone levels and growth factors, decreased obesity and central adiposity, reduced systemic inflammation, oxidative damage and changes in immune function (Irwin *et al.*, 2008b).

Hormones and other soluble signalling molecules are influenced by exercise. Elevated blood insulin concentrations have been associated with increased risk of breast cancer as they have an anabolic role, stimulating net protein synthesis and growth that may facilitate carcinogenesis. Exercise enhances insulin sensitivity, thereby reducing plasma insulin concentration and may result in a protective effect against cancers. High levels of IGFs have also been associated with an increased risk of breast cancer. IGFs are peptide hormones that are synthesised in direct response to growth hormones, stimulating cell

turnover in body tissues. Physical activity downregulates IGFs by increasing production of their binding proteins thus may link physical activity to decreased cancer risk (Irwin *et al.*, 2008b). Furthermore, participation in regular exercise may reduce the risk of hormone related cancers, such as breast cancer, by reducing exposure to oestrogens, for example, through delayed menarche, a decreased number of ovulatory cycles or increasing sex hormone binding globulin (Irwin *et al.*, 2008b).

Obesity may increase the risk of breast cancer in post-menopausal women due to abdominal adiposity. Imbalance between energy expenditure and energy intake leads to excess body fat which increased risk of breast cancer through changes in metabolic mediators that influences carcinogenesis via cell proliferation and apoptosis or indirectly through changes in downstream targets of weight loss such as oestrogen metabolism and particularly by reducing the highly metabolically active abdominal adiposity. Increased body fatness or adiposity is associated with increased concentrations of circulating oestrogens in women which may facilitate carcinogenesis (Leitzmann *et al.*, 2015a). Hence, lower levels of body fat in women who are physically active may lead to lower oestrogen exposure. Insulin resistance is also characterised by high adiposity, thus having a similar association with increased risk of cancer (Irwin *et al.*, 2008b).

Oxidative stress leads to damaged DNA and potential mutations. Physical activity whilst increasing the damage to DNA, also increases repair and may reduce oxidative damage by increasing a variety of antioxidant enzymes. There may be physiological adaptations that occur in response to long term exercise that prevent oxidative DNA damage as an important stimulus to upregulate antioxidant enzymes as well as non-enzymatic repair systems that work to prevent and or repair the damage induced by ROS (Fehrenbach and Northoff, 2001; Rogers *et al.*, 2008).

Exercise is able to inhibit the development of a tumour promoting environment by combating inflammation. Exercise may reduce pro-inflammatory mediators and reduce the state of low grade chronic inflammation, exerting anti-inflammatory effects (Irwin *et al.*, 2008b). The anti-inflammatory effects of regular exercise may be mediated via both a reduction in visceral fat mass (with a subsequent decreased release of adipokines) and the induction of an anti-inflammatory environment with each bout of exercise. Positive consequences of exercise include increased release of cortisol and adrenaline from the adrenal glands; increased production and release of IL-6 and other myokines from working skeletal muscle; reduced expression of TLRs on monocytes and macrophages (with subsequent inhibition of downstream pro-inflammatory cytokine production); inhibition of adipose tissue infiltration

by monocytes and macrophages; phenotypic switching of macrophages within adipose tissue to a more anti-inflammatory phenotype; a reduction in the circulating numbers of pro-inflammatory monocytes; and an increase in the circulating numbers of regulatory T-lymphocytes. All these factors are known to contribute to the generation of this anti-inflammatory environment (Gleeson *et al.*, 2011).

It is commonly suggested that exercise reduces the risk of cancer via immune enhancement (Rogers *et al.*, 2008). Immune cells are highly stress-responsive and are rapidly mobilised into peripheral blood by exercise (Shephard and Shek, 1999; Pedersen and Hoffman-Goetz, 2000; Bigley *et al.*, 2014; Dhabhar *et al.*, 2012; Butcher, 1990) where they are likely to encounter antigens (Dhabhar *et al.*, 2012). Infiltrating cytotoxic immune cells have been demonstrated as positive prognostic factors for disease outcome and overall survival in several cancers (Vivier *et al.*, 2012). Chronic exercise has been linked to improved NK cell cytotoxicity, prognosis, and survival in breast cancer patients (Mohamady *et al.*, 2013; Fairey *et al.*, 2005a). Indeed, some of the latest evidence in this field has focussed on NK cells. Tumour-bearing mice randomised to voluntary wheel running showed that NK cell infiltration correlated inversely to tumour burden whereas a depletion of NK cells enhanced tumour growth and blunted the beneficial effects of exercise (Pedersen *et al.*, 2016).

One area of immunology that has not received much attention in the context of exercise and cancer is cytotoxic T-lymphocytes which have an important role in cancer immune surveillance and are also an known to be strongly affected by exercise (Pedersen and Hoffman-Goetz, 2000). Human studies have demonstrated that T-lymphocyte responses to a number of tumour-associated antigens can be detected in both healthy and cancer patient participants (Schmidt *et al.*, 2003; Andersen *et al.*, 2007; Jäger *et al.*, 2001; Vella *et al.*, 2009; Muraro *et al.*, 2015a; Muraro *et al.*, 2011; Inokuma *et al.*, 2007b; Roscilli *et al.*, 2014b; Epel *et al.*, 2008; Stadler *et al.*, 2016; Kao *et al.*, 2001). Exercise-induced mobilisation of CD8+ T-lymphocytes towards the periphery may result in these lymphocytes coming across and responding to tumour-associated antigens presented to them (Figure 3.).

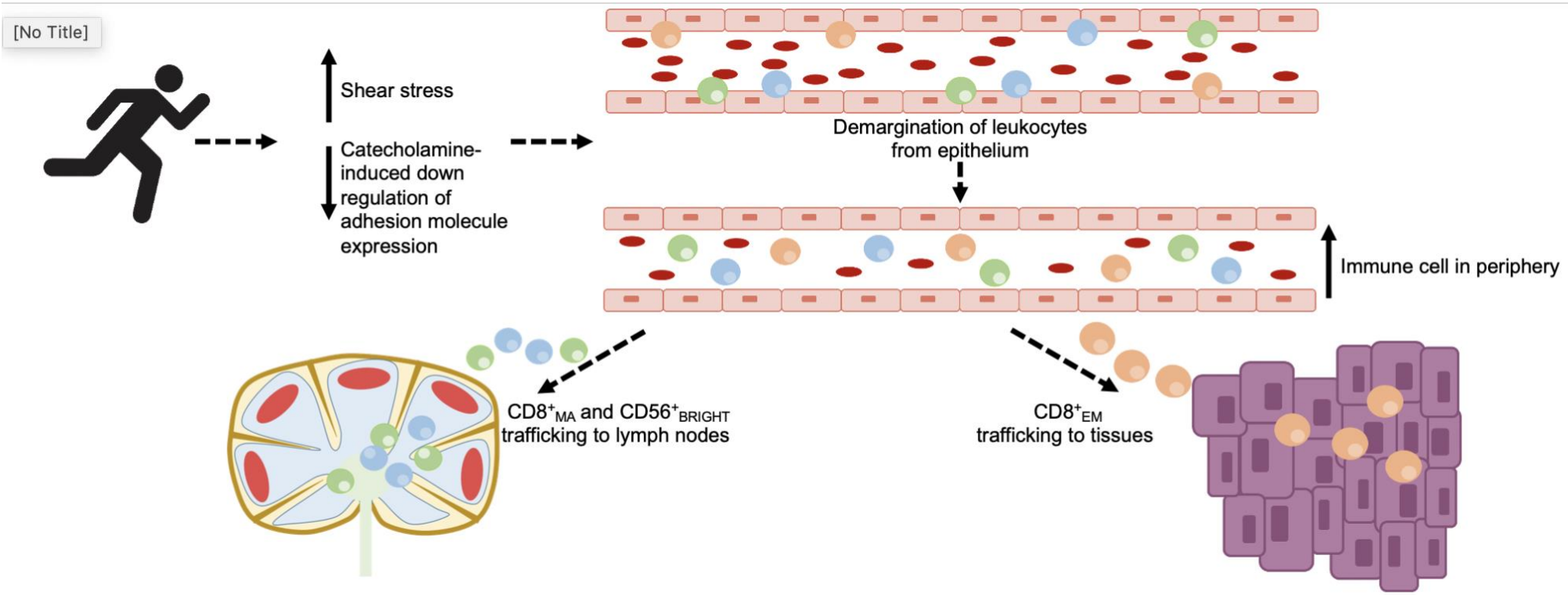

Figure 3. Exercise induced cancer immune surveillance. Exercise increases the demargination of leukocytes through sheer stress and catecholamine release. Leukocytes preferentially target differing organs with naïve T-lymphocytes travelling to the lymph nodes to encounter tumour antigens and effector memory T-lymphocytes circulating tissues to identify potentially cancerous cells.

CD; cluster of differentiation. NA; naïve. EM; effector memory

1.10 CONCLUSIONS

The incidence of cancer is increasing, in part due to our ageing population. Breast cancer is the most common female malignancy in the UK and although survival is improved many patients still do not respond well to treatment. Thus, there is a need to establish and investigate predictors of treatment responses and survival. It has been established that breast cancer is associated with unhealthy lifestyles, with being physically active reducing disease risk by nearly 25%, improving disease outcomes and lengthening survival. Many suggestions have been made to explain how exercise improves the risk and prognosis of breast cancer including, modulating hormone levels and growth factors, regulating weight, reducing inflammation and oxidative stress and finally through perturbations of immune function. Specifically, T-lymphocytes are known to both be crucial for cancer immune surveillance and are known to be strongly stimulated by exercise. However, it has yet to be studied in the setting of exercise, whether breast cancer risk and disease prognosis, can be linked to anti-cancer immune surveillance by CD8+ T-lymphocytes.

1.11 THESIS HYPOTHESES AND AIMS

The following hypotheses will be examined in this thesis:

(1) Healthy individuals and breast cancer patients will have T-lymphocytes that strongly recognise breast cancer tumour-associated antigens if the host exhibits the following characteristics:

- **a)** Higher cardiorespiratory fitness
- **b)** Lower body mass index
- **c)** Lower percentage body fat
- **d)** Lower energy intake

(2) Patients demonstrating the best clinical outcomes in terms of pathological response will exhibit stronger recognition of breast cancer tumour-associated antigens by T-lymphocytes and exhibit the following characteristics:

- **a)** Higher cardiorespiratory fitness
- **b)** Lower body mass index
- **c)** Lower percentage body fat
- **d)** Lower energy intake

(3) Markers of health (fitness, body composition, markers of inflammation) will be improved by a remotely monitored (through use of a fitness tracker) exercise intervention to an extent that is non-inferior to improvements elicited by a partly supervised exercise intervention in breast cancer survivors

In order to investigate these hypotheses, the aims of the thesis are to:

- **a)** Examine whether breast cancer specific T-lymphocyte responses can be detected in healthy women, and to explore potential relationships between this response and precisely measured aspects of lifestyle (cardiorespiratory fitness, diet, physical activity level, body composition)
- **b)** Explore potential relationship between physiological and lifestyle measurements (cardiorespiratory fitness, diet, physical activity level, body composition) and treatment outcome in breast cancer patients (e.g. pathological response)
- **c)** Examine whether the magnitude of breast cancer specific T-lymphocyte responses over the course of 18-weeks of chemotherapy are related to treatment outcomes (e.g. pathological response)
- **d)** Examine whether supervised and remote exercise interventions can improve health in breast cancer survivors

CHAPTER TWO: General Methods

This chapter describes the general methodology, measurements and laboratory techniques employed in the studies within this thesis. Where relevant, the text includes background information that is beyond the scope of other chapters. In addition, a justification of the methodology used, and a discussion of key analytical or interpretational decisions that have been made is included. Within each experimental chapter in the thesis, the methodology is described more concisely, and a description of each study design is provided.

2.1 RESEARCH VOLUNTEERS AND RECRUITMENT

Breast cancer in men is not examined in the present work because male breast cancer is very rare, with around 350 men diagnosed each year in the UK, compared with around 55,000 cases of breast cancer in women (CRUK, 2017). Thus, recruiting enough male participants would be impractical for this work that is being undertaken with a single collaborating hospital. In addition, tumour-associated antigens for male breast cancer have not been well characterised. Thus, a first step for male breast cancer research would be to establish a range of tumour-associated antigens for investigation – a task which is beyond the scope of this thesis. Specific information about volunteers is discussed within the respective experimental chapters.

2.2 SELF-REPORT QUESTIONNAIRES

2.2.1 General questionnaires

Healthy volunteers, patients and breast cancer survivors were required to complete a number of questionnaires as part of screening and baseline characterisation:

- *The International Physical Activity Questionnaire (IPAQ)* was administered requiring participants to recall information regarding their physical activity habits in the last seven days. Questions assessed included; job related physical activity, transportation physical activity, housework, house maintenance, caring for family, recreational sport and leisure time physical activity, and time spent sitting. This data was interpreted using the IPAQ scoring system. Test-retest indicates good stability and reliability is high ($\alpha > .80$) (Craig *et al.*, 2003; Hagstromer *et al.*, 2006; Lee *et al.*, 2011b; van Poppel *et al.*, 2010).

- *A general health questionnaire* and *physical activity readiness questionnaire* were administered to screen individuals prior to participation to ensure safety during exercise testing (ACSM, 2013).

- *A sex specific cancer risk factor questionnaire* was used to assess the age of first menstruation, child birthing age or absence, and family history of cancer as they are known risk factors for breast cancer (Ajithkumar, 2011).

- *The Depression, anxiety and stress questionnaire (DASS-21)* consists of 21 items to measure the negative emotional states of depression, anxiety and stress. Each item was scored from 0-3, whereby 0 represented 'did not apply to me at all', 1; 'applied to me to some degree or some of the time', 2; 'applied to me to a considerable degree or a good part of time' and 3; 'applied to me very much or most of the time' (Henry and Crawford, 2005). Scores for depression, anxiety and stress were calculated by summing the scores and multiplying by two for the relevant items (Lovibond and Lovibond, 1995; UNSW, 2014).This questionnaire has been shown to have good reliability (α >.76) (Le *et al.*, 2017).

2.3 PARTICIPANT CHARACTERISATION AND MEASUREMENT OF LIFESTYLE VARIABLES

2.3.1 Body composition

Body mass was assessed using electronic scales (Tanita InnerScan BC-543, Tanita Ltd, USA). Height was assessed using a stadiometer (Leicester height measure, Seca Ltd, UK). Values were used to calculate BMI and interpreted on the basis of <18.5 kg.m$_2$ (underweight), 18.5-24.9 kg.m$_2$ (normal weight), 25-29.9 kg.m$_2$ (overweight), and >30 kg.m$_2$ (obese).

2.3.1.1 Waist to hip ratio (W:H)

Android obesity, characterised by more fat on the trunk (abdominal fat) confers an increased risk of non-communicable disease, including cardiovascular disease, diabetes and cancer (Folsom *et al.*, 1993). Waist and hip circumference measurements were made using a tension sensitive, non-elastic tape, twice and averaged. The circumference of the hips was assessed horizontally at the maximal circumference of the hip/proximal thigh, just below the gluteal fold, with the participant standing, legs slightly apart (~10cm). Waist circumference

was measured twice at the narrowest part of the torso (above the umbilicus and below the xiphoid process) with the participant standing, arms at their sides and feet together with abdomen relaxed. If measurements were not within 5mm then a third assessment was made, and the median was recorded. The waist to hip ratio (W:H) (circumference of the waist divided by the circumference of the hips) was calculated.

2.3.1.2 Dual energy x-ray absorptiometry (DEXA)

DEXA was used to accurately quantify fat mass, fat-free soft tissue mass and BMD (Hologic Discovery W, USA) (Ellis, 2000). The participant was aligned so that their head and spine were aligned with the central long axis of the scanner with feet turned in and arms as wide as possible, flexed slightly at the elbow.

2.3.2 Exercise tests

2.3.2.1 Estimating cardiorespiratory fitness

Maximal oxygen uptake ($\dot{V}O_2max$) is the criterion measure of cardiorespiratory fitness and can either be expressed as litres of oxygen per min ($L.min^{-1}$) or standardised to body mass ($ml.kg^{-1}.min^{-1}$). Considering the patient population under investigation it was deemed more appropriate to use a submaximal exercise test to predict $\dot{V}O_2max$ in women undergoing cancer treatment and within the healthy population to allow for future comparison between the two groups. Age-predicted maximum heart rate (HR_{max}) was estimated using the following equation: 206.9 − (0.67 × age) (Gellish *et al.*, 2007a). Oxygen uptake at HR_{max} was extrapolated from the heart rate to exercise workload relationship, assuming a linear relationship.

After resting heart rate and blood pressure measurements were taken (Polar heart rate monitor RS400, Kempele, Finland, Bosch and Sohn, Germany), the sub-maximal exercise test was undertaken by walking on a treadmill (HP Cosmos Saturn, Nußdorf, Germany). The test consisted of four incremental stages each lasting five minutes to allow heart rate and oxygen consumption to plateau during each workload. Each stage comprised walking at a speed of 5.8 kph which was selected based on the average walking speed of older adults, (men 4.1 kph – 7.3 kph, women 3.5 kph – 7.0 kph and that moderate intensity activity (3 – 6 METs) can be achieved when walking 4.2 – 6.9 kph (Ainsworth *et al.*, 2011)). Exercise intensity was increased by adjusting the gradient of the treadmill, with the first stage being undertaken at a 0% gradient, and each subsequent stage increasing by 3% to reach a final

gradient of 9%. During the final minute of each stage, heart rate was measured and ratings of perceived exertion (RPE) were recorded using the Borg scale (Borg, 1982). Expired air samples were collected during the final minute of each stage using Douglas bags. Oxygen and carbon dioxide concentration within each bag was analysed using a calibrated gas analyser (Servomex Group Ltd, Jarvis Brook, UK) and volume and temperature of the air assessed using a gas dry gas meter (Harvard Bioscience, Inc., U.K).

2.3.2.2 Measuring $\dot{V}O_2$ max

Breast cancer survivors undertook a maximal incremental treadmill test (HP Cosmos Saturn, Nußdorf, Germany) to volitional exhaustion, rather than a sub-maximal exercise test. This choice was made given the importance of the maximal oxygen uptake and maximum heart rate values in prescribing training intensities during the exercise interventions. The test lasted ~12-20 minutes and followed the Bruce Protocol, changing both the speed and the percent gradient to increase the workload. Each stage lasted 3 minutes. Initial start pace was selected by the individual from either 2.7kph, 4.0kph, 5.3kph or 6.6kph, as a pace that was slower than their average walking pace. Every 3-minutes the speed was increased by 1.3kph until the speed reaches 6.6kph or self-selected fastest walking pace. At this point gradient was increased by 2% every 3 minutes until the participant reached exhaustion (Schneider *et al.*, 2004). Heart rate was monitored by telemetry (Polar heart rate monitor RS400, Kempele, Finland, Bosch and Sohn, Germany). During the final minute of each stage, heart rate was measured and RPE were recorded (Borg, 1982). Expired air samples were collected during the final minute of four stages including during the final minute of exercise using Douglas bags. Oxygen and carbon dioxide concentration within each bag was analysed using a calibrated gas analyser (Servomex Group Ltd, Jarvis Brook, UK) and volume and temperature of the air assessed using a gas dry gas meter (Harvard Bioscience, Inc., U.K).

2.3.3 Assessing dietary intake

Participants were provided with a set of digital electronic scales and a 3-day diet diary. Participants were asked to record everything they consumed on two typical weekdays and a typical weekend day (i.e., days the participant felt most closely represented 'normal' days). Diet diaries were analysed using commercially available software (Nutritics diet analysis software, Dublin, Ireland) for total energy content and macronutrient composition. A 3-day weighed food diary rather than 7-day diary was selected to minimise the demands placed upon participants. Research has shown that 3-day versus 7-day diaries helps with

recruitment and response rates, with the likelihood of misreporting food intake increasing with the length of observation period. In addition, comparisons of 3-day versus 7-day diet-diaries show that 3-day diaries are as accurate as 7-day for both micro- and macronutrients (Fyfe *et al.*, 2010).

2.3.4 Assessment of free-living sedentary activity and physical activity

Sedentary activity and physical activity were assessed using a BodyMedia Sensewear (APC Cardiovascular Ltd, UK) armband physical activity monitor which was worn for a full seven days (i.e., the day of fitting plus seven more days) by healthy and patient volunteers. Verbal and written instructions were provided concerning adjusting the armband for comfort and removing the unit prior to showering/bathing/water-based activity. Due to the inaccuracies associated with self-reporting physical activities, and due to the small proportion of water-based activities that took place, time spent with the armband removed was assumed to be equal to RMR (resting metabolic rate). A diary was provided for participants to record when the armband was removed and to record estimated sleep and wake up time. In order to convert energy expenditure to METs, which expresses the energy cost of activities, an age-specific Schofield equation estimated basal metabolic rate (BMR) on the basis of body mass, age and sex (Schofield, 1985). Activities with MET values of ≤1.8, rather than ≤1.5 were considered as sedentary because the Sensewear Armband slightly over-estimates energy expenditure compared to other devices (Scheers *et al.*, 2012). Activities with MET values of 1.8-2.99 were classified as light activity, 3.0-5.99 moderate, 6-10.19 vigorous (Haskell *et al.*, 2007) and >10.2 very vigorous (Thompson and Batterham, 2013).

2.4 BLOOD SAMPLING AND PROCESSING

Prior to blood sampling, participants refrained from exercising and drinking alcohol or caffeine for 24 hours and consumed only water from 22:00 the night before (i.e., participants fasted overnight and visited the laboratory without having eaten breakfast).

2.4.1 Collection of resting blood samples

Following a 15 minute rest in the supine position, approximately 50ml of blood was collected using venepuncture. Blood was collected into a sterile syringe containing 2.0IU of sodium heparin per ml of blood (preservative free, in water for injection) for isolation of PBMCs (see section 2.5.4). A further 8ml of blood was collected into a syringe without anti-coagulant and

aliquoted immediately into a 4ml Ethylenediaminetetraacetic acid (EDTA) vacutainer tube (Becton Dickinson, U.S) for preparation of plasma and a 4ml plain (anti-coagulant free) vacutainer tube (Becton Dickinson, U.S), for preparation of serum by allowing blood to clot at room temperature for 30 minutes prior to centrifugation.

2.4.2 Leukocyte differential

Prior to centrifugation of EDTA vacutainer tubes, approximately 100µl of blood was used to provide the full leukocyte differential, including total leukocyte count, absolute and relative counts for lymphocytes, along with other haematological variables (e.g., haematocrit and haemoglobin) with an automated haematology analyser (Sysmex, KX-21N, Kobe, Japan). Samples were analysed in triplicate and the median computed.

2.4.3 Preparation of plasma and serum

For preparation of plasma, EDTA vacutainers were centrifuged at 2000 × *g* for 10 minutes at 4°C and the supernatant (i.e., plasma) collected and stored in multiple aliquots at −80°C. For preparation of serum, after clotting for ~30-minutes at room temperature, samples were centrifuged at 2000 × *g* for 10 minutes at 4°C and the supernatant (i.e., serum) collected and stored in multiple aliquots at −80°C.

2.4.4 Isolation of peripheral blood mononuclear cells

Blood (with sodium heparin as an anti-coagulant) was diluted 1:1 with sterile Roswell Park Memorial Institute medium, (RPMI) warmed to 37°C and layered on top of lymphocyte separation media (Ficoll-Paque GE Healthcare Bio-sciences AB, GE Life Sciences, USA) for density gradient centrifugation. Samples were centrifuged at 500 × *g* for 30 minutes (acceleration 4, deceleration 3, temperature 20°C). PBMCs were aspirated from the interface between the plasma and Ficoll-Paque and washed in warm RPMI by centrifuging at 400 × *g* for 10 minutes (acceleration 6, deceleration 5, temperature 20°C). An additional wash step in RPMI centrifuging at 300 × *g* for 7 minutes (acceleration 6, deceleration 6, temperature 20°C) was undertaken to combine cells into a single centrifuge tube. A final wash step in RPMI centrifuging at 200 × *g* for 10 minutes (acceleration 6, deceleration 6, temperature 20°C) was employed to remove contaminating platelets. The supernatant was removed and PBMCs were re-suspended in an appropriate volume of RPMI (approximately half the volume of blood collected). Cells were counted using a haemocytometer and a light microscope (Primo Vert, Zeiss, Germany) by mixing 12µl of cell suspension with 12µl of a

solution consisting of 1.5% acetic acid (to lyse contaminating erythrocytes) and trypan blue (a membrane-impermeable dye which can only penetrate dead cells). The number of cells were calculated taking into account the total volume of the cell suspension and dilution factors. Samples were assayed fresh for functional assays, but the remaining cells were cryopreserved in freezing media (70% Foetal Bovine Serum (FBS), 20% RPMI, 10% Dimethyl sulfoxide (DMSO)) at −1°C per minute in a “Mr Frosty” freezing container (Nalgene, Thermo Fisher Scientific, Massachusetts). Samples were stored at −80°C.

2.5 ANALYTICAL TECHNIQUES

2.5.1 An overview of enzyme-linked immunosorbent spot (ELISpot)

ELISpot assays, which enable detection of antigen-specific immune cells at very low frequencies, consisted of four stages (Janetzki *et al.*, 2015). First, isolation and preparation of cells from blood samples. Second, the assay itself, where under sterile conditions, a 96-well polyvinylidene difluoride (PVDF) membrane plate was coated with a IFN-γ-specific antibody, then cells and stimuli (e.g., tumour-associated antigens or viral-antigens) were added to the plate, followed by an incubation overnight to allow for the cytokine of interest (e.g., IFN-γ), to be produced by stimulated cells. Third, cells were removed and the cytokine bound to the capture antibody was made visible using enzyme-linked immunoassay principles (Janetzki *et al.*, 2015). Fourth, each spot, which represented a single antigen-specific cell that secreted IFN-γ, was quantified using a micro-plate reader equipped with a high definition digital camera and appropriate software.

2.5.1.1 Tumour-associated antigens

Some tumour-associated antigens are self-antigens (i.e., antigens expressed on some normal tissues) that have become over-expressed on tumour cells, but some of these antigens differ from those expressed by non-cancerous cells due to mutation (Andersen and thor, 2002). Central and peripheral tolerance mechanisms in the immune system usually remove T-lymphocytes that recognise self-antigens. Central tolerance mechanisms target newly developing lymphocytes, occurring in the primary lymphoid organs whereas peripheral tolerance occurs once lymphocytes have matured and have entered into the periphery. The increased expression of tumour-associated antigens can ‘break tolerance’ and encourage CD8+ cytotoxic T-lymphocytes to respond to the over-expressed self-antigen (Vigneron, 2015; Xing and Hogquist, 2012). Tumour antigens can be loosely categorised as oncofetal antigens (typically only expressed in fetal tissues and in cancerous

somatic cells), oncoviral antigens (encoded by tumorigenic transforming viruses), overexpressed antigens (expressed by both normal and neoplastic tissue, with the level of expression highly elevated in neoplasia), cancer-testis antigens (expressed only by cancer cells and adult reproductive tissues such as testis and placenta), lineage-restricted antigens (expressed largely by a single cancer histotype), mutated antigens (only expressed by cancer as a result of genetic mutation or alteration in transcription), post-translationally altered antigens (tumor-associated alterations in glycosylation, etc.), or idiotypic antigens (highly polymorphic genes where a tumour cell expresses a specific 'clonotype') (Decker, 2003).

Tumour-associated antigens can be exploited therapeutically (e.g., for use in vaccine production, therapeutic antibody production, and cell-based adoptive immunotherapy). Thus, the ideal tumour-associated antigens possess the following; therapeutic functions (clinical data showing that a vaccine induces a clinical response in patients), immunogenicity (ability to elicit a T-lymphocyte response), oncogenicity (are known to be associated with the cancer process) and specificity (specific or over-expressed in mutated genes) (Cheever *et al.*, 2009).

The antigens examined in this thesis, have been prioritised as therapeutic targets for immunotherapy and are known to be targeted by T-lymphocytes (Cheever *et al.*, 2009); mammaglobin-A (MamA), receptor tyrosine-protein kinase ErbB-2 (extracellular (ERB ECD) and intracellular domains (ERB ICD)), mucin-1 (MUC1), survivin (SUR), carcinoembryonic antigen related adhesion molecule-5 (CEA), stromelysin-3 (MMP11), TCRgamma alternate reading frame protein (TARP), claudin-6 (Cl6) and cyclin B1 (CycB1) (Cheever *et al.*, 2009) (Table 1.).

Table 1. List of tumour-associated antigens used within the current thesis including rationale for use

Tumour-associated antigen	Rationale	References
Mammaglobin A (MamA	- Overexpressed in ~80% breast cancer - Elicits an immune response related with prognosis	(Fleming and Watson, 2000a; Watson *et al.*, 1999; Tiriveedhi *et al.*, 2013)
Carcinoembryonic antigen (CEA)	- Regulates cell proliferation and apoptosis - Elicits immune cell response	(Kuespert *et al.*, 2006; Inokuma *et al.*, 2007b)
Claudin-6 (Cl6)	- Involved in cell proliferation - Elicits immune cell response	(Xu *et al.*, 2012; Stadler *et al.*, 2016)
Cyclin B1 (CycB1)	- Immune cell responses detected in breast cancer patients	(Vella *et al.*, 2009)
Receptor tyrosine-protein kinase extracellular domain (ERB ECD)	- ERB gene is widely overexpressed in breast cancer - Immune responses related to treatment outcome	(Slamon *et al.*, 1989; Muraro *et al.*, 2011; Harao *et al.*, 2015)
Receptor tyrosine-protein kinase intracellular domain (ERB ICD)	- ERB gene is widely overexpressed in breast cancer - Immune responses related to treatment outcome	(Slamon *et al.*, 1989; Muraro *et al.*, 2011; Harao *et al.*, 2015)
Stromelysin-3 (MMP11)	- Expression related to breast cancer prognosis - Elicits immune cell response	(Peruzzi *et al.*, 2009; Cheng *et al.*, 2010; Roscilli *et al.*, 2014b)
TCRgamma alternate reading frame protein (TARP)	- Overexpressed in breast cancer -Elicits immune cell response	(Epel *et al.*, 2008)
Mucin 1 (MUC1)	- Highly expressed in epithelial cancers - Immune response related with favourable outcomes	(Muraro *et al.*, 2015b; Blixt *et al.*, 2011)
Survivin (SUR)	- Overexpressed in malignant tissue - Implicated in cell growth and death - Induces an immune response	(Adida *et al.*, 2000; Ambrosini *et al.*, 1998; Altieri, 2001; Ambrosini *et al.*, 1997; Schmidt *et al.*, 2003; Andersen *et al.*, 2007)

2.5.1.2 Tumour-associated antigens examined in this thesis

In cells that have undergone a malignant transformation, the proteins that are degraded into fragments, and presented on MHC-class I molecules, may have originally been hundreds or thousands of amino acids long. The exact positioning of the 8-15 amino acids from this larger protein that are presented by an individual's MHC-class I molecules is determined by two factors: the type of each MHC molecule and the amino acid-recognising sequence of the cleft within this structure. These parameters are subsequently determined by the individual's MHC or HLA (human leukocyte antigen) genes, and this phenotype is referred to as 'tissue type' or more formally known as HLA-type (Moser and Leo, 2010). Many hundreds of HLA-types exist, which for class-I, are within three broad categories of HLA-A, HLA-B and HLA-C, and all individuals possess a variant of each category. There are many sub-types within each category, and the prevalence of each HLA-type is dependent on race and geographical location. Some HLA-types are more common than others, for example HLA-A*02 and HLA-B*07 account for approximately 50% and 30% per cent of HLA-types within Caucasian populations. MHC molecules linked to a particular HLA-type recognise a different sequence and positioning of amino acids for the same tumour associated antigen. Some research groups focus on participants of an identical HLA-type (e.g., often HLA-A*02). This is only possible after HLA-type screening during the recruitment process (e.g., via immunofluorescent staining of HLA molecules expressed by lymphocytes using flow cytometry, or polymerase chain reaction amplification of HLA genes, often within leukocyte DNA, and subsequent visualisation on agarose gels (Campbell *et al.*, 2007). This strategy slows recruitment and depending on the precision of HLA-typing that is required, can be an expensive and a time-costly process. Moreover, by focusing on a single HLA-type, the applicability of the findings are limited to a narrow population of individuals. For these reasons, the present work uses a laboratory approach that is independent of HLA-type. Following blood preparation, lymphocytes are incubated with a solution of hundreds of short peptides 15 amino acids long that span the entire length of the tumour or viral antigen of interest overlapping by 11 amino acids. Thus, at least one of these peptides will be of the correct sequence to match any HLA-type.

2.5.1.3 Virus antigens

Prior work has shown that patients with low numbers of T-lymphocytes, that are only able to produce very small amounts of IFN-γ in response to stimulation with tumour-associated antigens, appear to exhibit shorter survival in a number of cancers. This observation could be due to global immune suppression, but alternatively, due to suppression (or just lack of)

cancer-specific immunity. Thus, in addition to examining T-lymphocyte responses to tumour-associated antigens, it is also important to examine, for control purposes, T-lymphocyte responses to antigens from infectious disease.

The ideal non-cancer antigens to assess are those derived from viral infections, and in particular, Herpes viruses because they infect most of the population and require uninterrupted immune surveillance that is maintained throughout life. Examples of common herpes viruses are VZV, EBV and CMV. Thus, examining T-lymphocyte responses to proteins derived from these infections, provides an indication of the ability to control chronic latent infections, and is an excellent marker of global (or at least, non-cancer-specific) immunity. Another family of viruses, influenza viruses, do not persist in the body, but as with the herpes viruses, initiate a marked clonal expansion of virus-specific T-lymphocytes (Murali-Krishna *et al.*, 1998) that acquire effector functions (Berke, 1995), allowing them to eliminate virus infected cells. After overcoming the initial virus insult, the expanded antigen-specific T-lymphocyte pool contracts through apoptosis leaving only 10% of the virus-specific T-lymphocytes that remain as memory cells, ready to respond to the same infection if it was ever encountered again (Murali-Krishna *et al.*, 1998). While the numbers of T-lymphocytes targeting Herpes viruses often remains high due to periodic viral reactivation, some influenza-specific T-lymphocytes also persist. Some of these cells recognise amino acid sequences that are conserved within different strains of flu. Thus, by assessing the ability of T-lymphocytes to target proteins expressed by cells infected with almost any strain of influenza, gives an indication of the ability to respond to flu. Thus, in the present work, anti-viral T-lymphocyte responses will be assessed in parallel with ‘anti-cancer’ responses, by stimulating cells with proteins derived from VZV, EBV, CMV and flu.

2.5.1.4 Positive and negative controls used within ELISpot

A number of other controls were used to help with interpretation of ELISpot data and for quality control purposes. An anti-CD3 antibody (OKT3) was used as a positive control which binds to an epitope in the CD3+ complex in both CD8+ and CD4+ T-lymphocytes eliciting a robust pro-inflammatory response, mimicking TCR engagement with antigen. As virus and tumour proteins were dissolved in PBS (0.2% Dimethyl sulfoxide; DMSO), a mixture free from peptide was used as a negative control with cells, example results shown in Figure 4. Further, a non-cell control was used to confirm that the immunoassay process itself does not produce spots.

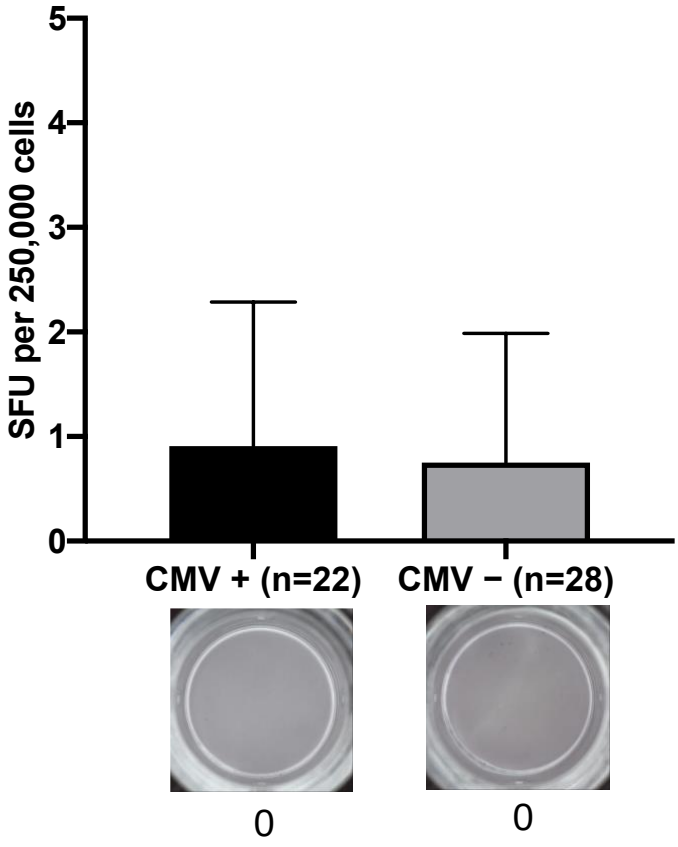

Figure 4. The relevant magnitude of non-specific T-lymphocytes IFN-γ secretion by with example ELISpot wells for negative control in ■ healthy CMV + (seropositive) n=22 and ■ healthy CMV – (seronegative) women n=28. Data show as means ± SD, data is taken from work undertaken during Chapter 4 of this thesis. *CMV; cytomegalovirus. SFUs; spot forming units*

2.5.2 Laboratory procedure for ELISpot

96-well PVDF membrane plates were coated with an anti-IFN-γ capture antibody (clone 1-D1K, 7.5 µg/ml). PVDF membranes were pre-wet with 50µl of 70% ethanol 30% sterile distilled water per well prior to coating. Outer wells were not used in the assay, but were filled with PBS (phosphate buffered saline) to avoid artefacts caused by evaporation (Schlingmann *et al.*, 2009; Streeck *et al.*, 2009). Plates were incubated at 4˚C overnight and subsequently blocked with FBS-supplemented culture medium for 1 hour at room temperature. 250,000 PBMCs were added to wells (Schmittel *et al.*, 1997; Streeck *et al.*, 2009) in 10% media (RPMI, 10% FBS, 1% penicillin-streptomycin). Cells were stimulated with 1 µg/mL of individual tumour-associated antigens or virus antigens in PBS (0.2% Dimethylsulfoxide; DMSO). An anti-CD3 (clone OKT3) antibody was used as a positive control (4 µg/ml). The plate was incubated for 16-20 hours at 37˚C, 5% CO_2. Following

incubation, plates were washed (8 x 200µl with 0.05% tween solution in PBS). An anti-IFN-γ (clone 7-B6-1) biotin-conjugated detection antibody was added (1 µg/ml) and plates were incubated for 3 hours at room temperature. Plates were washed (8 x 200µl 0.05% tween with PBS) followed by addition of a streptavidin-alkaline phosphate enzyme (1:1000 dilution) and incubated at 1.5 hours at room temperature. Plates were washed (8 x 200µl 0.05% tween with PBS and 3 x 200µl PBS) and substrate made up from distilled water and colour reagents (Biorad alkaline phosphatase conjugate substrate kit, California) was then added (100µl per well) and left to develop in a dark at room temperature for 30 minutes. Colour development was stopped by gently rinsing with tap water.

2.5.3 Analysis of ELISpot plates

Spots were enumerated using an automated ELISpot reader system and software (AID-diagnostika, Ebinger Straßberg).

2.5.3.1 Camera settings

All study samples were analysed with the same set of parameters. The area of interest was selected as the size of the well and adjusted to exclude the outermost part of the well in order to exclude artefacts. Camera settings were as follows; brightness 0, sharpness 0, white balance (R) 456, white balance (b) 310, hue 128, saturation 256, and pan 0.

2.5.3.2 Count settings

Manufacturer settings and algorithms were used to define spots using several parameters including; overall spot size, spot diffuseness, overall spot crowdedness and overall background staining in the well. To distinguish true single cell spots from artefacts, minimum and maximum threshold values were defined for each parameter to permit the exclusion of weak bystander responses and clusters containing multiple cells. A true single cell spot satisfied the criteria of being within a standard range of acceptable values for each parameter.

To be counted, a spot had to have a specified difference of 20-77 arbitrary units between the intensity of the peak of the spot and its surrounding background based on brightness (ranging from 0, white, to 255, black). Any spots falling above or below these values were excluded. The size function, in this case set at 40-863 pixels, set the minimum size an object needed to have to be recognised as a spot. Any object that was smaller or larger than these

values was not considered a spot. The gradient function, set at 1-90 arbitrary units, was a further parameter for characterising spots. The intensity of a true ELISpot follows a near Gaussian distribution with a maximum intensity in the middle of the spot and decreasing intensity towards the edges. The gradient value represents the angle defined by a tangent from the maximum intensity to the background intensity between the middle of the spot and the edges. Any spots falling above or below these values were excluded. Small basic algorithms were used with sensitivity setting 60 using algorithm C to detect small spots according to manufacturer instructions. Well saturation was set at 70% (i.e., spots could be counted even if 70% of the well was covered) however wells over this limit were marked as being too saturated (or too numerous to count; TNTC).

2.5.3.3 Spot data analysis

Cells were plated in duplicate, thus values derived from two independent wells containing 250,000 cells were averaged for each antigen. Counts were summed to derive the response frequency.

2.5.4 Laboratory procedure of ELISAs

To determine serostatus towards CMV (DIESSE Diagnostica Senese, Siena, Italy), EBV (VIDIA, Czech Republic) and VZV (TestLine Clinical Diagnostics, Czech Republic) immunoenzymatic methods were used: ELISAs (enzyme linked immunosorbent assay). Polystyrene strips were coated with a specific viral antigen containing immunodominant epitopes of the virus. Anti-viral antibodies present in the sample serum bound to the immobilised antigen. After washing to eliminate proteins that have not reacted, incubation was performed with conjugate composed of human IgG monoclonal antibodies conjugated to horseradish peroxidase. The amount of the bound antibody is measured after addition of a chromogenic substrate. The colour, which developed after addition of sulphuric acid solution, was proportional to the concentration of specific antibodies present in the serum. Plates were read at 450nm using a plate reader (SPECTROstar plate reader, BMG Labtech, Great Britain). Results were analysed quantitatively. A calibration curve was constructed by plotting the IU/ml of standards (x-axis) versus the absorbance of the standard wells (y-axis) using a logarithmic scale. Positivity was determined at IgG concentration of sample 4.4IU/ml, negativity at 125IU/ml for EBV and VZV. Positivity was determined at IgG concentration of sample > 1.2IU/ml, negativity at < 0.8IU/ml for CMV. Any samples lying between thresholds or had results higher than recordable were rerun at a higher dilution.

2.5.5 Laboratory procedure for erythrocyte sedimentation rate (ESR)

ESR results can be elevated in many conditions including malignant tumours and is an established screening test for inflammatory illness amongst clinicians (Pincherle and Shanks, 1967). A review in 2011 confirmed that the reference method for measurement of ESR should be based on the Westergren method using diluted blood (Altintas *et al.*, 2011; 1973). ESR is based on the principle that over time red blood cells sediment. During the initial phase, defined as the lag phase, the process follows a Rouleau pattern and sedimentation is generally slow. The rate then accelerates in the second phase (decantation phase) and then slows again in the final packing phase as the red blood cells pile up towards the lower part of the tube. The initial phase is impacted by plasma proteins such as fibrinogen and IgM. Whole blood samples were obtained by venepuncture over a maximum period of 30 seconds. 1ml of blood was collected in an EDTA anticoagulant tube and mixed with saline solution via inversion 8 times. Once the blood/ saline solution had returned to the bottom of the reservoir the Dispette2 pipet (Guest Scientific, Switzerland) was inserted. The pipette and reservoir were then placed in a levelled ESR stand so that the pipette was at 90 degrees for 60 minutes. After 60 minutes the result was read by eye where the red cell column had dropped in mm, leaving clear plasma above. Samples were tested within 2 hours from collection and at 18-25°C in accordance with International Council for Standardization in Haematology recommendations.

2.5.6 Laboratory procedure for clinical chemistry analyser

Plasma samples were analysed for non-esterified fatty acids (NEFA), glucose, triglycerides, total cholesterol, lipoproteins, CRP and glycerol using a Daytona automated analyser (Randox Laboratories, Crumlin, NI) according to manufacturer guidelines using commercially available immunoassays.

Glucose was determined using a colorimetric without deproteinization method whereby glucose was determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide produced, reacted with the catalysed peroxidase, phenol and 4-aminophenazone and produced a red-violet quinonimine dye whose intensity (measured at 505nm) was directly proportional to the glucose concentration. NEFA was determined using a similar colorimetric method. Triglycerides were determined using a colorimetric method whereby triglycerides were determined after enzymatic hydrolysis with lipases where quinonimine formed from hydrogen-peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase was quantified. Cholesterol was determined after

enzymatic hydrolysis and oxidation. Quinonimine was formed from hydrogen peroxide and 4-aminoantipyrine, in the presence of phenol and peroxidase, was subsequently quantified. Glycerol was determined using a direct colorimetric procedure using the quinonimine chromogen system in the presence of glycerol kinase, peroxidase and glycerol phosphate oxidase. To measure CRP, CRP was reacted with a buffer and anti-CRP coated latex. The formulation of antibody-antigen complex resulted in an increase in turbidity which was measured as the amount of light absorbed at 570nm. The concentration of CRP was determined using a standard curve.

2.6.7 Laboratory procedures for R-PLEX

Leptin, osteopontin, resistin and RANTES were measured using R-PLEX (Meso Scale Diagnostics, Maryland, USA) assays. MSD GOLD singleplex streptavidin plates with integrated high binding carbon electrodes to deliver an electric impulse to the well were coated with 25µl of coating solution (200µl of biotinylated capture antibody in 3.3ml of coating diluent). The plate was sealed and incubated at room temperature for 1 hour with shaking to allow the capture antibody to bind to the plate surface. The plate was then washed 3 times with 150µl of PBS plus 0.05% Tween. 25µl of assay diluent was added to each well and the plate was tapped before 2µl of calibrator standard or serum sample was added to each well. The plate was sealed with an adhesive plate seal and incubated with shaking at room temperature for 1 hour to allow the sample to bind to the capture reagent with an electrochemiluminescent label (MSD GOLD SULFO-TAG) to complete a sandwich immunoassay. The plate was then washed 3 times with 150µl PBS plus 0.05% Tween. 50µl of detection antibody, which contained electrochemiluminescent labels, was added to each well. The plate was then sealed and incubated with shaking for 1 hour. The plate was then washed again in 150µl PBS plus 0.05% Tween before adding 150µl MSD GOLD Read Buffer to each well. The plate was analysed on an MSD MESO QuickPlex SQ 120 (Meso Scale Diagnostics, Maryland, USA) whereby an electric voltage was applied to the plate electrodes, activating the electrochemiluminescent labels in the detection antibody causing the captured labels to emit light. The intensity of light emitted was proportional to the amount of analyte and provided a quantitative measure of the analyte in the sample.

CHAPTER 3: T-lymphocytes release IFN-γ in response to tumour-associated antigens in healthy women regardless of lifestyle characteristics

3.1 INTRODUCTION

The immune system plays a critical role protecting the body against cancer (Jakobisiak *et al.*, 2003; Decker, 2003). The theory of 'immune surveillance' suggests immune cells, particularly T-lymphocytes, can identify and eliminate malignancies through recognition of specific tumour-associated antigens (Burnet, 1970; Thomas, 1982; Jäger *et al.*, 2001; Tian *et al.*, 2011; Gross, 1943; Foley, 1953). Anti-cancer immune responses are generated through the cancer immunity cycle (Chen and Chen, 2013; Chen and Mellman, 2017). Many categories of tumour-associated antigens have been identified (Criscitiello, 2012); oncofoetal (e.g. CEA), over-expressed (e.g. HER2, MUC1, SUR, CycB1), oncoviral, cancer testis, lineage restricted, mutated, and post-translationally altered antigens (Zarour, 2003; Scanlan *et al.*, 2002; Jager *et al.*, 2003; Neville *et al.*, 1975; Finn, 2017; Vigneron, 2015; Piura and Piura, 2010; Linley *et al.*, 2011; Cheever *et al.*, 2009).

In cancer patients (such as lung, head and neck, prostate, blood and breast cancer), T-lymphocyte specificity to various tumour-associated antigens have been linked to longer survival and improved prognosis (Cobbold *et al.*, 2013a; Muraro *et al.*, 2015a; Muraro *et al.*, 2011; Inokuma *et al.*, 2007b; Roscilli *et al.*, 2014b; Epel *et al.*, 2008; Stadler *et al.*, 2016; Kao *et al.*, 2001; Criscitiello, 2012; Galvis-Jimenez *et al.*, 2013). Breast cancer patients demonstrating a pCr, (whereby no residual tumour cells are detectable), have shown to exhibit an increased number of IFN-γ producing circulating and intra-tumour CD8+ T-lymphocytes after stimulation with antigens MamA, HER2 and MUC1 (Muraro *et al.*, 2015a; Muraro *et al.*, 2011; Blixt *et al.*, 2011).

Previously, CD8+ T-lymphocytes from healthy donors have also elicited antigen-specific activity against tumour-associated antigens such as SUR, HER2, CEA and CycB1 (Schmidt *et al.*, 2003; Andersen *et al.*, 2007; Inokuma *et al.*, 2007b; Fujiwara *et al.*, 2004; Nagorsen *et al.*, 2005; Sotiropoulou *et al.*, 2003; Vella *et al.*, 2009; Inokuma *et al.*, 2007a). It is unknown what triggers this immune response to tumour-associated antigens in health individuals. Previously, little information has been provided on characteristics of healthy donors, (e.g., lifestyle variables), which may help decipher why these responses may occur. Tumour-specific memory T-lymphocytes may confer protection against cancer, but it is currently unknown whether healthy women possess T-lymphocytes with specificity to a range of tumour-associated antigens and what the magnitude of this response is.

Lifestyle factors are important modifiable determinants of cancer risk (Kushi *et al.*, 2012b; Leitzmann *et al.*, 2015b; Friedenreich and Orenstein, 2002; Thune *et al.*, 1997) and are known to affect the function of the immune system, including T-lymphocyte cytokine production and number and phenotype of T-lymphocytes within peripheral blood. Evidence has shown that moderate intensity aerobic exercise training and regular physical activity are beneficial for immune function, with individuals who lead an active lifestyle often exhibiting stronger immune responses to vaccination compared to those who are inactive (Pascoe *et al.*, 2014; Simpson, 2011; Simpson and Guy, 2010; Turner, 2016; Simpson *et al.*, 2012).

Assessing cell-mediated immunity to viral antigens analyses the efficiency of the immune system, a major defence in terms of tumour initiation (Gonzalez *et al.*, 2018). Some viruses (e.g. CMV, EBV and VZV), establish lifelong latency within the host (Dunn *et al.*, 2002b; Larsson *et al.*, 1998) which creates sufficient immunocompetency to resist reinfection. However, in immunocompromised hosts, (e.g. HIV positive patients, or organ transplant patients), there is increased risk of viral reinfection and poor disease prognosis (Moss and Khan, 2004; Adler *et al.*, 1995; Abate *et al.*, 2012). An increase in viral-specific CD8+ T-lymphocyte response has been shown to increase with age alongside immunosenescence, an age-related decline in cell-mediated immune function (Khan *et al.*, 2002; Hodes, 1997; Appay *et al.*, 2002; Moss and Khan, 2004; Rothberg *et al.*, 2007). Increasing numbers of resident memory T-lymphocytes specific for viruses such as EBV and CMV in seropositive individuals, may deplete the number of naïve T-lymphocytes available from the total T-lymphocyte pool to respond to new antigens, such as tumour-associated antigens (Looney *et al.*, 1999; Karrer *et al.*, 2003). It is therefore unknown whether a larger anti-viral immune response represents an improvement in response to tumour-associated antigens, or in fact, negatively affects this.

Blood biomarkers such as RANTES, leptin, resistin and osteopontin have recently received attention surrounding their implications in breast cancer, aspects of lifestyle and/or immune cell function. RANTES is an inflammatory chemokine expressed on T-lymphocytes and certain tumour cells (Soria and Ben-Baruch, 2008). RANTES plays a role in immune surveillance and has been detected in breast cancer and associated with its progression (Balkwill, 2012) by contributing to immune evasion mechanisms that promote immune tolerance (Kershaw *et al.*, 2013; Araujo *et al.*, 2018; Liu *et al.*, 2015). Leptin and resistin are adipokines (Sarmento-Cabral *et al.*, 2017) which may increase breast cancer risk (Niu *et al.*, 2013; Cleary *et al.*, 2003; Cleary *et al.*, 2004; Gong *et al.*, 2016; Assiri *et al.*, 2015;

Munoz-Palomeque *et al.*, 2018; Gunter *et al.*, 2015; Kang *et al.*, 2007; Lee *et al.*, 2012; Zeidan *et al.*, 2018; Dalamaga *et al.*, 2013; Deshmukh *et al.*, 2015; Lee *et al.*, 2016; Wang *et al.*, 2018). Osteopontin is also expressed by T-lymphocytes (Murry *et al.*, 1994; Kunii *et al.*, 2009; Kruger *et al.*, 2014; Shurin, 2018) and is overexpressed in breast cancer, alongside being implicated in inflammation (Irby *et al.*, 2004; Pietrowska *et al.*, 2009). Osteopontin's relationship to the immune system leads to immune evasion and pro-tumourigenic changes to the tumour microenvironment, favouring immunosuppressive leukocytes at the site of cancer (Zhao *et al.*, 2018; Rangaswami *et al.*, 2006; Castello *et al.*, 2017).

Understanding the factors that may affect T-lymphocyte response to tumour-associated antigens may help to devise novel strategies towards assessing cancer risk. The main aim of this study was to examine whether breast cancer specific T-lymphocyte responses can be detected in healthy women, and to explore potential relationships with these responses and aspects of lifestyle, anti-viral immunity and blood biomarkers. We hypothesise that women with healthier lifestyles will have increased T-lymphocyte responsiveness to tumour-associated antigens.

3.2 METHODS

3.2.1 Participants and study design

50 healthy volunteers (43 ± 12 years, BMI 24.8 ± 4.9 kg.m$_{2,}$ predicted $\dot{V}O_2$ max 37.1 ± 8.9 ml.kg.min^{-1}), were recruited following local advertisements, to take part in a cross-sectional study. Participants were female, aged 25-69 years, free from cardiovascular disease, autoimmune or inflammatory disease, cancer or any form of diabetes. Ethical approval was granted by a local NHS research ethics committee (reference: 15/SW/0004). Participants were informed verbally and in writing about the rationale, nature and demands of the study (and of their right to withdraw) before providing written informed consent. Participants subsequently completed a general health questionnaire, a physical activity readiness questionnaire, a sex specific cancer risk factor questionnaire, the DASS-21 (Lovibond and Lovibond, 1995; UNSW, 2014) and IPAQ (IPAQResearchCommittee, 2016).

3.2.2 Procedures

Participants arrived at the laboratory between 07:00-11:00 following an overnight fast and after refraining from exercise, alcohol and caffeine for the previous 24 hours. Participants

rested for 15 minutes in the supine position, during which heart rate and blood pressure were measured using an automated sphygmomanometer (Bosch and Sohn, Germany). Following this period of rest, a blood sample was collected by venepuncture of an antecubital vein. Approximately 40ml of blood was collected into a sterile syringe containing sodium heparin (2.0 international units/ml) for isolation of PBMCs. A further 10ml of blood was collected into a syringe free from anti-coagulant and aliquoted immediately into a 5ml EDTA vacutainer tube (Becton Dickinson, U.S) for preparation of plasma and a 5ml plain vacutainer tube (Becton Dickinson, U.S), for preparation of serum.

Assessment of body composition (DEXA, W:H and BMI), cardiorespiratory fitness (sub-maximal exercise test), habitual physical activity (IPAQ and Sensewear), habitual diet and depression, anxiety and stress were measured in line with methodology explained in Chapter 2 of the current thesis.

3.2.3 Biochemical and immunological procedures

A three-part leukocyte differential was measured in EDTA blood using an automated haematology analyser (Sysmex, KX-21N, Kobe, Japan). For preparation of plasma, EDTA vacutainers were centrifuged at 2000 × *g* for 10 minutes at 4°C and the plasma collected and stored in multiple aliquots at −80°C. For preparation of serum, after clotting for ~30 minutes at room temperature, samples were centrifuged at 2000 × *g* for 10 minutes at 4°C and the serum collected and stored in multiple aliquots at −80°C. PBMCs were separated from heparinised whole blood by density gradient centrifugation. The cells were washed in sterile RPMI and resuspended in culture medium (RPMI 1640 containing 10% FCS, 1% antibiotic). Cells were counted using trypan blue (1.5% acetic acid to lyse contaminating erythrocytes) using a haemocytometer and light microscope (Primo Vert, Zeiss, Germany).

3.2.3.1 ELISpot

ELISpot was conducted on fresh PBMCs. Cells were stimulated overnight with breast cancer tumour associated antigens; MamA, ERB ECD, ERB ICD, MMP11, MUC-1, TARP, CycB1, Cl6, survivin and CEA. Virus antigens were examined as controls; VZV (IE63 and gE), EBV (BZLF1 and EBNA1), CMVs (pp65 and IE1), influenza-A (NP1 and MP) (JPT Peptide Technologies, Berlin, Germany) at a concentration 1µg/ml and 250,000 cells/well (PBMCs) in a 37° C humidified CO_2 incubator. Cells stimulated with an anti-CD3 (clone OKT3) antibody served as a positive control, and cells stimulated with PBS (0.2% dimethylsuphoxide) or incubated in media only served as negative controls. Each condition

was measured in duplicate. Plates were analysed using an automated ELISpot reader system (AID-diagnostika, Ebinger Straßberg). Spot forming units (SFUs) were enumerated to obtain the frequency of reactive cells.

3.2.3.2 Enzyme-linked immunosorbent assays for viral serostatus

IgG antibodies specific for latent viruses were assessed in serum using commercially available kits; VZV (TestLine Clinical Diagnostics, Czech Republic.), EBV (VIDIA, Czech Republic) and CMV (DIESSE, Italy) to define serostatus positivity. The criteria for defining positivity was >125 IU/L, >44 IU/L and >1.2IU/L for VZV, EBV and CMV respectively.

3.2.3.3 Quantifying a T-lymphocyte response to tumour-associated antigens

Spot counts were averaged from duplicate wells. Responses were deemed positive if the mean number of spots from the stimulated wells was greater than the number of spots in viral wells where participants possessed positive serostatus, as determined by ELISA towards the virus. This strategy is shown in Table 2. Participants are listed (1-50) on the left-hand side of the table and SFUs in response to viral antigens are listed under EBV, CMV and VZV viral antigens. If a participant had positive serostatus towards a virus as determined by ELISA, SFUs are listed in green. If a participant had negative serostatus towards a virus, SFUs are listed in red. For example, participant number 1 had positive serostatus to EBV, CMV and VZV and participant 2 had positive serostatus to EBV and VZV but negative serostatus for CMV. If an individual had positive serostatus towards a virus, the lowest spot count towards either viral antigen was calculated and determined the minimum ‘positive’ response towards the virus. Finally, the lowest spot count from the minimum ‘positive’ responses from all viral antigens was calculated (second column from the right). This was then deemed the minimal ‘positive’ response for the individual. An SFU equal or higher than this value in response to any tumour-associated antigen was deemed positive and an SFU below this value was deemed a negative response. For example; participant 1 had a minimum ‘positive’ response of 2 as the lowest number of SFUs to a viral antigen they were seropositive towards, which was in response to VZV IE63. Thus, in any tumour-associated antigen eliciting a response of 2 or more SFUs, it was determined that they had a ‘positive’ response to the antigen. When the minimum ‘positive’ response was deemed as 0 this was substituted with the next lowest SFU.

For some participants minimum ‘positive’ response was the same as the average of the negative control (participants 3, 27, 35 and 38) and in three participants (40, 44 and 45) the

negative control was higher than the calculated minimum ‘positive’ response. Whilst some strategies incorporate the negative control when calculating a ‘positive’ response we opted for a less conservative approach as T-lymphocyte response to tumour-associated antigens is poorly defined in the literature and was much lower than response to viral antigens. However, for completeness and thoroughness, multiple strategies previously employed to calculate positive responses to viral antigens were also used to define positivity including >2 x average spot count in the negative control wells and >4 x average spot count in the negative control wells. The results from these strategies are presented in Appendix 1 and the overall conclusions and findings remained relatively similar as reported in the current results section. Other strategies for determining positive responses are >50 x 10_6 PBMCs however as response to tumour-associated antigens was generally low, this approach was not included in analysis.

Table 2. Strategy for defining individualised positive responses to tumour-associated antigens using spot forming units in response to viral antigens and serostatus.

	Spot count (SFUs per 250,000 cells)										
Participant no.	**CMV pp65**	**CMV IE1**	**Minimal CMV**	**EBV EBNA1**	**EBV BZLF1**	**Minimal EBV**	**VZV IE63**	**VZV gE**	**Minimal VZV**	**Minimal 'positive' response to tumour-associated antigen**	**Average of 4 x negative control wells**
1	471	27	27	22	3	3	2	4	2	**2**	*1*
2	4	2	X	6	12	6	11	5	5	**5**	*0*
3	3	1	X	10	1	1	4	5	4	**1**	*1*
4	2	2	X	3	6	3	5	0	X	**3**	*1*
5	11	3	X	24	8	8	11	6	6	**6**	*1*
6	TNTC	372	372	35	9	9	9	15	9	**9**	*0*
7	5	1	X	8	44	8	2	11	2	**2**	*1*
8	107	381	107	12	4	4	1	2	1	**1**	*0*
9	43	78	43	1	1	X	1	1	1	**1**	*0*
10	2	0	X	1	2	X	1	2	1	**1**	*0*
11	0	0	X	1	8	1	0	1	0	**1**	*0*
12	0	1	X	1	1	1	2	0	0	**1**	*0*
13	2	0	X	5	6	5	8	3	3	**3**	*0*
14	2	0	X	1	5	1	5	4	4	**1**	*0*
15	0	0	X	6	3	3	0	3	0	**3**	*0*
16	2	2	X	10	11	10	3	3	3	**3**	*2*
17	1	0	X	27	18	18	3	14	3	**3**	*0*
18	181	58	58	4	115	4	1	1	X	**4**	*0*
19	114	197	114	23	4	4	2	2	2	**2**	*0*
20	63	1	X	4	3	3	2	2	2	**2**	*0*
21	258	27	27	15	7	7	2	2	2	**2**	*0*
22	4	2	X	42	399	42	1	0	0	**42**	*0*
23	2	0	X	2	2	2	1	4	1	**1**	*0*

24	3	0	0	0	1	0	0	0	0	**1**	*0*
25	5	6	x	6	363	6	3	9	3	**3**	*1*
26	2	10	X	2	10	2	16	19	16	**2**	*1*
27	6	11	X	169	146	146	4	7	4	**4**	*4*
28	0	0	X	1	3	1	0	0	0	**1**	*0*
29	19	15	15	2	8	2	1	3	1	**1**	*0*
30	1	5	1	3	61	3	4	3	3	**1**	*0*
31	3	6	X	9	8	8	3	2	2	**2**	*0*
32	286	180	180	40	11	11	5	4	4	**4**	*3*
33	65	15	X	13	3	3	5	0	0	**3**	*1*
34	0	188	X	6	2	2	0	1	0	**2**	*0*
35	4	1	1	56	10	10	8	7	7	**1**	*1*
36	108	328	108	24	2	2	4	13	4	**2**	*0*
37	250	21	21	3	9	3	5	3	3	**3**	*1*
38	TNTC	316	316	11	112	11	6	5	5	**5**	*5*
39	34	254	34	22	3	3	6	10	6	**3**	*1*
40	204	196	196	28	104	28	2	1	1	**1**	*4*
41	314	18	18	3	15	3	6	0	0	**3**	*1*
42	270	27	27	2	2	X	5	1	1	**1**	*1*
43	2	4	X	26	59	26	6	14	6	**6**	*1*
44	7	3	X	22	5	5	3	4	3	**3**	*5*
45	3	2	X	3	3	3	1	1	1	**1**	*2*
46	0	2	X	3	0	0	0	0	0	**3**	*0*
47	166	151	151	45	6	6	1	4	1	**1**	*1*
48	166	61	61	45	44	44	28	23	23	**23**	*1*
49	0	0	0	0	1	0	0	0	0	**1**	*0*
50	1	2		2	0	0	1	0	0	**1**	*0*

Green; positive serostatus as determined by ELISA. Red; negative serostatus as determined by ELISA.

SFUs; spot forming units. TNTC; too numerous to count. EBV, Epstein Barr Virus. EBNA1, Epstein Barr nuclear antigen 1. BZLF1, BamHIZ leftward reading frame 1. MP1, matrix protein1. NP, nucleoprotein. CMV, Cytomegalovirus. pp65, phosphoprotein 65. ie1, immediate-early protein 1. VZV, Varicella Zoster Virus. ie63, immediate-early protein 63. gE, glycoprotein E. Min; minimum.

3.2.3.4 Quantification of leptin, osteopontin, resistin and RANTES

Leptin, osteopontin, resistin and RANTES were measured from serum using R-PLEX (Meso Scale Diagnostics, Maryland, USA) assays in accordance with manufacturer guidelines.

3.2.4 Statistical analysis

Data were tested for normal distribution using the Kolmogorov Smirnov test. Analysis of variance (ANOVA) was used to examine differences in key outcome variables between groups. To measure congruency between measurement techniques, paired t-tests and bivariate correlations were conducted. Pearson's correlations were conducted on normally distributed data. Effects sizes (Cohen's *d)* were calculated and thresholds of 0.2-0.5, 0.5-0.8 and >0.8 for small, medium and large effect sizes were accepted respectively in line with Cohen's *d* (Lakens, 2013). Bland-Altman plots were also employed to investigate agreement between measurement techniques. Chi-squared tests were used to compare ordinal data. Statistical analyses were conducted using SPSS (Statistical Package for Social Science) version 22. Statistical significance was accepted at $p<0.05$. Graph Pad Prism 8 was used for producing graphical figures. Data is presented as means ± SD.

3.3 RESULTS

3.3.1 T-lymphocytes from healthy women release IFN-γ in response to stimulation by tumour-associated antigens

IFN-γ secreting T-lymphocytes in response to tumour-associated antigens were present in 43/50 healthy women with only 14% of women lacking an immune response to any of the 10 tumour-associated antigens (Figure 5A). On average, 4 ± 3 of the 10 tumour-associated antigens elicited IFN-γ secretion from T-lymphocytes of healthy women but this ranged from a response to only 1 tumour-associated antigen to a response to all 10 tumour-associated antigens.

3.3.2 Immunogenicity of tumour associated antigens

MamA and CEA were the most immuno-dominant antigens with 52% of women eliciting an immune response towards them followed by ERB ECD (50% women showing an immune response), CycB1 (48%), ERB ICD and TARP (46%), MUC1 (44%), SUR and Cl6 (38%). MMP11 was the least immune-dominant antigen with only 28% of women eliciting an

immune response towards this antigen (Figure 5). The magnitude of the immune response varied between tumour-associated antigens (Figure 6.) where on average, number of IFN-γ responding cells from the 250,000 PBMC pool in response to tumour-associated antigens was the largest towards survivin, MUC1, ERB ICD, ERB ECD and CEA (an average of 6 responding cells) and the smallest towards MMP11 (an average of 2 IFN-γ secreting cells ± 3). However, the number of SFUs did not significantly differ between the 10 tumour-associated antigens ($p>0.05$). The highest individual number of SFUs per 250,000 PBMCs ranged for each antigen from 51 responding cells (stimulated by antigen CEA) to 13 responding cells (MMP11). The lowest positive individual response to each antigen was 1 which was the same for all the 10 antigens.

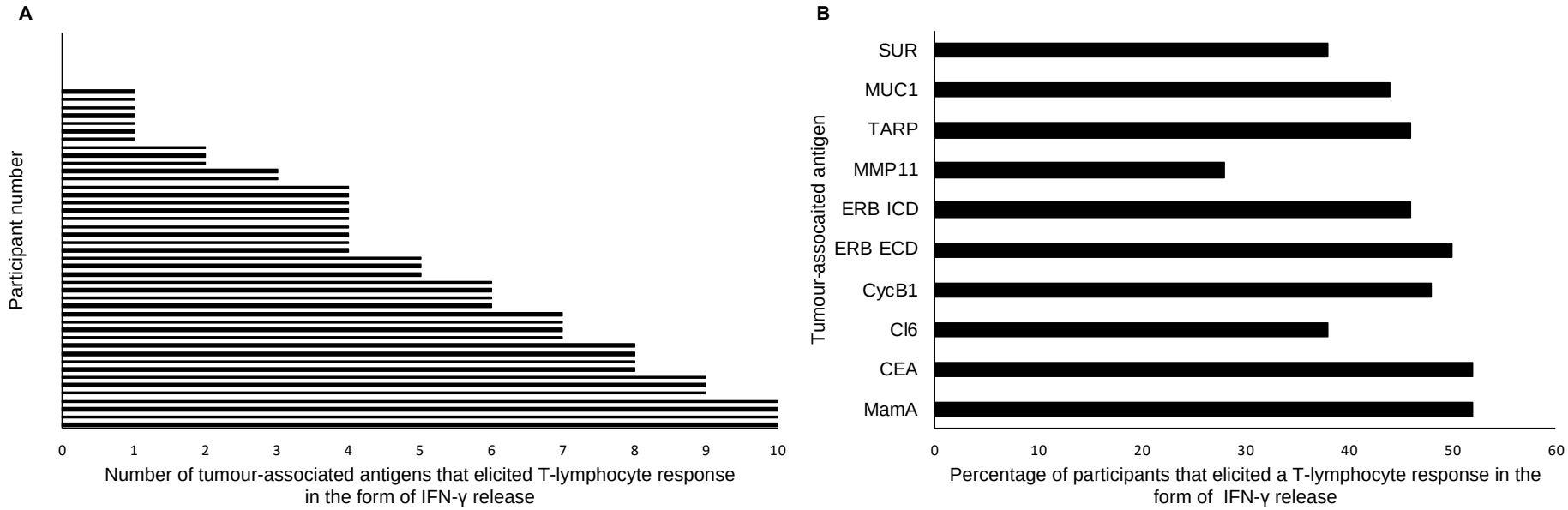

Figure 5A. Participants ranked from showing positivity to all 10 tumour-associated antigens to participant lacking positivity to any of the tumour-associated antigens. 5B. Tumour associated antigens in order of immunodominance. N=50. Total number of tumour-associated antigens = 10. 250,000 PBMCs per well were tested using ELISpot, against 10 tumour-associated antigens; *MamA, mammaglobin A. CEA, carcinoembryonic antigen. Cl6, claudin-6. CycB1, cyclin-B1. ERB ECD, receptor tyrosine-protein kinase erbB-2 extracellular domain. ERB ICD, receptor tyrosine-protein kinase erbB-2 intracellular domain. MMP1, stromelysin-3. TARP, TCRgamma alternate reading frame protein. MUC1, mucin-1. SUR, survivin. IFN-γ; interferon gamma.*

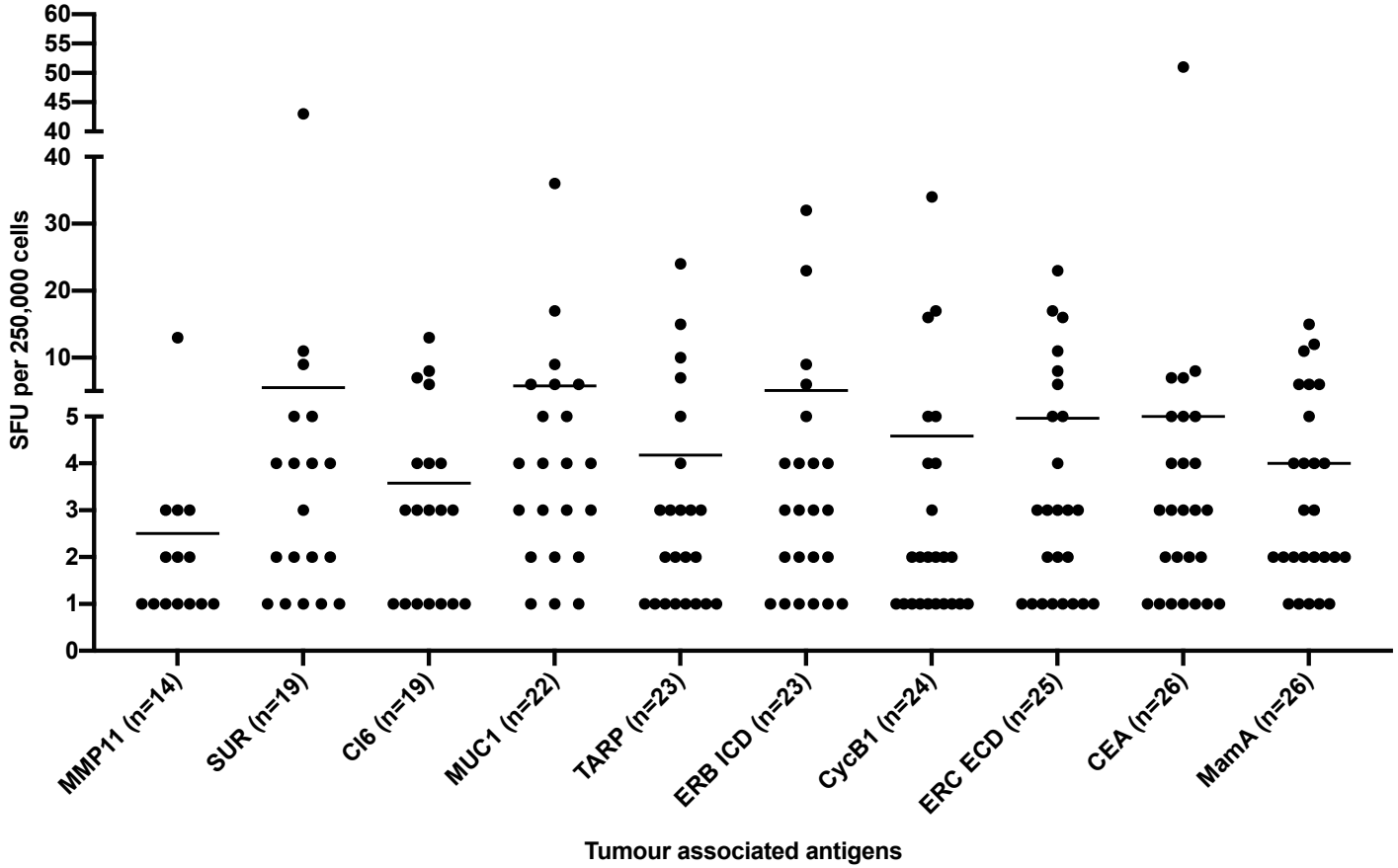

Figure 6**.** The magnitude of specific T-lymphocyte response compared against 10 tumour-associated antigens following overnight culture. N=43, only positive responses shown. Data show as individual positive responses (averaged from 2 wells) and means. N= represents number of women that elicited a response towards the tumour-associated antigens. *SFU, spot forming units. MamA, mammaglobin-A. CEA, carcinoembryonic antigen. Cl6, claudin-6. CycB1, cyclin-B1. ERB ECD, receptor tyrosine-protein kinase erbB-2 extracellular domain. ERB ICD, receptor tyrosine-protein kinase erbB-2 intracellular domain. MMP1, stromelysin-3. TARP, TCRgamma alternate reading frame protein. MUC1, mucin-1. SUR, survivin.*

3.3.3 T-lymphocyte IFN-γ release in response to stimulation by viral antigens

No significant differences in IFN-γ secretion by T-lymphocytes in response to viral antigens were found between individuals that responded to 1 or more tumour-associated antigens and individuals that did not respond to any tumour-associated antigens in terms of IFN-γ secretion by T-lymphocytes in response to viral antigens for Flu, EBV, CMV and VZV (Figure 7.). Individuals that possessed tumour-associated antigen specific T-lymphocytes, had on average 6 ± 8 SFUs vs 5 ± 3 SFUs and 21 ± 61 SFUs and 6 ± 7 SFUs IFN-γ secreting cells per 250,000 cells in responders and non-responders in response to antigens MP1 and NP respectively. In response to CMV pp65 responders and non-responders had an average of 188 ± 141 SFUs vs 123 ± 82 SFUs per 250,000 cells respectively. In response to CMV IE1 responders and non-responders, on average had 139 ± 136 vs 37 ± 30 SFUs per 250,000 cells respectively. For EBV antigen EBNA1 responders had on average 16 ± 29 SFUs vs non-responders 20 ± 14 and for BZLF1 this was 26 ± 64 SFUs compared with 86 ± 144 per 250,000 cells for responders and non-responders respectively. In response to stimulation to VZV antigens; 4 SFUs were observed on average in response to IE63 for both responders and non-responders and 4 ± 5, vs 6 ± 7 SFUs per 250,000 cells in response to gE for responders and non-responders respectively. The range of SFUs towards each antigen was always large. For Flu antigens MP1 and NP the range was 38 and 372 SFUs per 250,000 cells respectively. For EBV antigens EBNA1 and BZLF1 the range was 169 and 363 SFUs per 250,000 cells respectively. CMV antigens produced a range of 471 and 381 SFUs per 250,000 cells for pp65 and IE1 respectively and VZV had a smaller range of 28 and 23 SFUs per 250,000 cells in response to antigens IE63 and gE respectively (Figure 7).

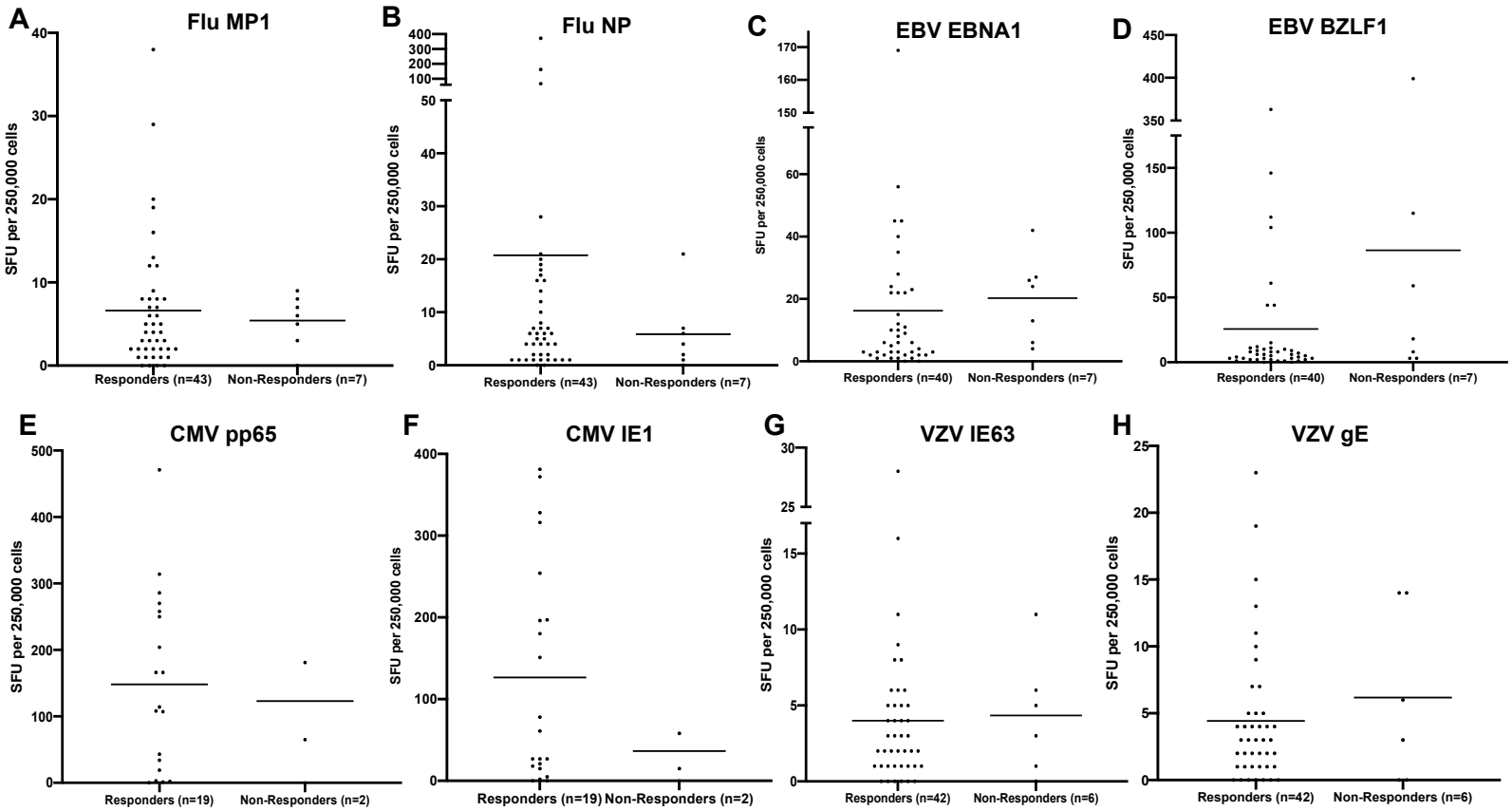

Figure 7. The magnitude of specific T-lymphocytes stimulated by A. Flu MP1, B. FLU NP, C. EBV EBNA1, D. EBV BZLF1, E. CMV pp65, F. CMV IE1, G VZV IE63, H VZV gE viral antigens. Data show as individual positive responses as deemed by ELISA and means. Total responders (n=43) and total non-responders (n=7) to tumour associated antigens. *SFUs, spot forming units. EBV, Epstein Barr Virus. EBNA1, Epstein Barr nuclear antigen 1. BZLF1, BamHIZ leftward reading frame 1. MP1, matrix protein1. NP, nucleoprotein. CMV, Cytomegalovirus. pp65, phosphoprotein 65. ie1, immediate-early protein 1. VZV, Varicella Zoster Virus. ie63, immediate-early protein 63. gE, glycoprotein E.*

3.3.4 Differences in lifestyle and psychological characteristics between responders and non-responders

No significant differences in general characteristics; age, height, weight, predicted $\dot{V}O_2$ max and blood pressure were found between individuals who responded to one or more tumour-associated antigens (n=43) and individuals who did not respond to any tumour-associated antigens (n=7) (Table 3.)

Table 3. Physiological characteristics of women who show positivity to at least one tumour-associated antigen and those negative to all tumour-associated antigens

Characteristics	**Responder to tumour-associated antigens (n=43)**	**Non-responder to tumour-associated antigens (n=7)**	**Difference**	**ANOVA statistic**
Age (years)	43 ± 12	40 ± 12	3	F(1,48)=0.399, p=0.531
Height (m)	1.67 ± 0.08	1.64 ± 0.07	0.04	F(1,48)=1.395, p=0.243
Weight (kg)	70.2 ± 16.5	63.5 ± 9.9	6.7	F(1,48)=1.079, p=0.304
Predicted $\dot{V}O_2$ max ($ml.kg.min^{-1}$)	36.3 ± 8.8	41.7 ± 33.9	-5.4	F(1,48)=2.308, p=0.135
Systolic blood pressure (mmHg)	116 ± 17	117 ± 12	-1	F(1,48)=0.003, p=0.958
Diastolic blood pressure (mmHg)	74 ± 11	75 ± 8	-1	F(1,48)=0.053, p=0.818

*N=50, * P<0.05.* Data shown as means ± SD

m; metres. kg; kilograms. $\dot{V}O_2$ max; maximal oxygen consumption. ml; millilitres. min; minute. mmHg; millimetres of mercury.

BMD and T-score were significantly higher in non-responders by ~0% and 351% respectively (effect size 0.3 for both) (Table 4.). Further analysis demonstrated this difference did not dependent on whether individuals had a normal or overweight BMI 18.5><25kg.m$_2$ (interaction effect of BMI status and response status of BMD, p=0.447). Whilst not reaching significance (p=0.057), Z-score was also elevated in non-responders (1.53 ± 1.19 versus 0.56 ± 1.22 in responders). All other measures of body composition were largely similar between groups, including BMI, visceral fat and FMI (fat mass index) (Table 4.). Appendix 2 demonstrates a significant correlation was observed between DEXA and Tanita measured body fat percentage (p=0.001), r=470, r$_2$=0.221 and Bland Altman plots are provided.

1162 Table 4. Body composition of women who respond to at least one tumour-associated antigen and those who did not respond to any tumour
1163 associated antigens

Body composition	**Responder to tumour-associated antigens (n=43)**	**Non-responder to tumour-associated antigens (n=7)**	**Difference**	**ANOVA statistic**
BMI (kg.m2)	25.0 ± 5.1	23.7 ± 3.4	1.3	F(1,48)=0.437, p=0.512
W:H	0.78 ± 0.04	0.79 ± 0.11	- 0.02	F(1,48)=0.571, p=0.453
Body fat % (DEXA scan)	32 ± 8	31 ± 5	1	F(1,48)=0.135, p=0.715
Body fat % (Tanita scales)	32 ± 8	30 ± 7	2	F(1,46)=0.792, p=0.378
Fat mass index (kg.m2)	7.81 ± 2.97	7.89 ± 2.03	- 0.07	F(1,48)=0.004, p=0.949
BMD (g/cm2)	1.15 ± 0.13	1.26 ± 0.12	- 0.11	F(1,48)=4.4049, p=0.050*
T-score	0.52 ± 1.54	1.79 ± 1.40	- 1.26	F(1,48)=4.132, p=0.048*
Z-score	0.56 ± 1.22	1.53 ± 1.19	- 0.97	F(1,48)=3.803, p=0.057
Breast fat (%)	29 ± 11	29 ± 11	0	F(1,48)=0.001, p=0.978
Android fat (%)	30 ± 10	30 ± 10	0	F(1.48)=0.000, p=0.987
Gynoid fat (%)	37 ± 7	35 ± 3	2	F(1,48)=0.634, p=0.430
Visceral fat body fat (%)	32 ± 11	32 ± 10	0	F(1,48)=0.002, p=0.961
Visceral fat outer wall middle (%)	30 ± 10	30 ± 9	0	F(1,48)=0.000, p=0.914
Visceral fat cavity inner fat (%)	27 ± 9	27 ± 8	0	F(1,48)=1.015 p=0.914
Fat mass ratio Trunk:Limb	0.760 ± 0.151	0.831 ± 0.275	-0.07	F(1,48)=1.056, p=0.309
Android:Gynoid ratio	0.798 ± 0.177	0.857 ± 0.233	-0.06	F(1,48)=0.640, p=0.428

1164 *N=50, * P<0.05.* Data shown as means ± SD
1165 *BMI; body mass index. kg; kilograms. m; metres. W:H; waist to hip ratio. DEXA; dual energy X-ray. BMD; bone mineral density. cm; centimetres*.

Physical activity data from Sensewear armbands were obtained from 48/50 participants. 2/50 participants did not have >80% wear time therefore data was not included. No significant differences between responders and non-responders were seen in physical activity measured objectively by Sensewear or self-reported IPAQ (Table 5). As recorded by Sensewear, number of MET minutes spent undertaking light, moderate vigorous (>6 METS) and very vigorous activities per day for responders and non-responders respectively was 106 ± 58 minutes and 72 ± 15, 126 ± 68 and 119 ± 33, 26 ± 23 minutes and 36 ± 16, and 0 ± 1 and 1 ± 2. To assess the validity of IPAQ we investigated the relationship between IPAQ subjectively measured and Sensewear objectively measured physical activity. A significant correlation was seen between Sensewear measured and IPAQ self-reported light physical activity, $p=0.039$, $r=0.306$, $r_2=0.094$ however there was no significant correlation when looking at measured and reported moderate and vigorous activity ($p=0.084$, $r=0.258$ and $p=0.878$, $r=0.025$ respectively). The mean differences and limits of agreements between IPAQ and Sensewear from the Bland-Altman plots for physical activity were 169 ± 355, 92 ± 339 and 319 ± 571 minutes per day for light, moderate and vigorous activity per day respectively (Figure 8A, 10B and 10C respectively.). Lower limits of agreement were −527, −572, −801 minutes per day for light, moderate and vigorous activity respectively and upper limits of agreement were 865, 756 and 319 minutes per day for light, moderate and vigorous activity respectively.

Table 5. Physical activity levels of women who respond to at least one tumour-associated antigen and those negative to all tumour-associated antigens

Physical Activity Levels	**Responder to tumour-associated antigens n=43**	**Non-responder to tumour-associated antigens n=7**	**Difference**	**ANOVA statistic**
<1.8 METS (mins/day)	703 ± 114	733 ± 43	-31	F(1,46)=0.492, p=0.486
1.8><3 METS (mins/day)	106 ± 58	72 ± 15	34	F(1,46)=2.282, p=0.138
3><6 METS (mins/day)	126 ± 68	119 ± 33	7	F(1,46)=0.077, p=0.783
>6 METS (mins/day)	26 ±23	36 ±16	-10	F(1,46)=1.215, p=0.276
>10.2 METS (mins/day)	0 ± 1	1 ± 2	0	F(1,46)=0.709, p=0.404
Energy expenditure (kcal/day)	2498 ± 421	2405 ±230	94	F(1,46)=0.327, p=0.570
PAL	1.74 ± 0.27	1.77 ± 0.15	-0.03	F(1,46)=0.073, p=0.788
Steps per day	10121 ± 3287	11688 ± 4672	-1476	F(1,46)=1.064, p=0.308
Sedentary time (% of waking)	74 ± 11	78 ± 4	-4	F(1,46)=0.665, p=0.419
IPAQ Light MET (mins/week)	620 ± 788	431 ± 334	189	F(1,48)=0.384, p=0.538
IPAQ Moderate MET (min/week)	398 ± 611	274 ± 238	124	F(1,48)=0.277, p=0.601
IPAQ Vigorous MET (mins/week)	307 ± 531	141 ± 105	165	F(1,48)=0.663), p=0.420
Total MET (mins/week)	1325 ± 1599	847 ± 434	2440	F(1,48)=0.660, p=0.420

*N=50, * P<0.05.* Data shown as means ± SD

tumour-associated antigens; tumour-associated antigen. *MET; metabolic equivalent. kcal; kilocalorie. PAL; physical activity level. IPAQ; international physical activity questionnaire.*

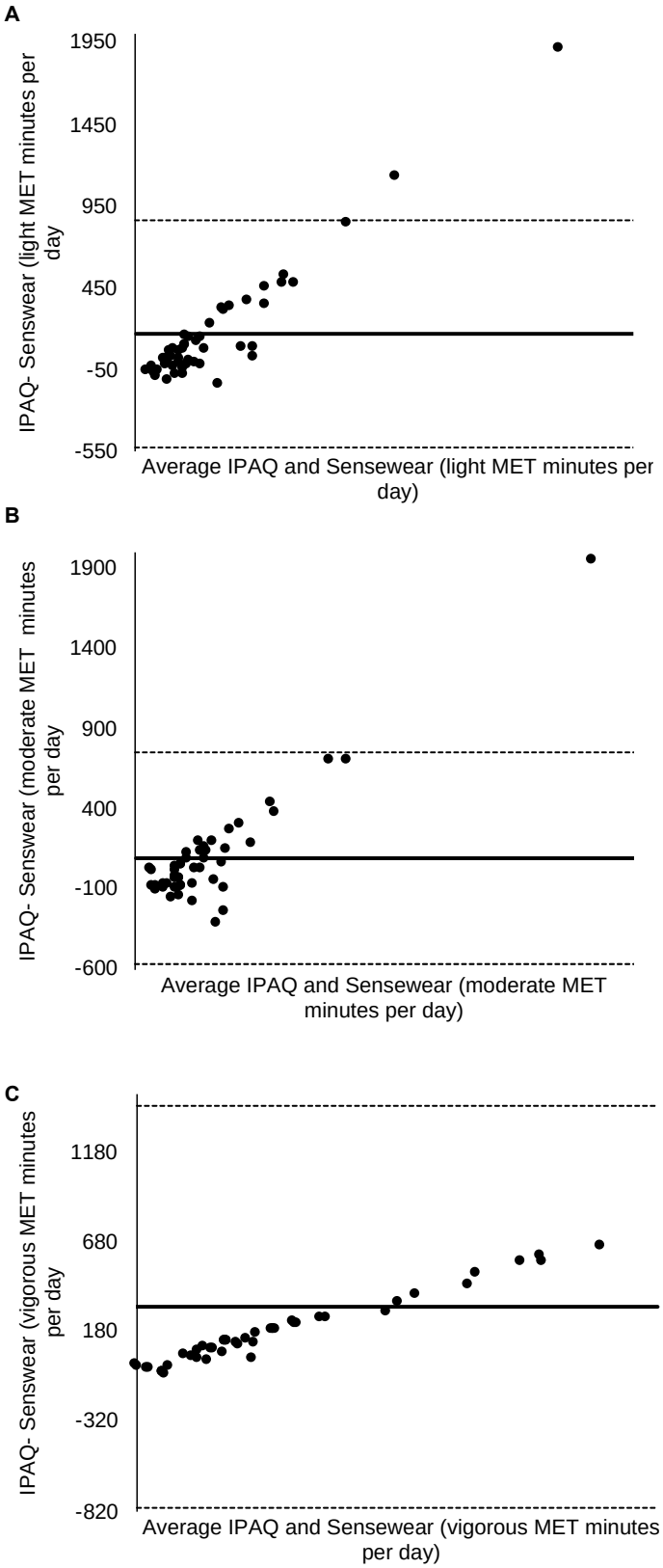

Figure 8. Bland-Altman plot between two measures of physical activity; Self-reported IPAQ and Sensewear armband for A. Light activity per day, B. Moderate activity per day and C. Vigorous activity per day.

IPAQ; international physical activity questionnaire. MET; metabolic equivalent.

No significant differences were observed between responders and non-responders regarding nutritional intake (Table 6.). Intake of macronutrients (carbohydrates, proteins and fats), were consistent across groups. Responders reported a 1.3 ± 0.8 g.kg.day^{-1} sugar intake per day versus 1.2 ± 0.6 g.kg.day^{-1} in non-responders and 0.4 ± 0.2 g.kg.day^{-1} and 0.5 ± 0.4 g.kg.day^{-1} saturated fat intake. Overall responders reported a total of energy intake of 1933 ± 482kcal per day versus 1689 ± 530kcal per day in non-responders. No significant differences were observed in reported micronutrient intake per day between groups.

No significant differences were seen between depression, anxiety or stress between responders and non-responders (Table 7.) No significant differences were seen between groups in terms of thresholds for depression anxiety and stress (X_2 (2) = 0.557, p=0.757, X_2(2) = 726, p=0.696 and X_2(2) = 533, p=0.766 respectively). In responders 39 participants had normal scores of depression and anxiety (thresholds 0-9 and 0-7 respectively), 3 participants had moderate scores for depression and anxiety (10-13 and 8-9 respectively) and 1 participant had severe depression and anxiety (21-27 and 15-19 respectively). In terms of stress, in responders 41 participants recorded normal levels (0-14), 1 participant recorded mild (15-18) and two participants scored moderate (19-25). All non-responders recorded normal scores for anxiety and stress and one non-responder recorded a moderate depression score with all other participants reporting normal scores.

Table 6. Nutritional intake of women who responded to at least one tumour-associated antigen and those negative to all tumour associated antigens

Nutrition intake	Responder to tumour-associated antigens (n=43)	Non-responder to tumour-associated antigens (n=7)	Difference	ANOVA statistic
Energy Intake (kcal)	1933 ± 482	1689 ± 530	244	F(1,48)=1.501, p=0.227
Carbohydrate (g.kg.day^{-1})	3.1 ± 1.2	3.0 ± 13	0.1	F(1,48)=0.090, p=0.765
Protein (g.kg.day^{-1})	1.2 ± 0.5	1.1 ± 0.4	0.1	F(1,48)=0.226, p=0.637
Fat (g.kg.day^{-1})	1.2 ± 0.4	1.1 ± 1.7	0.1	F(1,48)=0.259, p=0.613
Sugars (g.kg.day^{-1})	1.3 ± 0.8	1.2 ± 0.6	0	F(1,48)=0.015, p=0.903
Saturated fat (g.kg.day^{-1})	0.4 ± 0.2	0.5 ± 0.4	- 0.1	F(1,48)=1.001, p=0.322
Vitamin A (mg.day^{-1})	1022 ± 622	1006 ± 600	16	F(1,48)=0.004, p=0.951
Vitamin C (mg.day^{-1})	112 ± 102	114 ± 46	-2	F(1,48)=0.002, p=0.966
Vitamin D (mg.day^{-1})	5 ± 4	2 ± 1	3	F(1,48)=3.124, p=0.083
Vitamin E (mg.day^{-1})	11 ± 10	7 ± 2	4	F(1,48)=0.948, p=0.335
Calcium (mg.day^{-1})	788 ± 412	591 ± 174	196	F(1,48)=1.522, p=0.223
Sodium (mg.day^{-1})	2139 ± 1230	1810 ± 713	330	F(1,48)=0.472, p=0.496
Iron (mg.day^{-1})	11 ± 7	10 ± 11	0	F(1,48)=0.027, p=0.870
Zinc (mg.day^{-1})	11 ± 2	7 ± 2	3	F(1,48)=0.606, p=0.440

*N=50, * P<0.05.* Data shown as means ± SD

g; grams. kg; kilograms. kcal; kilocalorie.

Table 7. Psychological measures of women who responded to at least one tumour-associated antigen and those negative to all tumour associated antigens

Psychological measures	**Responder to tumour-associated antigens (n=43)**	**Non-responder to tumour-associated antigens (n=7)**	**Difference**	**ANOVA statistic**
Depression	3 ± 6	4 ± 5	-1	F(1,47)=0.056, p=0.813
Anxiety	2 ± 3	1 ± 1	1	F(1,47)=0.450, p=0.506
Stress	7 ± 6	6 ± 5	1	F(1,47)=0.135, p=0.736

*N=50, * P<0.05.* Data shown as means ± SD

44% of responders were also CMV seropositive compared with 14% of non-responders. Significant differences were found between groups when assessing specific IgG antibody response to VZV which was 56% lower in the non-responders (687 ± 542IU/ml) vs responders (1558 ± 951IU/ml), $p<0.05$, effect size 0.3 (Table 8.). Regarding haematological measures platelet counts were significantly lower ($F(1,48)= 4.693$, $p=0.035$, effect size 0.3) in non-responders (mean of 182 ± 72 vs 120 ± 50 x 10^9/L respectively for responders and non-responders). No other significant differences were found regarding cell counts (Table 10.). Osteopontin was significantly higher in responders compared with non-responders (average of 25.16 ± 8.88 ng/ml vs 17.08 ± 2.76 ng/ml). No differences in levels of resistin, leptin and RANTES were observed ($p > 0.05$) (Table 8.).

Table 8. Haematological and anti-viral characteristics of women who responded to at least one tumour associated antigens and those negative to all tumour associated antigens

Characteristic	**Responders (n=43)**	**Non-responders (n=7)**	**Difference**	**ANOVA statistic**
Total leukocytes (x 10_9/L)	5.3 ± 1.6	4.9 ± 0.6	0.4	F(1,48)=0.445, p=0.508
Platelets (x 10_9/L)	182 ± 72	120 ± 50	-62	F(1,48)=4.693, p=0.035*
Lymphocytes (x 10_9/L)	1.6 ± 0.5	1.5 ± 0.4	0.1	F(1,48)=0.201, p=0.656
Monocytes# (x 10_9/L)	0.4 ± 0.2	0.3 ± 0.1	0.1	F(1,48)=2.439, p=0.125
Neutrophils (x 10_9/L)	3.3 ± 1.3	3.1 ± 0.7	0.2	F(1,48)=0.159, p=0.692
EBV positive (number, %)	41/43, 95%	7/7, 100%	-5%	F(1,48)=0.504, p=0.481
EBV specific IgG (IU/ml)	436 ± 230	436 ± 256	0	F(1,45)=0.057, p=0.813
CMV positive (number, %)	19/43, 44%	1/7, 14%	30%	F(1,48)=0.585, p=0.448
CMV specific IgG (IU/ml)	14 ± 7	13 ± 5	1	F(1,19)=0.053, p=0.820
VZV positive (number, %)	41/43, 95%	6/7, 86%	9%	F(1,48)=2.254, p=0.140
VZV specific IgG (IU/ml)	1558 ± 951	687 ± 542	869	F(1,46)=4.748, p=0.034*
RANTES (ng/ml)	10.96 ± 5.91	11.79 ± 5.29	-0.83	F(1,48)=0.122, p,0.728
Resistin (ng/ml)	1.01 ± 0.49	0.90 ± 0.25	0.11	F(1,48)=0.483, p=0.491
Leptin (ng/ml)	15.29 ± 14.58	9.76 ± 6.03	5.53	F(1,48=0.312, p=0.579
Osteopontin (ng/ml)	25.16 ± 8.88	17.08 ± 2.76	8.12	F(1,48)=6.221, p=0.016*

*N=50, * P<0.05,* data shown as means ± SD #*Monocytes, eosinophils and basophils, however eosinophils and basophils only make up a small fraction (0.1-1.6 X 10_9/L according to manufacturer's guidelines,* Sysmex, KX-21N, Kobe, Japan*). WBC, white blood cell. EBV, Epstein Barr Virus. EBNA1, Epstein Barr nuclear antigen 1. BZLF1, BamHIZ leftward reading frame 1. MP1, matrix protein1. NP, nucleoprotein. CMV, Cytomegalovirus. pp65, phosphoprotein 65. IE1, immediate-early protein 1. VZV, Varicella Zoster Virus. IE63, immediate-early protein 63. gE, glycoprotein E.*

3.4.5 Correlations in lifestyle and psychological characteristics and T-lymphocyte response to tumour-associated antigens within responders only

A significant negative correlation was observed between stress and the average magnitude of T-lymphocyte response to tumour-associated antigens was observed (p=0.020) whereby as stress increased, magnitude of T-lymphocyte response decreased (Table 9). A significant correlation was seen in those who were EBV positive in terms of EBV IgG levels and the number of tumour-associated antigens positive towards. No other significant differences were observed (Table 9).

Table 9. Correlations between variables and number positive responses towards 10 tumour-associated antigens and correlations between variables and the magnitude of T-lymphocyte response to tumour-associated antigens

Variables	**Correlation with number of tumour-associated antigens responded to**	**Correlation with average T-lymphocyte response to tumour-associated antigens (SFUs/ 250,000 cells)**
Age (years)	r_s=0.107, p=0.494	r_s=0.187, p=0.229
Predicted $\dot{V}O_2$ Max (ml.kg.min^{-1})	r_s= –0.100, p=0.524	r_s= –0.177, p=0.256
Systolic Blood Pressure (mmHg)	r_s= –0.075, p=0.635	r_s= –0.004, p=0.978
Diastolic Blood Pressure (mmHg)	r_s=0.017, p=0.911	r_s=0.026, p=0.870
BMI (kg.m$_2$)	r_s=0.190, p=0.223	r_s=0.043, p=0.783
W:H	r_s=0.138, p=0.372	r_s=0.050, p=0.749
Body fat (%)	r_s= –0.076, p=0.628	r_s=0.101, p=0.518
FMI (kg.m$_2$)	r_s= –0.010, p=0.947	r_s=0.121, p=0.439
BMD (g.cm$_2$)	r_s= –0.058, p=0.710	r_s= –0.108, p=0.489
T-score	r_s= –0.055, p=0.725	r_s= –0.113, p=0.471
Z-score	r_s= –0.025, p=0.872	r_s= –0.100, p=0.522
Depression	r_s=0.004, p=0.979	r_s= –0.231, p=0.141
Anxiety	r_s=0.075, p=0.635	r_s= –0.273, p=0.130
Stress	r_s= –0.197, p=0.211	r_s= –0.358, p=0.020*
PAL	r_s=0.094, p=0.560	r_s= –0.012, p=0.942
Energy Expenditure (kcal per day)	r_s= 0.185, p=0.246	r_s= –0.057, p=0.726
Sedentary time (% per day)	r_s= –0.190, p=0.223	r_s= –0.058, p=0.717
Energy Intake (kcal per day)	r_s=0.136, p=0.384	r_s= –0.062, p=0.694
EBV IgG (IU/ml)	r_s=0.464, p=0.003*	r_s= –0.232, p=0.150
CMV IgG (IU/ml)	r_s= –0.410, p=0.081	r_s= –0.214, p=0.380
VZV IgG (IU/ml)	r_s=0.028 p=0.862	r_s= –0.001, p=0.994
RANTES (ng/ml)	r_s=0.151, p=0.333	r_s= 0.249, p=0.107
Resistin (ng/ml)	r_s=0.104 p=0.512	r_s= –0.105, p=0.506
Leptin (ng/ml)	r_s=–0.086, p=0.585	r_s= 0.042, p=0.788
Osteopontin (ng/ml)	r_s=–0.066, p=0.674	r_s= 0.004, p=0.978

Total N=43, positive responses only. EBV, CMV and VZV IgG only measured in those who were seropositive. ml; millilitre. kg; kilogram. min; minute. mmHg; millimetres of mercury. m; metres. g; grams. cm; centimetres. kcal; kilocalorie. IU; international units. ng; nanograms.

3.4 DISCUSSION

The aim of this study was to examine whether T-lymphocyte responses can be detected in healthy women when stimulated by tumour-associated antigens, and to explore potential relationships with aspects of lifestyle. This study reports that T-lymphocyte responses can be detected in healthy women with no current malignant disease, whereby immune responses to at least one of ten tumour-associated antigens assessed were present in 43/50 (86%) healthy women.

Previous studies focus on the immune response of T-lymphocytes in cancer patients rather than healthy women with no known presence of disease, however, some studies have used blood from healthy donors to act as controls and the data from these donors is comparable to the data of the current study. Evidence exists with varying relevance to support the finding that all the tumour-associated antigens used within this study can generate an immune response in healthy individuals. MMP11 has previously induced a humoral immune response (Roscilli *et al.*, 2014a). A study demonstrated purified CD8+ T-lymphocytes from 4 HLA-A*0201 positive donors responded to MMP11 peptide, MPP-4, producing ~10 spots per 20,000 CD8+ cells (relative to 500 SFUs per million PBMCs) (Al Qudaihi *et al.*, 2010), much higher than the average count of 8 ± 12 per million in the current study. However, in the current study, antigens stimulated a PBMC mix rather than CD8+ T-lymphocytes alone thus we cannot confirm that the 250,000 cells are all CD8+ T-lymphocytes unlike previous work. On top of this, previously, cells were stimulated with the peptide 3-8 times in vitro for 7-10 days, rather than stimulated once and cultured overnight. Repeated exposure to the peptide may elicit increased responses and allow both naïve and memory T-lymphocyte responses to occur, explaining the larger response generated (Chudley *et al.*, 2014).

Previously, MUC1 specific T-lymphocytes have been detected in healthy individuals but only in ~10% of the cohort (n=32) (Bonertz *et al.*, 2009). In the current study we reported that 44% of women had MUC1 specific T-lymphocytes. Differing methods for determining a true response may explain differences, as in the previous work responses were defined by significantly increased spot numbers in triplicate wells of test antigen relative to negative control antigen, a different strategy compared to the current study. Healthy donor cells have also been shown to induce CD8+ T-lymphocyte responses in vitro in response to SUR (Schmidt *et al.*, 2003), mirrored in the current study with 38% of women responding towards the antigen.

Previous research regarding TARP shows that when dendritic cells are pulsed with TARP peptides $TARP_{1-14}$ and $TARP_{14-27}$, they are able to stimulate CD4+ T-lymphocytes in healthy volunteers (Kobayashi *et al.*, 2005). In the current study, T-lymphocytes from 23 women responded to TARP. Intracellular and extracellular domain of ERB (ERB ICD and ERB ECD respectively) was used in the current study, which is often combined in the form of HER2. One study demonstrated that healthy participants do not possess T-lymphocytes able to respond to HER2 stimulation, disagreeing with our finding that 46% and 50% women respond to ERB ICD and ERB ECD respectively (Nagorsen *et al.*, 2000). Discrepancies may be due to differences in number of cells within the ELISpot well (167,000 vs 250,000), however a strong response was seen towards the positive control so it may instead be due to the low sample size in the previous work which did not capture individuals who possessed specific T-lymphocytes. Other research agrees with our finding that healthy donors can elicit T-lymphocyte responses towards HER2 antigens. Anti-HER2 CD4+ responses have been generated after 24h-36h ELISpot in healthy donors at a magnitude of average of 260 ± 24 SFUs per million cells (Datta *et al.*, 2015). This is much higher than the average SFU seen in the current study for both ERB ICD and ERB ECD. Differences may be due to the use of CD4+ T-lymphocytes alone in the previous research. Interestingly, this is the only study found that reported any characteristics of healthy donors and reported no differences in anti-HER2 CD4+ responses in those over and under 50 years old (Datta *et al.*, 2015).

In the current study CycB1 elicited T-lymphocyte specific responses in 48% of participants. Previously it has been reported anti-CycB1-specific IgG antibody and memory CD4+ and CD8+ T-lymphocytes were found in young and old healthy individuals, aged 25-79 years old, who have no history of cancer (Vella *et al.*, 2009). The average age of women in our study was 43 years old, ranging from 25-69 years old, and positive responses were seen across the age range. Another study that measured healthy donor response to CycB1 peptide CB204 found 10/11 donors had a response, with the average number of SFUs 25 per 100,000 PBMCs, much higher than those observed in the current study which is likely due to the 7-8 days culture of the ELISpot compared to only a 24h incubation in our study (Andersen *et al.*, 2011).

CEA and MamA elicited the highest proportion of immune responses in the current cohort with 52% of participants responding towards them. However, previous research on CEA has shown that the majority of donors (9/11) had less than 1 SFU per 200,000 cells (Arlen *et al.*, 2000). A lack of T-lymphocytes secreting IFN-γ was also shown in eight HLA-A2 positive after a 24h ELISpot (Nagorsen *et al.*, 2000). Finally, CD3+ response to MamA after

a 48h incubation showed an average of 3 ± 2 SFUs per million cells for CD8+ and 2 ± 2 SFUs for CD4+ in 6 healthy females (Jaramillo *et al.*, 2002). Whist results from the present study combine total PBMC response, results are similar with an average of 16 ± 8 SFUs per million PBMCs. No literature was available regarding CD3+ cells from healthy individuals responding to CI6.

The ELISpot technique quantifies the number of IFN-γ producing T-lymphocytes on a single cell basis in response to stimulation from the antigen. Measuring single cell IFN-γ secretion is a way to measure T-lymphocyte function as IFN-γ is often used to track CD8 T-lymphocyte responses (Slota *et al.*, 2011; Lalvani *et al.*, 1997; Herr *et al.*, 1998). Individuals that demonstrate anti-tumour-associated antigen T-lymphocytes responses must either possess specific naïve T-lymphocytes, or, have come across the antigen before via malignant cells or other events (e.g. infection or inflammation) and therefore possess memory T-lymphocytes specific for the presented tumour-associated antigens (Klebanoff *et al.*, 2006). Having such responses can be down to luck, or a number of other factors, both modifiable or not (Pennock *et al.*, 2013a; Gerlach *et al.*, 2010; Chang *et al.*, 2014).

In the current study it is unknown whether these responses are from naïve or memory T-lymphocytes. As the incubation in the current study is an overnight culture, positive responses are likely to represent memory rather than naïve T-lymphocyte responses due to reduced time and exposure for naïve cells, which must first undergo proliferation and differentiation before they can express cytokines (Berard and Tough, 2002; Whitmire *et al.*, 2008; Sallusto *et al.*, 2004; Matesic *et al.*, 1998). The magnitude of the responses within wells containing CMV, EBV and VZV peptides, whereby it is known individuals possess protective memory responses, confirm it is likely that responses represent memory T-lymphocytes responses (Gourley *et al.*, 2004). This is supported by vaccine evidence whereby an increased number of SFUs is observed after HER2/neu vaccination in breast cancer patients (Disis *et al.*, 2009; Knutson *et al.*, 2001), alongside the successful use of monoclonal antibody therapies (e.g. Trastuzumab) and adoptive T-cell therapies (Ahmed *et al.*, 2015; Slamon *et al.*, 2001; Piccart-Gebhart *et al.*, 2005; Denkert *et al.*, 2015b).

The potential significance of having anti-tumour immune responses before the onset of cancer is speculative. A positive response to tumour-associated antigens may be protective in terms of breast cancer risk, with a larger response conferring decreased risk of disease. This approach is supported by literature that reports better prognosis in breast cancer patients who possess higher immune responses to tumour-associated antigens when

stimulated via ELISPOT, flow cytometry, and cytotoxicity assays (Tiriveedhi *et al.*, 2014; Muraro *et al.*, 2015a; Blixt *et al.*, 2011; Muraro *et al.*, 2015c; Tiriveedhi *et al.*, 2013). However, it is unknown how patient responses compare to healthy counterparts and whether their response to tumour-associated antigens may in fact be involved in the development of cancer. Furthermore, one of the hallmarks of cancer is the ability of cancer cells to avoid immune destruction whereby an immune response towards cancer cells results in the inability for the cancer cells to survive (Hanahan and Weinberg, 2011). Therefore a memory T-lymphocyte response towards such antigens may thus confer protection (Hanahan and Weinberg, 2011; Klebanoff *et al.*, 2006; Reading *et al.*, 2018; Mami-Chouaib *et al.*, 2018).

The immunogenicity of tumour-associated antigens may relate to whether the antigen was abnormally expressed or an overexpressed self-antigen (Cheever *et al.*, 2009). After differentiating from a haematopoietic stem cell in bone marrow, T-lymphocytes that are exposed to self-proteins are stimulated to undergo apoptosis in the thymus whereby, in theory, no naïve T-lymphocytes and subsequent memory T-lymphocytes should be present towards the self-antigen (Dzhagalov *et al.*, 2013). This suggests stronger immune responses and an increased number of individuals producing responses to non- or altered self-antigens, compared to overexpressed self-antigens. The over-expressed self-antigen, survivin, was one of the least immunodominant antigens in the current study, demonstrating this may be the case. In the current study MamA was the most immunodominant antigen. MamA is highly expressed in 40-80% breast cancers but is generally absent within normal tissues (Fleming and Watson, 2000b; Kundu *et al.*, 1996) and is proven to be highly immunogenic, generating both CD4+ and CD8+ recognition (Tiriveedhi *et al.*, 2013; Jaramillo *et al.*, 2002; Kim *et al.*, 2016; Fleming and Watson, 2000b). As MamA is expressed at very low levels in normal tissues and is often a target for immunotherapy and vaccination, the immunodominance in the current study is not surprising (Al Joudi, 2014; Tiriveedhi *et al.*, 2013).

CEA was as immunodominant as MamA. CEA is only expressed in tumours and in the testes, therefore tolerance and autoimmunity are not much of an obstacle when looking at CEA, explaining the higher immunogenicity. Furthermore, recent cancer treatment such as vaccination or immunotherapy stimulate cellular anti-tumour immune responses by expansion of CD8+ T-lymphocytes capable of destroying tumour cells expressing CEA (Parmiani *et al.*, 2007; Curigliano *et al.*, 2009; Schaefer *et al.*, 2006; Acres *et al.*, 2007).

ERB ECD was the third most immunodominant following MamA and CEA. The ErbB-2 gene is one of the most widely overexpressed genes in breast cancer, with the HER2 tumour-associated proteins being expressed in 10% to 30% of human primary breast cancers (Slamon *et al.*, 1989) due to increased transcription and gene amplification (Coulie *et al.*, 2014). This, alongside the knowledge that HER2 is targeted by immunotherapeutic interventions (Denkert *et al.*, 2015b), supports the heightened immunogenicity of this antigen compared to others (Banchereau and Palucka, 2005). Surprisingly, MUC1, which is expressed in 90% of all cancers (Chen *et al.*, 2016), and has proven immunogenicity (Bullo *et al.*, 2003; Kimura and Finn, 2013; Finn, 2008; Yuan *et al.*, 2010), only elicited immune responses within 44% of women in the present study. A likely reason for smaller numbers of women demonstrating T-lymphocyte responses towards this particular antigen may be that it is an overexpressed self-antigen and in some people MUC1 specific T-lymphocytes may have undergone apoptosis in the thymus during development (Dzhagalov *et al.*, 2013).

T-lymphocyte responses to CMV, EBV and VZV peptides, on average, were much higher than responses to tumour-associated antigens. Mean response to tumour-associated antigens ranged from 2-6 SFUs/250,000 cells compared to the average response to FLU MP1; 6 ± 7 SFUs, FLU NP; 18 ± 57 SFUs. An even larger average response was seen after stimulation with EBV peptides, EBNA1 (17 ± 27 SFUs) and BZLF1 (35 ± 81 SFUs) and CMV peptides pp65 (182 ± 137 SFUs) and IE1 (182 ± 137 SFUs). This is likely due to exposure to viruses at a young age and the effect of cumulative re-exposure throughout life, causing repeated reactivation of specific T-lymphocytes and further differentiation of specific memory cells (Long *et al.*, 2019; Steain *et al.*, 2014; Spitaels *et al.*, 2016). Such repeated activation of tumour-associated antigens may not have occurred in participants of the current study as they lack a cancer diagnosis, explaining why on average SFUs are smaller in response to tumour-associated antigens. However, 8 individuals had a response >20 SFUs per 250,000 PBMCs towards a specific tumour-associated antigens which may suggest that in these individuals they have had an increased amount of repeated exposure to this antigen (Slota *et al.*, 2011).

Measuring T-lymphocyte responses to viral antigens offers a way of assessing cell-mediated immunity. Previously in heart transplant patients, impaired CMV pp65-specific immune responses have been defined as <50 SFUs/200,000 cells (Moss and Khan, 2004; Adler *et al.*, 1995; Abate *et al.*, 2012). In the present study, when assessed per 200,000 cell basis, 5/21 CMV seropositive individuals had < 50 SFUs in response to pp65, 4 of whom

were responders to tumour-associated antigens. It was also suggested that SFU of >100 per 200,000 cells was defined as a high response (Moss and Khan, 2004; Adler *et al.*, 1995; Abate *et al.*, 2012). 12/21 participants in the current study demonstrated such responses to CMV pp65. Research in kidney transplant patients used different thresholds of <30 spots per 200,000 cells in response to pp65 and <10 spots per 200,000 cells in response to IE1 (Lee *et al.*, 2017b). Only four participants in our study had <30 SFUs per 200,000 cells towards pp65 and only two participants had <10 SFUs in response to IE1. The lower number of SFUs in response to IE1 versus pp65 was also observed in this study, with, on average, 53 more SFUs in response to CMV antigen pp65 compared with IE1. A study that assessed T-lymphocyte response in healthy individuals also demonstrated increased activation on stimulation with pp65 (median 399 SFUs per 200,000 PBMC (range 12–864 SFUs per 200,000 PBMC) versus IE1 median of 26 SFUs per 200,000 PBMC (range 1-96 SFUs per 200,000 PBMC) (Barabas *et al.*, 2017) comparable to our results. The differences in response to the two peptides pp65 and IE1 may be explained by immune evasion mechanisms of IE1 (Gilbert *et al.*, 1993), due to reduced presentation of the antigen due to its stability, size and nuclear reactivity (Scheller *et al.*, 2008; Delmas *et al.*, 2005).

Decreased EBV-specific immunity measured by EBV DNA has also been observed in individuals who had undergone cell transplantation compared with healthy donors (Baldanti *et al.*, 2011; Baldanti *et al.*, 2008; Macedo *et al.*, 2005), suggesting a lower EBV specific immunity has negative health consequences. When CD8+ T-lymphocyte responses in healthy donors have previously been elicited with stimulation from EBV peptides, an average of 58 ± 70 SFUs per 100,000 and 27 ± 15 SFUs per 100,000 cells were seen in response to EBV antigens BMLF1 and EBNA3 respectively (Macedo *et al.*, 2005). Response on a per 100,000 cell basis, in the current study were lower, for EBNA average SFUs were 6 ± 10 and for BZLF1 were 14 ± 33. These differences may be due to the use of different antigens but, as demonstrated by the large SDs, is that within a cohort, responses have large variability. Another study demonstrated that the median number of net spots per million PBMCs in response to BZLF was 1075 and for EBNA1 was 1375 (Calarota *et al.*, 2013). In our study the median and mean number of spots was much lower for both peptides (mean 67 ± 108 SFUs per million PBMCs, median 30 SFUs per million PBMCs for EBNA1 and mean 140 ± 325 SFUs per million PBMCs, median 28 SFUs per million PBMCs for BZLF1). In a study with a similar sample size of 50 (median age 44 years), 92% of participants were seropositive for EBV like the overall 98% seropositive in the current study. In these participants median EBNA specific T-lymphocyte response was median 890 spots per million PBMCs, again much higher than observed in our study

(Cassaniti *et al.*, 2019).

The beneficial effect of an increased number of viral specific T-lymphocytes is demonstrated by increased SFUs produced in response to VZV after vaccination (Smith *et al.*, 2003). A study has shown that response to VZV pre-vaccination was on average, 142 SFUs per million cells, which is much higher than observed in the current study, perhaps again due to the use of differing stimulants. The median spot count previously seen in 151 healthy donors was 88 SFUs per 400,000 PBMCs compared to 4 ± 45 SFUs per 400,000 PBMCs and 4 ± 36 SFUs per 400,000 PBMCs in response to IE1 and gE antigens respectively (Tyring *et al.*, 2012). The likely reasons for our results showing much lower numbers of spots compared to previous work is the use of live VZV virus to stimulate cells in previous studies.

As far as the authors are aware this is the first study to investigate links between lifestyle measurements (such as fitness and body composition) and immune response to tumour-associated antigens. Previously, literature has shown improvements in immune responses with physical stress such as exercise (Dhabhar *et al.*, 2012; Dhabhar and Viswanathan, 2005; Edwards and Booy, 2013; Rosenberg *et al.*, 1988; Silberman *et al.*, 2003). In the current study it was found that there were no differences in number of tumour-associated antigens responded to between those who were fitter and those who were less fit. On top of this, no differences were seen in measures of physical activity between individuals who did and did not respond to tumour-associated antigens. Furthermore, no differences were seen in average predicted $\dot{V}O_2$ max in those who did and did not respond to any of the tumour-associated antigens rather than post-exercise. Evidence suggests T-lymphocyte function and mobilisation is improved during and immediately post-exercise, when T-lymphocytes capable of IFN-γ production, are resident in the peripheral blood, migrating towards tissues. Therefore, as the sample was taken at rest, it is possible that T-lymphocytes are resident in tissues, rather than blood. Secondly the lack of differences in T-lymphocyte response to tumour-associated antigens in participants with differing lifestyle characteristics may be due to the relatively low average age of participants in the current study. It has been suggested that an active lifestyle (repeated bouts of exercise which therefore cause increases in cardiorespiratory fitness) may be able to delay immunosenescence (Campbell and Turner, 2018), hence fitness may play a more important role in T-lymphocyte function in older adults. A lack of a relationship between immune function and fitness in the current study could also be down to limitations in measurements,

such as the use of a submaximal exercise test to predict fitness, rather than an exercise test to volitional exhaustion (Wicks and Oldridge, 2016).

In the present study the only aspects of lifestyle characteristics that differed significantly between individuals that did and did not respond to tumour-associated antigens were BMD and T-score. There are many reasons why other aspects of lifestyle were not different between groups. There are several more robust breast cancer risk factors that may be more influential over the immune response to tumour-associated antigens than lifestyle factors. Such risk factors which were not assessed include genetic mutations, responsible for 5-10% of all breast cancers (Claus *et al.*, 1996). These are much more prevalent risk factors compared to inadequate exercise and obesity which are reportedly accountable for only 1 and 8% of breast cancers respectively (Parkin, 2011; Brown *et al.*, 2018). However, menopausal status, pregnancy, age of first menstruation (Brinton *et al.*, 1988) and age of first full-term pregnancy (Eliassen *et al.*, 2006; Pike *et al.*, 1979) were measured within the current study, but, due to the low response rate (5/7) in the non-responders group, it is difficult to determine whether these have an effect. In those that this was measured in, no significant differences were seen in any of these established risk factors between responders and non-responders. Interestingly, when adding age of menarche, menopausal status, previous pregnancy and age of first pregnancy as covariates, the significant difference in BMD and T-score between groups is no longer present (data not shown). This relationship was not affected by age of menopause, but this may be due to the reduced number of women eligible for this assessment (n=14).

In the current study it was observed that BMD was significant higher in non-responders compared to responders by around 10% and T-score (a comparison of a person's bone density with that of a healthy 30-year-old of the same sex) by 351%. Osteoporosis occurs when the rate of osteoclastic bone breakdown exceeds that of osteoblastic bone formation (Weitzmann and Ofotokun, 2016). The adaptive immune system has previously been proven to have an impact on regulating bone health, a term coined ‘osteoimmunology’ (Greenblatt and Shim, 2013). Activated T-lymphocytes affect bone health through inflammatory cytokine secretion, such as TNF-α, which promotes bone destruction demonstrated in people with inflammatory conditions (Srivastava *et al.*, 2018; Cenci *et al.*, 2000; Kong *et al.*, 1999; Kawai *et al.*, 2011). This is further shown in T-lymphocyte deficient mice who have normal or elevated BMD (Andersen *et al.*, 2007; Cenci *et al.*, 2000).

BMD was significantly higher in non-responders to tumour-associated antigens whilst osteopontin was significantly lower in this group, supporting previous research that showed serum osteopontin levels were higher in individuals with osteoporosis ($p < 0.001$) (Wei *et al.*, 2016b). As osteopontin is expressed by T-lymphocytes (Murry *et al.*, 1994; Kunii *et al.*, 2009; Kruger *et al.*, 2014; Shurin, 2018) and has been implicated in inflammation, tumour progression, and metastasis (Zhao *et al.*, 2018; Rangaswami *et al.*, 2006; Castello *et al.*, 2017), this finding demonstrates that serum levels of osteopontin may reduce T-lymphocyte activity in response to tumour-associated antigens. However, it has been suggested that osteopontin levels at the higher ranges may promote tumourgenesis (Cook *et al.*, 2005; Bandopadhyay *et al.*, 2014) so it is difficult to say at this stage whether high or low levels are more beneficial and whether there is a link to T-lymphocyte response to tumour-associated antigens.

Another aspect of lifestyle measured in this study was dietary intake. Evidence has previously shown that nutritional intake can modulate immune function (Moreira *et al.*, 2007). Whilst certain nutrients may help improve immune functions, other nutrients in excess may reduce immune function (Boynton *et al.*, 2007). In the current study no differences were seen in terms of nutrient intake in women who responded to tumour-associated antigens and those who did not respond to tumour-associated antigens. The likely reason for this is for inaccuracies and bias linked with self-reported measures of diet. Furthermore, rather than nutrient intake itself being responsible for improvements in immune function, it may instead be the contribution of a healthy diet on body weight.

In terms of total leukocyte counts, no differences were seen in total leukocyte or leukocyte subset counts and T-lymphocyte response to tumour-associated antigens. As a set number of T-lymphocytes are stimulated to respond to each antigen, this is expected. However, what may have affected the response is the phenotype of T-lymphocytes within each well (predominance of memory or naïve T-lymphocyte), which was not measured in the current study. One finding of the present study is that platelet counts were significantly lower in non-responders (mean of 181 vs 120 x 10^9/L respectively for responders and non-responders). Alongside the innate immune system, platelets act as one of the immune system's first responders (Morrell *et al.*, 2014). However, continued platelet activation can lead to adverse effects from excessive immune stimulation and inflammation. Platelets influence adaptive immunity via recruitment and activation of dendritic cells, increasing activation (Langer *et al.*, 2007) expression of T-lymphocyte costimulatory molecules CD80 and CD86, which leads to a stronger and more rapid T-lymphocyte response. This may explain why in

participants who did not elicit T-lymphocyte responses to tumour-associated antigens platelet counts were lower compared to responding counterparts. However normal platelet counts range from 100,000-450,000 platelets per ml of blood, so all individuals in both groups are well within the normal range.

Significant differences were found between responders and non-responders when assessing specific IgG antibody response to VZV, which was 56% lower in the non-responders vs responders. VZV specific IgG antibody protects the host from VZV infection, thus re-exposure to the virus can lead to increased IgG levels. Whilst ageing causes a decline in VZV specific immunity this is likely due to the decline in T-lymphocyte function rather than a reduction in antibodies (Miller, 1980; Berger *et al.*, 1981; Burke *et al.*, 1982; Levin *et al.*, 1992; Levin *et al.*, 2003). Thus, an increased IgG may be suggestive of a more robust immune response which can be maintained throughout the lifespan (Arvin *et al.*, 1983). This suggests a stronger immune response towards VZV in responders, or may also suggest recent reactivation of the virus (De Paschale and Clerici, 2012). It was also demonstrated that there was a significant positive correlation between EBV IgG and the number of tumour-associated antigens responders were positive towards, suggesting a stronger immune response may be related to improved ability to T-lymphocytes to recognise and respond to tumour-associated antigens.

Finally, no significant differences were found in terms of leptin, resistin and RANTES. Previous literature has linked RANTES to CD8+ T-lymphocyte function however activation is dependent on self-aggregation of RANTES and other leukocyte populations such as monocytes and neutrophils, which may explain why no differences were seen in the current study. Leptin and resistin are both associated with T-lymphocyte function and it is well established that they are increased with increasing adiposity (Sarmento-Cabral *et al.*, 2017). Previously increased leptin levels have been found to affect naïve T-lymphocytes but not memory T-lymphocytes (Lord *et al.*, 1998; Lord *et al.*, 2002). As the response measured in the current study is likely a memory T-lymphocyte response, this is a good explanation as to why no differences were seen in T-lymphocyte function with differing leptin levels. Resistin has been shown to induce the expansion of T-reg cells, turning off an immune response, suggesting that with increasing resistin (and obesity), T-lymphocyte response may be reduced (Son *et al.*, 2010). Furthermore, leptin and resistin levels may not have affected T-lymphocyte function in the current study due to the lack of extreme body compositional values (obese and morbidly obese) in the population sampled.

Consideration should be taken when interpreting the results of the current study as only 7/50 women were defined as non-responders compared to 43/50 women defined as responders, causing an imbalance between the two groups and the potential for extreme individual values in non-responders to influence the mean. A limitation of the current study is that it is unknown whether the IFN-γ production is from CD4+ or CD8+ T-lymphocytes. Previously, it has been suggested that antitumor immune responses are predominantly via CD8+ cytotoxic T-lymphocytes and tumour growth is regulated by CD8+ lymphocytes within the tumour microenvironment (Chen *et al.*, 2016; de La Cruz-Merino *et al.*, 2017; Zhang and Chen, 2018). However, response to viral antigens has previously been shown to be dependent on CD4+ (Smith *et al.*, 2001). Another limitation of the current study is that blood samples were taken from peripheral blood which may represents a very different immune compartment than that within the lymph and tissues, where antigen exposure to T-lymphocytes is likely to take place (Peeters *et al.*, 2011). A blood sample on another day from the same individual may contain, by chance, different cells, and therefore demonstrate a different immune response. Finally, in terms of lifestyle measurements, the range of women included did not represent or include many women at the extreme ends of the population, only 5/50 participants (10%) with a BMI in the obese category (>30kg.m$_2$), 2/50 participants (4%) with a predicted VO_2max within the 10$_{th}$ percentile based on age and only 4/50 (8%) of women with a DEXA assessed body fat percentage over 40%.

From this study there are a range of suggestions for future research. Cell separation techniques should be used to separate CD4+ and CD8+ T-lymphocytes and subsequently stimulate cells with tumour-associated antigens. This will determine which cells, or the proportion of cells, that are responsible for the immune response so that these can be investigated to either target in immunotherapy treatment strategies or help create strategies to improve immune cell functionality. Multiple blood samples should be taken from the same individual on different days and, where possible, cell samples should be taken from within the tissue and lymph to determine a more robust estimate of an individual's immune response to tumour-associated antigens to confirm the robustness of the methodology. Knowing whether cells from the tissue, lymph and blood produce similar results is important in terms of cancer defence as immune cells at the site of cell mutation or in the lymph are those that are first recognising and responding to tumour-associated antigens. As T-lymphocytes migrate to the tissue and exercise in a phenotype dependent manner (Campbell *et al.*, 2009), it is important to know if peripheral lymphocytes are a good representative of infiltrating lymphocytes. In relation to immune responses to tumour-associated antigens and their relationship with lifestyle variables, it is suggested that more

extreme populations (i.e. obese vs lean) are investigated and post-acute exercise blood samples should be analysed. Finally, to determine the relationship between immune responses to tumour-associated antigens and breast cancer risk, there is a need for longitudinal research to follow women over a number of years to assess whether individuals with higher immune responses to tumour-associated antigens have a reduced incidence of breast cancer diagnoses compared to those with lower or no responses.

In conclusion, this research confirms that healthy women have responses to tumour-associated antigens.

CHAPTER 4: Lifestyle and T-lymphocyte IFN-γ release in response to tumour-associated and viral antigens in Cytomegalovirus seropositive and seronegative healthy women: An observational study

4.1 INTRODUCTION

CMV is a near ubiquitous herpesvirus, present in at least 70% of humans by the age of 35 years (Froberg, 2004), with the majority showing few clinical symptoms of primary infection (Landolfo *et al.*, 2003). Upon infection, CMV elicits a series of robust immune responses initiated by CD4+ and CD8+ T-lymphocytes to control viral replication (Jackson *et al.*, 2017; Crough and Khanna, 2009). As CMV encodes numerous proteins and microRNAs that function to evade the immune system, the virus is never fully cleared and remains latent in hematopoietic stem cells, monocytes/macrophages, dendritic cells, endothelial cells lining the surface of blood, and lymphoid vessels (Reeves and Sinclair, 2008; Goodrum, 2016) despite competent immune responses (Wills *et al.*, 2015). Through establishing latency, CMV persists within the host throughout life, repeatedly reactivating during periods of immunosuppression or inflammation (Hummel and Abecassis, 2002), leading to subsequent antigen presentation to T-lymphocytes. This in turn stimulates an inflammatory immune response further increasing the frequency of CMV-specific T-lymphocytes (Jackson *et al.*, 2017; Sansoni *et al.*, 2014; Pawelec *et al.*, 2010; Solana *et al.*, 2012).

As humans age there are natural detrimental changes in immune function, a term coined immunosenescence (Denkinger *et al.*, 2015; Kline and Bowdish, 2016). Evidence suggests that CMV infection accelerates immunosenescence in humans (Almanzar *et al.*, 2005; Komatsu *et al.*, 2003; Weltevrede *et al.*, 2016). In CMV seropositive individuals as much as 10% of the total CD8+ T-lymphocyte and up to 50% of the IFN-γ producing CD8+ compartment is phenotypically specific to CMV (Sylwester *et al.*, 2005; Vescovini *et al.*, 2007). This is a large proportion considering the large variety of pathogens humans encounter (Crough and Khanna, 2009) and may leave less room for phenotypes of other immune cells. This may cause a lower diversity of the TCR repertoire (Goronzy *et al.*, 2007; Nikolich-Žugich, 2008; Aiello *et al.*, 2017), a reduced number of naïve T-lymphocytes and therefore a limited immune response to novel antigens, which may include tumour-associated antigens. It has been reported that older (>66 years old) CMV seropositive individuals have low numbers of naïve CD8+ T-lymphocytes (Ouyang *et al.*, 2004; Strindhall *et al.*, 2013; Wikby *et al.*, 2002). Furthermore, previous observational studies have shown an increase in ACM and susceptibility to new infections in elderly humans who are CMV

seropositive but research is inconsistent (Wikby *et al.*, 2002; Strindhall *et al.*, 2013; Ouyang *et al.*, 2004; Olsson *et al.*, 2001; Hadrup *et al.*, 2006).

The term cancer immunosurveillance describes the process whereby the immune system acts as a critical component of cancer defence (Burnet, 1970; Thomas, 1982; Burnet, 1957; Thomas, 1959). This is demonstrated by an increased cancer risk in individuals with immunodeficiencies such as HIV or in solid transplant patients who are given immune supressing drugs (Gatti and Good, 1971; Kinlen *et al.*, 1985; Salavoura *et al.*, 2008; Van Der Meer *et al.*, 1993; Engels *et al.*, 2011). Furthermore, in cancer patients, the quantity and quality of tumour-infiltrating lymphocytes is positively correlated with patient prognosis and survival (Zhang *et al.*, 2003; Mahmoud *et al.*, 2011; Al-Shibli *et al.*, 2008; Kawai *et al.*, 2008; Naito *et al.*, 1998; Cho *et al.*, 2003; Hiraoka *et al.*, 2006). This suggests that the anti-cancer immune response, specifically the adaptive immune response, is tailored to recognise tumour-associated antigens and elicit a cytotoxic response against cancer cells. Finally, the fact that cancer cells, in order to survive, must develop strategies to evade the immune response, further supports the importance of the immune system in cancer defence (Corthay, 2014; Dunn *et al.*, 2002a; Shankaran *et al.*, 2001a).

The immune system plays an important role in cancer defence and protection, therefore the accelerated immunosenescence reported in CMV seropositive individuals may act as a mechanism through which cancer risk may be elevated in this population (Foster *et al.*, 2011). This is supported by evidence demonstrating an increased risk of cancer with ageing (White *et al.*, 2014). Furthermore, it has been suggested that CMV has a pathogenic role in incidence of cancer (Söderberg-Nauclér, 2006) whereby CMV can facilitate cancer cells to fulfil the requirements of the hallmarks of cancer (Hanahan and Weinberg, 2011). This can be achieved through increased release of proinflammatory cytokines, IL-6 and TNF-α from effector T-lymphocytes (Alonso Arias *et al.*, 2013) and enhanced tumour growth and progression by changing the tumour microenvironment (Richardson *et al.*, 2004; Cox *et al.*, 2010; Mohamed *et al.*, 2014; Söderberg-Nauclér, 2006; Cinatl Jr *et al.*, 1996; Harkins *et al.*, 2010; Taher *et al.*, 2013; Bishop *et al.*, 2015; Soroceanu and Cobbs, 2011). However, research is inconsistent, and some studies have shown no relationship between CMV and breast cancer (Antonsson *et al.*, 2012).

Lifestyle factors have been linked to cancer risk, with an increased risk with increasing age, body composition and decreased physical activity (Kushi *et al.*, 2012a; Leitzmann *et al.*, 2015a; Friedenreich and Orenstein, 2002; Thune *et al.*, 1997). On top of this, increased

exercise, physical activity and improved body composition have also been demonstrated to improve aspects of immune function (Simpson and Guy, 2010; Simpson, 2011). Regular exercise elicits a strong anti-inflammatory response (Gleeson *et al.*, 2011) and improved immune response to vaccination, T-lymphocyte proliferation and cytokine production in response to mitogens has been demonstrated to be stronger in those who undertake regular exercise training (Pascoe *et al.*, 2014; Haq and McElhaney, 2014; Kohut *et al.*, 2002; Kohut *et al.*, 2004; Woods *et al.*, 2009a; de Araújo *et al.*, 2015; Simpson *et al.*, 2012). Exercise can also delay or reduce the impact of immunosenescence (Simpson *et al.*, 2012; Kohut and Senchina, 2004; Woods *et al.*, 2002).

Exercise causes the mobilisation of late-stage differentiated T-lymphocytes in the blood with subsequent homing of these cells to peripheral tissues (Campbell *et al.*, 2009; Turner *et al.*, 2010) whereby they are more likely to undergo apoptosis (Turner and Brum, 2017). The decrease in number of late-stage differentiated T-lymphocytes (which may be specific to CMV) (Simpson and Guy, 2010; Simpson, 2011) may then create 'space' for the naïve T-lymphocyte pool to expand, reversing the decreased number of naïve T-lymphocytes (and consequential increase in late stage differentiated T-lymphocytes) seen in CMV driven immunosenescence. However, there is limited evidence on whether lifestyle differences exist within CMV seropositive and how this impacts measures of immune function.

Psychological stressors such as depression, anxiety and stress can also impact the immune system (Rector *et al.*, 2014). Infection with CMV has been previously associated with mood and wellbeing through increased serum concentrations of cytokines such as TNF- α and IL-6 (Phillips *et al.*, 2008) or by psychological stress driving CMV replication (Rector *et al.*, 2014). Previous research has also demonstrated that individuals reporting higher levels of depression were more likely to be CMV seropositive (Miller *et al.*, 2005) and within CMV seropositive individuals, those with higher levels of CMV specific antibodies were more likely to report being depressed and/or anxious (Trzonkowski *et al.*, 2003).

When considering body composition, obesity has been related to impaired lymphocyte proliferation (Nieman *et al.*, 1999), shorter leukocyte telomere length (Müezzinler *et al.*, 2014), and a skewing of the T-lymphocyte pool toward a regulatory and Th2-phenotype (van der Weerd *et al.*, 2012). Decreases in visceral and subcutaneous adipose tissue (Tchernof and Després, 2013) through diet and/or exercise can reduce inflammation and contribute to the maintenance of redox balance (Radak *et al.*, 2008; Gleeson *et al.*, 2011) and therefore contribute to a reduction in the proportion of late-stage differentiated T-

lymphocytes (Henson *et al.*, 2014; Campbell and Turner, 2018) and CMV reactivation. As evidence suggesting individuals with a healthier lifestyle (fitter, more physically active, reduced body fat) seem less likely to possess immunosenescent profile (Turner, 2016), leading such lifestyles may be able to limit the immunosenescence effects of CMV activation and reactivation. It is unknown whether lifestyle factors can affect CD8+ T-lymphocyte response to viral or tumour-associated antigens and whether this differs within CMV seropositive women. As exercise beneficially alters the immune system, it may be something CMV seropositive individuals can take advantage of to delay or reduce the accelerated immunosenescence related to the virus.

This study aims to identify differences in immune responses towards tumour-associated and viral antigens in CMV seropositive and CMV seronegative healthy individuals. This study also aims to examine whether lifestyle differences e.g. fitness, dietary intake and body composition in CMV seropositive women affect T-lymphocyte response to tumour-associated and viral antigens. It is hypothesised that CMV seropositivity will lead to impaired immune responses towards tumour-associated and non-CMV viral antigens due to accelerated immunosenescence reported in this population. It is also hypothesised that, due to improvements in immune function with healthier lifestyles, that individuals with less healthy lifestyle profiles (decreased fitness, increased body fat, increased levels of depression, anxiety and stress) will have reduced T-lymphocyte function in response to tumour-associated and viral antigens.

4.2 METHODS

Participants, study design, procedures, assessment of body composition, cardiorespiratory fitness, habitual physical activity, habitual diet, biochemical and immunological procedures mirrored those in Chapter 3. To avoid repetition, methods are not repeated within this chapter.

4.2.1 Statistical analysis

Data were tested for normal distribution using the Kolmogorov Smirnov test. ANOVA was used to examine differences in key outcome variables between groups. Cohen's *d* effects sizes were calculated and thresholds of 0.2-0.5, 0.5-0.8 and >0.8 for small, medium and large effect sizes respectively in line with (Lakens, 2013). Spearman's' rank and Pearson's correlations were used for normal and non-normally distributed variables as appropriate.

Multiple linear regression was run in a force entry manner. Chi-squared tests was used when comparing nominal and ordinal data and odds ratios were calculated. Statistical analyses were conducted using SPSS version 22. Statistical significance was accepted at $p < 0.05$. Figures were created using GraphPad Prism.

4.3 RESULTS

4.3.1 There are no lifestyle or psychological differences between CMV seropositive and CMV seronegative healthy women

No significant differences ($p>0.05$) were observed in characteristics between healthy women who were CMV seropositive compared to healthy women who were CMV seronegative (Table 10.) On average, both CMV seropositive and seronegative groups had normal blood pressure (<120/80 mmHg).

Table 10. Lifestyle characteristics of women who are CMV seropositive and those who are CMV seronegative

Characteristics	**CMV Seropositive (n=22)**	**CMV Seronegative (n=28)**	**Difference**	**ANOVA statistic**
Age (years)	45 ± 12	41 ± 12	4	F(1,48) = 1.142, p=0.290
Height (m)	1.68 ± 0.07	1.66 ± 0.08	0.02	F(1,48) = 0.658, p=0.421
Body mass (kg)	73.5 ± 18.8	65.9 ± 12.5	7.6	F(1,48) = 2.901, p=0.095
Predicted $\dot{V}O_2$ max ($ml.kg.min^{-1}$)	36.3 ± 10.2	37.7 ± 7.9	−1.4	F(1,48) = 0.279, p=0.599
Systolic blood pressure (mmHg)	118 ± 17	115 ± 16	3	F(1,48) = 0.631, p=0.431
Diastolic blood pressure (mmHg)	76 ± 9	73 ± 12	3	F(1,48) = 1.425, p=0.238

N=50 Data shown as means ± SD.

m; metres. kg; kilogram. ml; millilitres. min; minutes. $\dot{V}O_2$; oxygen uptake. mmHg; millimoles of Mercury.

No significant differences were seen in body composition in women who were seronegative and women who were seropositive (Table 11.). BMI was 26.0 ± 5.9 $kg.m_2$ in seropositive women versus 23.9 ± 3.8 $kg.m_2$ in seronegative women, making on average, BMI in seropositive women overweight and in seronegative women normal. W:H was the same between groups. No significant differences were seen in BMD, T-score and Z-score between groups ($p>0.05$) (Table 11.)

1841 Table 11. Body composition of women who are CMV seropositive and those who are CMV seronegative

1842

Body composition characteristics	**CMV Seropositive (n=22)**	**CMV Seronegative (n=28)**	**Difference**	**ANOVA statistic**
BMI (kg.m2)	26.0 ± 5.9	23.9 ± 3.8	2.1	F(1,48) = 2.352, p=0.132
W:H	0.78 ± 0.05	0.78 ± 0.06	0.00	F(1,48) = 0.012, p=0.915
DEXA measured Body fat (%)	32.5 ± 7.5	31.1 ± 7.1	1.4	F(1,48) = 0.492, p=0.486
Fat mass index (kg.m2)	8.26 ± 3.14	7.48 ± 2.59	0.78	F(1,48) = 0.927, p=0.340
BMD (g/cm2)	1.17 ± 0.13	1.16 ± 0.14	0.01	F(1,48) = 0.072, p=0.790
T-score	0.78 ± 1.51	0.63 ± 1.64	0.15	F(1,48) = 0.109, p=0;742
Z-score	0.77 ± 1.19	0.64 ± 1.31	0.13	F(1,48) = 0.138, p=0.712
Breast fat (%)	29 ± 10	29 ± 11	0	F(1,48) = 0.000, p=0.999

1843 N=50 Data shown as means ± SD.

1844 *BMI; body mass index. m; metres. kg; kilogram. W:H; waist to hip ratio. BMD; bone mineral density. g; grams. cm; centimetres.*

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No significant differences were seen in IPAQ measured physical activity levels between CMV seropositive and CMV seronegative women (Table 12.) In terms of Sensewear measured physical activity, no differences were observed in moderate, vigorous or very vigorous activity per day between groups. Minutes per day of light activity was significantly higher in CMV seropositive individuals by ~34 minutes per day, p=0.027 (Table 12.). No significant differences were observed in step count (10961 ± 3256 and 10194 ± 3802 steps per day in CMV seropositive and seronegative respectively) and sedentary time (73 ± 11 and 76 ± 10% wake time in CMV seropositive and seronegative respectively).

Table 12. Physical activity levels of women who are CMV seropositive and those who are CMV seronegative

Physical Activity	**CMV Seropositive (n=21)**	**CMV Seronegative (n=27)**	**Difference**	**ANOVA statistic**
Energy expenditure (kcal/day)	2555 ± 417	2405 ± 230	105	F(1,47)=1.158, p=0.287
PAL	1.75 ± 0.27	1.75 ± 0.23	0.00	F(1,47)=0.002, p=0.963
Steps per day	10961 ± 3256	10194 ± 3802	767	F(1,47)=0.896, p=0.349
Sedentary time (% of waking)	73 ± 11	76 ± 10	−3	F(1,47)=0.593, p=0.445
<1.8 METS (mins/day)	746 ± 117	760 ± 101	−14	F(1,47)=0.148, p=0.702
1.8><3 METS (mins/day)	120 ± 59	86 ± 50	34	F(1,47)=5.195, p=0.027*
3><6 METS (mins/day)	130 ± 72	124 ± 62	6	F(1,47)=0.127, p=0.723
>6 METS (mins/day)	23 ± 21	31 ± 21	−8	F(1,47)=1.383, p=0.246
>10.2 METS (mins/day)	1 ± 2	1 ± 2	0	F(1,47)=0.076, p=0.784
IPAQ Light activity (MET mins per day)	113 ± 142	63 ± 59	50	F(1,48)=2.905, p=0.095
IPAQ Moderate activity (MET mins per day)	52 ± 55	57 ± 99	−5	F(1,48)=0.047, p=0.828
IPAQ Vigorous activity (MET mins per day)	53 ± 648	31 ± 51	22	F(1,48)=1.150, p=0.289

N=50 Data shown as means ± SD. *p<0.05

kcal; kilocalories. PAL; physical activity level.

No significant differences were seen in macronutrient intake in CMV seropositive women compared to CMV seronegative women ($p>0.05$) (Table 13.). Energy expenditure was 1929 ± 435 kcal per day in seropositive women and 1874 ± 536 kcal in seronegative women. Intake of carbohydrates, fats and proteins relative to body weight were the same between groups. Sugar intake was 0.04 ± 0.02 g.kg. day^{-1} in seropositive women and 0.05 ± 0.02 g.kg. day^{-1} in seronegative women whilst saturated fat intake was 0.38 ± 0.12 g.kg. day^{-1} in seropositive women and 0.40 ± 0.19 g.kg. day^{-1}per day in seronegative women ($p>0.05$) (Table 13.). A significant difference was observed in vitamin C intake between groups which was higher in CMV seropositive individuals. No other differences in micronutrient intake were observed.

Table 13. Nutritional intake of women who are CMV seropositive and those who are CMV seronegative

	CMV Seropositive (n=22)	**CMV Seronegative (n=28)**	**Difference**	**ANOVA statistic**
Energy Intake per day	1929 ± 435	1874 ± 536	55	F(1,48) = 0.155, p=0.696
Carbohydrate per day (g.kg.day^{-1})	2.84 ± 1.14	3.31 ± 1.17	−0.47	F(1,48) = 2.105, p=0.506
Protein per day g.kg.day^{-1})	0.44 ± 0.17	0.41 ± 0.24	0.03	F(1,48) = 0.291, p=0.592
Fat per day g.kg.day^{-1})	1.60 ± 1.88	1.09 ± 0.56	0.51	F(1,48) = 1.858, p=0.179
Sugars (g.kg.day^{-1})	0.04 ± 0.02	0.05 ± 0.02	−0.01	F(1,48) = 0.448, p=0.291
Saturated fat (g.kg.day^{-1})	0.38 ± 0.12	0.40 ± 0.19	−0.02	F(1,48) = 0.103, p=0.749
Vitamin A (mg.day^{-1})	1074 ± 620	977 ± 615	97	F(1,48) = 0.302, p=0.585
Vitamin C (mg.day^{-1})	150 ± 125	84 ± 50	66	F(1,48) = 6.526, p=0.014*
Vitamin D (mg.day^{-1})	5.3 ± 4.6	3.6 ± 3.9	1.7	F(1,48) = 1.962, p=0.168
Vitamin E (mg.day^{-1})	12.7 ± 13.4	8.7 ± 4.8	4.0	F(1,48) = 2.122, p=0.152
Calcium (mg.day^{-1})	702 ± 260	806 ± 471	−104	F(1,48) = 0.850, p=0.361
Sodium (mg.day^{-1})	2159 ± 1512	2041 ± 839	118	F(1,48) = 0.123, p=0.728
Iron (mg.day^{-1})	13.2 ± 8.0	9.7 ± 4.5	3.5	F(1,48) = 3.906, p=0.054
Zinc (mg.day^{-1})	11.7 ± 14.3	7.9 ± 3.4	3.8	F(1,48) = 1.927, p=0.172

N=50 Data shown as means ± SD. * p<0.05

g; grams.

No significant differences were seen between CMV seropositive and seronegative individuals in terms of depression and anxiety (Table 14.). However, a significant difference in stress was found ($p<0.05$, effect size 0.3), whereby levels of stress were significantly higher in those who were CMV seronegative (9 ± 6) when compared with those who are seropositive (5 ± 5) (Table 14.). Averages for depression, anxiety and stress in both groups were within normal ranges. In those that were CMV seropositive, 2 people had over normal scores for depression, 1 for anxiety and 0 for stress, but all lay within the mild category. Within those who were CMV seronegative, 2 people had moderate and 1 person severe scores for depression, 1 had a moderate score for anxiety and 1 person had a mild and 2 people had moderate scores for stress.

Table 14. Psychological scores of women who are CMV seropositive and those who are CMV seronegative

Psychological scores	CMV Seropositive (n=22)	CMV Seronegative (n=27)	Difference	ANOVA statistic
Depression	3 ± 4	4 ± 6	−1	F(1,47) = 0.535, p=0.468
Anxiety	1 ± 3	2 ± 3	1	F(1,47) = 0.670, p=0.417
Stress	5 ± 5	9 ± 6	−4	F(1,47) = 5.896, p=0.019*

N=50 Data shown as means ± SD. *p<0.05

4.3.2 Haematological cell counts and levels of blood biomarkers in CMV seropositive and CMV seronegative healthy women

No differences were observed between cell counts when comparing CMV seropositive and CMV seronegative women (p>0.05) (Table 15.). Lymphocyte count was 1.7 ± 0.6 x 10^9/L in CMV seropositive women versus 1.5 ± 0.4 x 10^9/L in seronegative women. Monocyte count 0.4 ± 0.2 x 10^9/L and 0.3 ± 0.1 x 10^9/L in CMV seropositive and seronegative women respectively (p>0.05). Neutrophil count, on average, was the same between groups at 3.3 x 10^9/L. Most women in both groups were seropositive for EBV and VZV. In those who were CMV seropositive, 91% and 96% were also EBV and VZV seropositive respectively and 82% and 97% respectively in those who were CMV seronegative. No differences in EBV and VZV specific IgG were observed between groups (p>0.05) (Table 15.). Levels of circulating RANTES, leptin and osteopontin were not significantly different between women who were CMV seropositive or seronegative. A significant difference was seen in resting levels of circulating resistin which was significantly lower in women who were CMV seronegative by 0.27ng/ml (p<0.05), effect size = 0.3 (small).

Table 15. Haematological characteristics of women who are CMV seropositive and those who are CMV seronegative

Characteristic	CMV Seropositive n=22	CMV Seronegative n=28	Difference	ANOVA statistic
Total leukocyte count (x 10^9/L)	5.2 ± 1.7	5.1 ± 1.3	0.1	F(1,48) = 0.146, p=0.704
Platelets (x 10^9/L)	198 ± 87	157 ± 54	41	F(1,48) = 3.109, p=0.084
Lymphocyte (x 10^9/L)	1.7 ± 0.6	1.5 ± 0.4	0.2	F(1,48) = 0.662, p=0.420
Monocytes# (x 10^9/L)	0.4 ± 0.2	0.3 ± 0.1	0.1	F(1,48) = 1.193, p =0.280
Neutrophil (x 10^9/L)	3.3 ± 1.3	3.3 ± 1.2	0.0	F(1,48) = 0.000, p=0.992
EBV positive (%)	20/22, 91%	23/28, 82%	3, −9%	F(1,48) = 0.767, p=0.386
EBV specific IgG (IU/ml)	643 ± 358	540 ± 435	−103	F(1,48) = 0.710, p=0.404
VZV positive (number, %)	21/22, 96%	27/28, 97%	6, 1%	F(1,48) = 0.029, p=0.865
VZV specific IgG (IU/ml)	1423 ± 1055	1574 ± 942	−151	F(1,48) = 0.273, p=0.604
RANTES (ng/ml)	10.63 ± 6.56	11.90 ± 5.94	−1.27	F(1,48) = 0.513, p=0.477
Resistin (ng/ml)	1.14 ± 0.63	0.87 ±0.19	0.27	F(1,48) = 4.986, p=0.030*
Leptin (ng/ml)	13.15 ± 14.55	15.58 ± 13.36	−2.43	F(1,48) = 0.377, p =0.542
Osteopontin (ng/ml)	23.73 ± 9.36	24.30 ± 8.41	−0.57	F(1,48) = 0.052, p=0.821

N=50, * P<0.05, data shown as means ± SD. #Monocytes, eosinophils and basophils, however eosinophils and basophils only make up a small fraction, (0.1-1.6 X 10^9/L according to manufacturer's guidelines, Sysmex, KX-21N, Kobe, Japan).

L; litre. EBV; Epstein Barr virus. VZV; Varicella zoster virus. ng; nanogram. ml; millilitre.

4.3.3 T-lymphocyte anti-viral response in CMV seropositive and CMV seronegative healthy women

No significant differences were seen in T-lymphocyte anti-viral response towards FLU, EBV and VZV peptides ($p>0.05$) (Figure 9). The number of IFN-γ producing T-lymphocytes in response to FLU peptide MP1 was 7 ± 7 SFUs per 250,000 PBMCs and 6 ± 8 SFUs per 250,000 PBMCs for CMV seropositive and seronegative women respectively. In response to EBV peptides, T-lymphocyte response to EBNA1 was 19 ± 17 SFUs per 250,000 PBMCs in CMV seropositive individuals compared to 12 ± 34 SFUs per 250,000 PBMCs in seronegative individuals. No significant differences were seen in the number of T-lymphocyte IFN-γ producing cells in CMV seropositive individuals versus those who are CMV seronegative in response to both VZV peptides, IE63 and gE ($p>0.05$) (Figure 9).

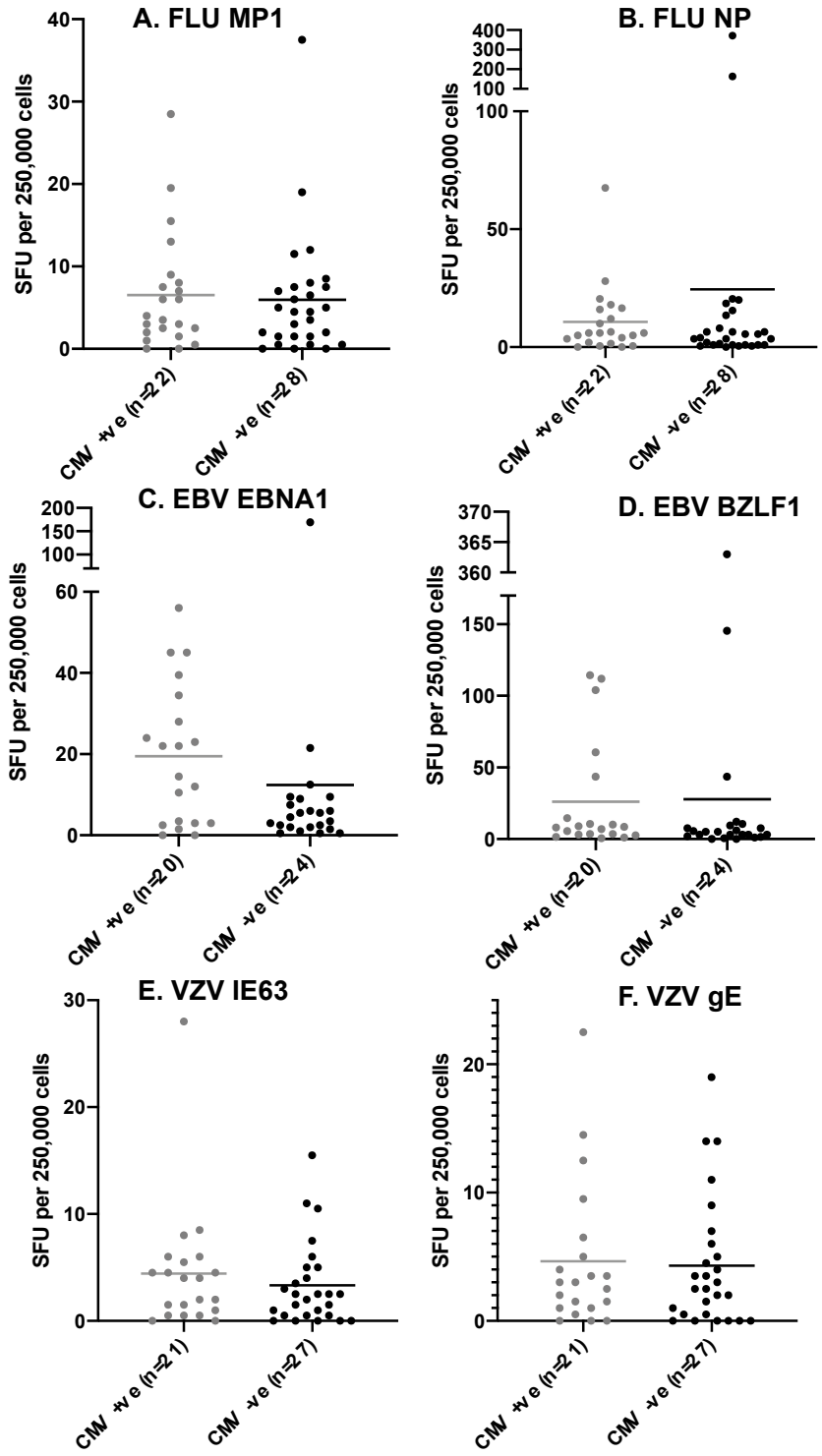


Figure 9. The magnitude of specific T-lymphocytes compared against A. Flu MP1, B. FLU NP, C. EBV EBNA1, D. EBV BZLF1, E VZV IE63, F VZV gE viral antigens. Data show as individual positive responses as deemed by ELISA and means. Total CMV +ve (seropositive) shown in grey (n=22) and CMV -ve (seronegative) shown in black (n=28). *SFU, spot forming units. EBV, Epstein Barr Virus. EBNA1, Epstein Barr nuclear antigen 1. BZLF1, BamHIZ leftward reading frame 1. MP1, matrix protein1. NP, nucleoprotein. VZV, Varicella Zoster Virus. ie63, immediate-early protein 63. gE, glycoprotein E. CMV; cytomegalovirus*

4.3.4 T-lymphocyte response towards tumour-associated antigens are larger in CMV seropositive healthy women

Significant differences were seen in terms of responsiveness towards tumour-associated antigens between CMV seropositive and seronegative women. Half of the tumour-associated antigens showed a significantly higher number of women positive towards tumour-associated antigens if they were also CMV seropositive (Figure 10). This trend was seen across all tumour-associated antigens, other than ERB ECD, where there was no difference. The biggest difference was seen in positivity towards ERB ICD (68% of women responding to the antigen in women who were CMV seropositive compared to 29% in those who were CMV seronegative, $\chi(1) = 7.782$, $p = .005$), odds ratio = 5.4, followed by MamA ($\chi(1) = 6.762$, $p = .009$), odds ratio = 4.8, Cl6 ($\chi(1) = 4.565$, $p = .033$), odds ratio = 3.6) and Survivin ($\chi(1) = 4.565$, $p = .033$) then by CEA ($\chi(1) = 4.121$, $p = .042$, odds ratio = 3.6) and CycB1 $\chi(1) = 3.848$, $p = .050$, odds ratio = 3.4). No significant differences were seen in terms of positivity to ERB ECD, MMP11, TARP or MUC1 between groups (Figure 10) ($p>0.05$). In seropositive individuals MamA was the most immunodominant antigen compared to ERB ECD in seronegative individuals (73% and 50% of women responding respectively). MMP11 was the least immunodominant antigen in both groups (41% and 18% of women responded in seropositive and seronegative groups respectively).

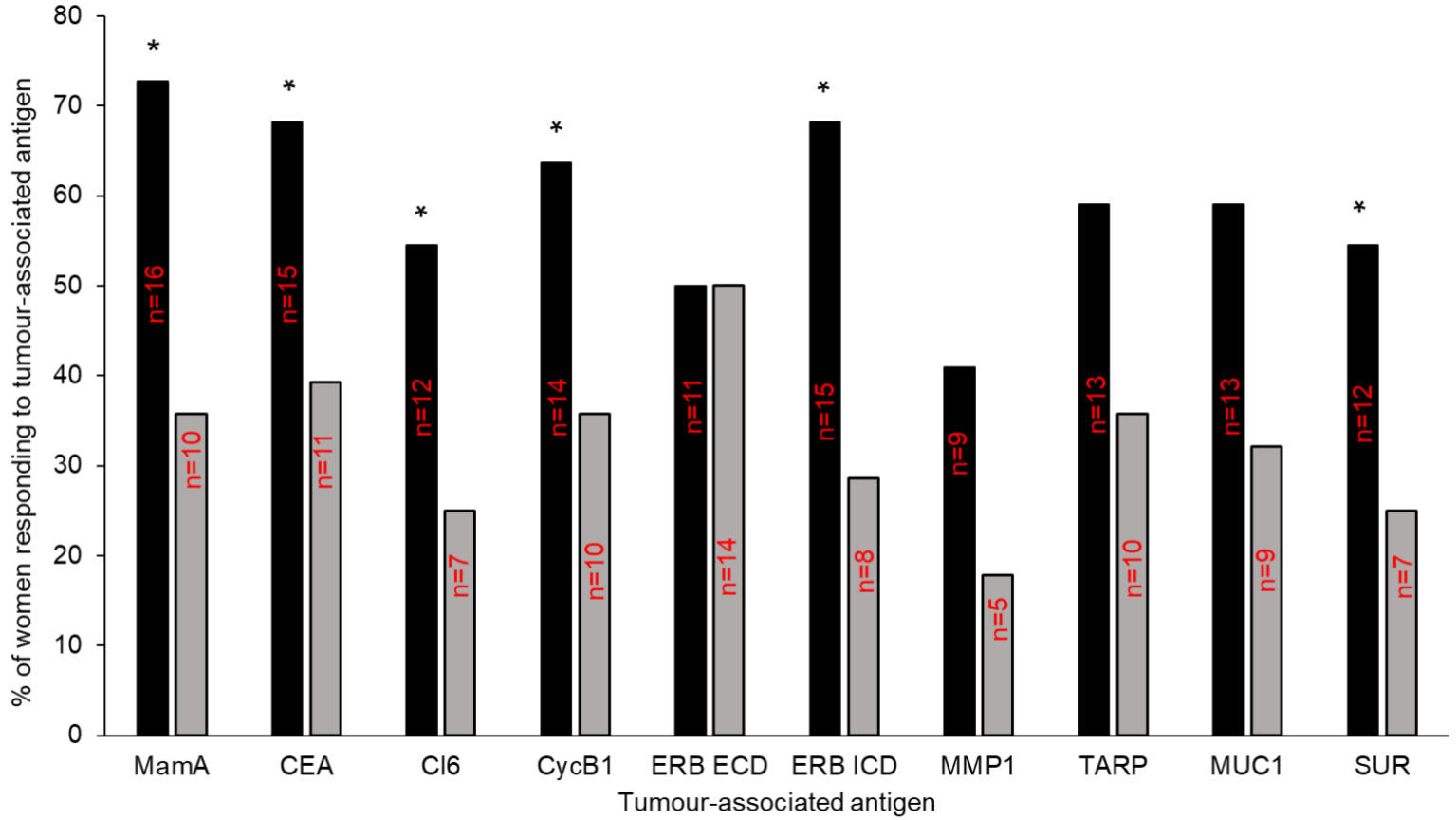


Figure 10. Immunodominance of tumour-associated antigens in women who are positive and negative for CMV
CMV positive, N=22. *CMV negative, N=28, 250,000 PBMCs per well tested using ELISpot, against 10 tumour associated antigens: MamA, mammaglobin A. CEA, carcinoembryonic antigen. Cl6, claudin-6. CycB1, cyclin-B1. ERB ECD, receptor tyrosine-protein kinase erbB-2 extracellular domain. ERB ICD receptor tyrosine-protein kinase erbB-2 intracellular domain. MMP1, stromelysin-3. TARP, TCRgamma alternate reading frame protein. MUC1, mucin-1. SUR, survivin. * P<0.05, difference between groups*

Significant differences were found between different tumour-associated antigens and the number of cells responding between CMV seropositive and seronegative women only for ERB ICD ($F(1,23)=5.674$, $p=0.026$, effect size 0.3) (Figure 11F.), where on average number of IFN-γ secreting T-lymphocytes was 3 x higher in those who were CMV seropositive versus those who were CMV seronegative (6 ± 9 SFUs/250,000 versus 2 ± 1 SFUs/250,000 respectively) (Figure 11). On average, 3 more T-lymphocytes responded to tumour-associated antigens in seropositive individuals. This ranged from a difference of 1 SFUs, seen towards tumour-associated antigens Cl6 and MMP11 (Figure 11C and 11G) to a difference of 5 SFUs, seen toward CycB1 and ERB ECD (Figure 11D and 11E).

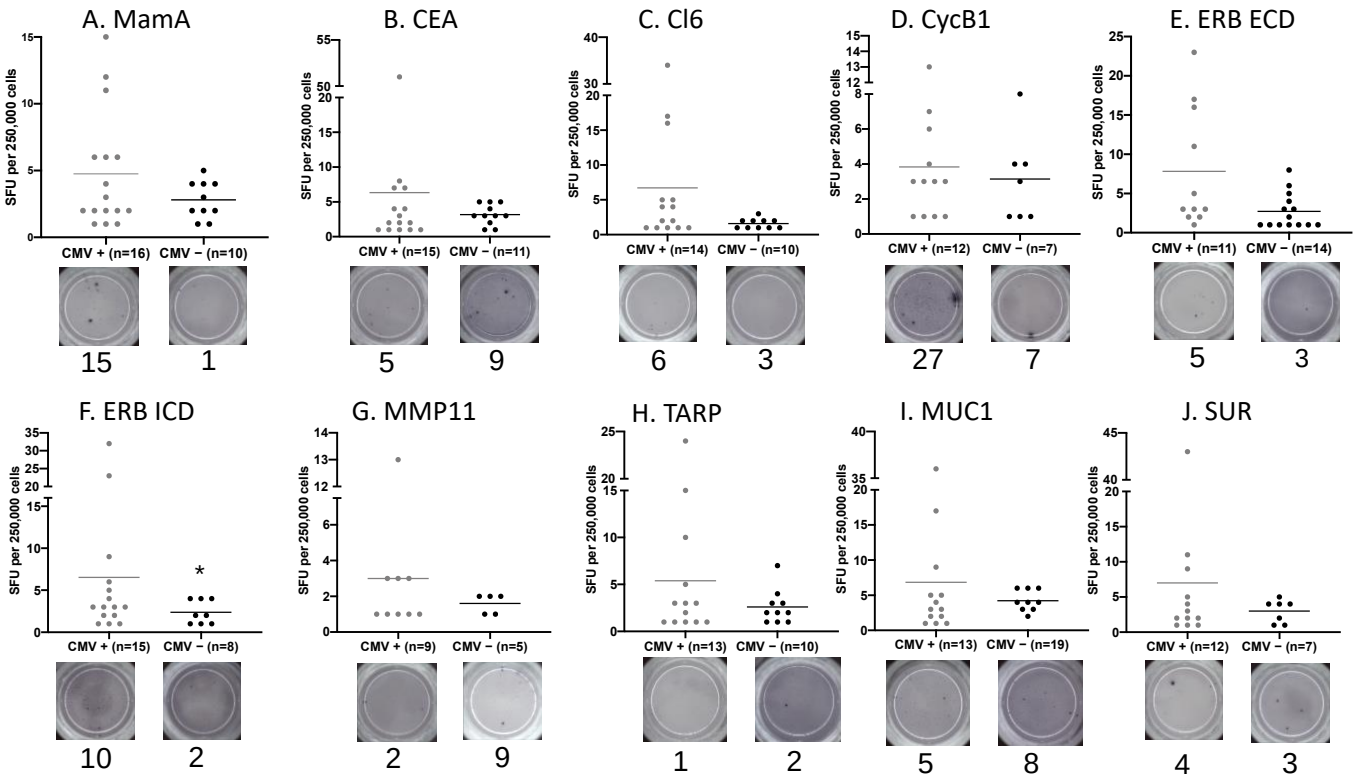


Figure 11. The relevant magnitude of specific T-lymphocytes when stimulated by tumour-associated associated antigens with example ELISpot wells. Data show as means ± SD. Individual positive responses as deemed by ELISA. CMV + (seropositive) (n=22) and CMV – (seronegative) (n=28). *250,000 PBMCs per well tested using ELISpot. SFU, spot forming units. A. MamA, mammaglobin-A. B. CEA, carcinoembryonic antigen. C. Cl6, claudin-6. D. CycB1, cyclin-B1. E. ERB ECD, receptor tyrosine-protein kinase erbB-2 extracellular domain. F. ERB ICD, receptor tyrosine-protein kinase erbB-2 intracellular domain. G. MMP1, stromelysin-3. H. TARP, TCRgamma alternate reading frame protein. I. MUC1, mucin-1. J. SUR, survivin. CMV; cytomegalovirus. *P<0.05, differences between groups.*

4.3.5 Correlations between CMV specific IgG antibody, lifestyle and haematological factors in CMV seropositive participants

No significant correlations were seen between CMV specific IgG antibody level and haematological variables including cell counts and circulating RANTES, resistin, osteopontin and leptin (P>0.05) (data not shown). A significant, strong correlation was reported between age and IgG antibody level (r=0.646, p=0.01) (Figure 12.), whereby level of IgG is seen to increase with age. No significant correlations were seen between IgG antibody level and any other lifestyle measures or IgG antibody level and T-lymphocyte response towards any of the breast cancer antigens (p>0.05), data not shown.

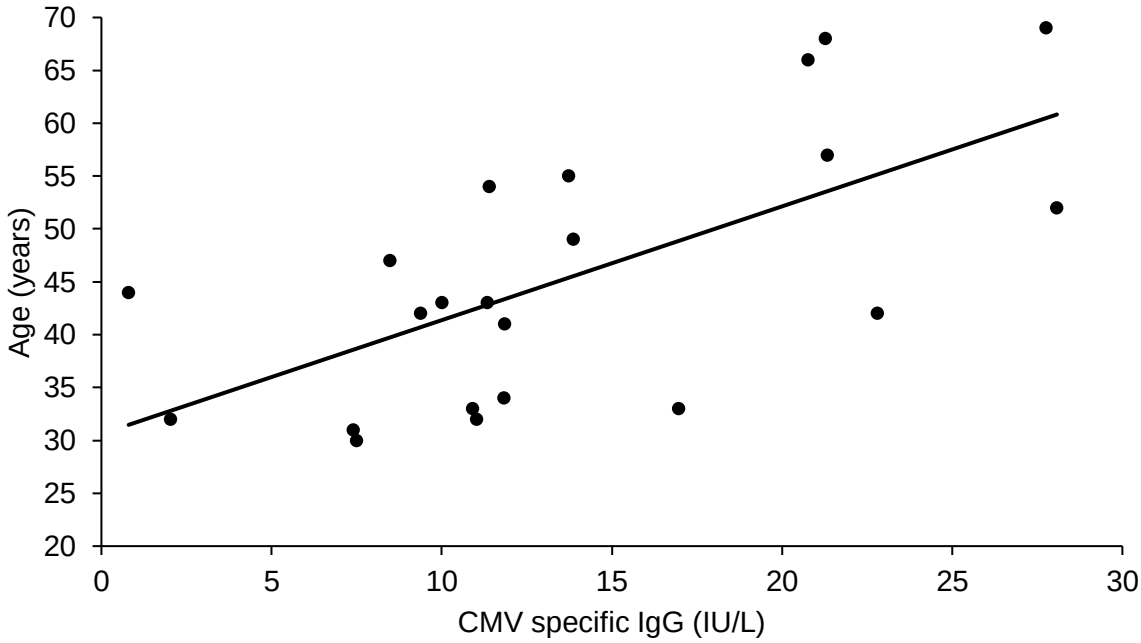


Figure 12. Significant correlations between CMV specific IgG antibody and with age. Individual data points shown, n=22.

CMV; cytomegalovirus, IgG; Immunoglobulin G. IU; international units. L; litres

A significant correlation was seen between IgG antibody level and T-lymphocyte response to CMV antigen IE1 (r=0.554, p=0.008, r_2=0.307) and towards CMV antigen pp65 (r=0.452, p=0.035, r_2=0.204). No other significant correlations were seen between anti CMV specific IgG level and T-lymphocyte response to EBV peptides BZLF1 or EBNA1 (r=0.061, p=0.799) and r=0.32 and p=0.169 respectively), VZV peptide IE1 or gE (r=0.033, p=0.895 and r=0.037, p=0.883 respectively) or to FLU peptides MP1 or NP (r=0.103, p=0.117 and r=−0.225, p=0.339 respectively).

A multiple linear regression was calculated to predict CMV specific IgG based on age, fitness, body fat, and T-lymphocyte response to CMV antigens IE1 and pp65. A significant regression equation was found ($F(5,16)=5.531$, $p=0.024$) with an $R_2=0.525$. Participants predicted CMV specific IgG was equal to −8.284+0.417 (age, years) + 0.119 ($\dot{V}O_2$ max, $ml.kg.min^{-1}$) + −0.072 (body fat, %) + (0.014 T-lymphocyte response to pp65, SFUs) + −0.002 (T-lymphocyte response to IE1, SFUs). Only age was a significant predictor of CMV specific IgG. Entered alone, age predicted 41.8% of variation in CMV specific IgG.

4.3.6 Effect of lifestyle and psychological factors on T-Lymphocyte IFN-γ release in response to viral antigens among CMV+ individuals only

No significant differences were seen in T-lymphocyte response to viral antigens when CMV seropositive women were split by age over and under 42 years, although on average, CMV seropositive individuals over 42 years old had a higher number of responding T-lymphocytes to all antigens for FLU, EBV and VZV (Table 17). No significant differences were seen in SFUs when grouped by predicted $\dot{V}O_2$ max (fit versus unfit) (Table 17.). When comparing those with normal blood pressure versus those with hypertension, a significantly higher number of T-lymphocytes responded to VZV antigen gE when looking at systolic and diastolic blood pressure ($F(1,19) = 9.228$, $p=0.007$ and $F(1,19) = 5.117$, $p=0.036$ respectively). No significant differences were found in T-lymphocyte response to viral antigens when groups were split by CMV specific IgG (split to create relatively equal groups) ($p>0.05$).

For thoroughness, correlations were also run on whole data sets (data not shown due to large ranges in SFUs and extreme outliers). Significant correlations were seen between age and T-lymphocyte response to VZV peptide IE63 ($r=0.450$, $p=0.041$, $n=21$, $r_2=0.203$). Significant correlations were also found between both systolic and diastolic blood pressure and VZV peptide gE ($r=0.525$, $p=0.015$, $n=21$, $r_2=0.276$ and $r=0.596$, $p=0.004$, $n=21$, $r_2=0.355$ respectively). For systolic blood pressure a significant correlation was also seen towards T-lymphocyte response to the other VZV peptide IE63 ($r=0.482$, $p=0.027$, $n=21$, $r_2=0.232$). Diastolic blood pressure was also correlated with the magnitude T-lymphocyte response towards EBV EBNA1 ($r=0.492$, $p=0.035$, $n=20$, $r_2=0.242$).

Table 17. Differences in number of T-lymphocytes responding to viral stimulation in CMV seropositive individuals only, grouped by lifestyle thresholds

	Age (years)		**Predicted $\dot{V}O_2$ max (ml.kg.min^{-1})**		**Systolic blood pressure (mmHg)**		**Systolic blood pressure (mmHg)**		**CMV specific IgG (IU/L)**	
	≤ 42 (n=10)	***> 42 (n=12)***	***≤ 50th percentile (n=9)***	***> 50th percentile (n=13)***	***< 120 (n=13)***	***≥ 120 (n=9)***	***< 80 (n=17)***	***≥ 80 (n=5)***	***< 12 (n=13)***	***≥12 (n=9)***
FLU MP1	6 ± 8	7 ± 6	7 ± 6	6 ± 8	6 ± 8	7 ± 6	6 ± 8	7 ± 4	13 ± 6	9 ± 7
FLU NP	6 ± 6	15 ± 19	10 ± 8	11 ± 18	10 ±18	12 ± 8	10 ± 16	14 ± 10	13 ± 13	9 ± 8
EBV EBNA1	21 ± 21	19 ± 16	22 ± 19	27 ± 42	14 ± 16	27 ± 17	17 ± 16	28 ± 19	11 ± 18	22 ± 16
EBV BZLF1	19 ± 39	31 ± 40	25 ± 37	2 ± 3	37 ± 47	10 ± 14	33 ± 44	6 ± 4	11 ± 13	42 ± 52
VZV IE63	2 ± 3	6 ± 7	7 ± 8	4 ± 5	2 ± 2	7 ± 8	4 ± 7	6 ± 2	13 ± 5	8 ± 4
VZV gE	2 ± 2	6 ± 7	6 ± 7	4 ± 5	2 ± 2	8 ± 7*	3 ± 5	9 ± 5*	13 ± 5	8 ± 5

Total n=22. Data shown as means ± SD, SFUs/250,000 PBMCs. * p<0.05 between lifestyle groups.

$\dot{V}O_2$ max; maximal oxygen consumption. mmHg; millimoles of mercury.MP1; matrix protein1. NP; nucleoprotein. VZV; Varicella Zoster Virus. IE63; immediate-early protein 63. gE; glycoprotein E. EBV; Epstein Barr Virus. EBNA1; Epstein Barr nuclear antigen 1. BZLF1; BamHIZ leftward reading frame 1. CMV; cytomegalovirus, IgG; Immunoglobulin G. IU; international units. L; litres

When investigating body composition, no significant differences were observed in those who were lean (as determined by BMI, body fat percentage and W:H ratio) and those who were overweight ($p>0.05$) (Table 18.) in terms of T-lymphocyte response to viral antigens. A significant difference was seen in terms of number of IFN-γ secreting T-lymphocytes in response to EBV antigen EBNA1 which was significantly higher in CMV seropositive individuals with a Z score ≤ 0.0 (34 ± 18 versus 13 ± 13 SFUs/250,000 PBMCs), $F(1,18) = 8.972$, $p=0.008$) (Table 18.). No other significant differences were seen ($p>0.05$). No significant correlations were found between any body composition variables and T-lymphocyte response to viral antigens.

Table 18. Differences in number of T-lymphocytes responding to viral stimulation in CMV seropositive individuals only, grouped by body compositional thresholds

	BMI (kg.m$_2$)		**DEXA measured body fat (%)**		**W:H**		**Z-score**	
	< 25.0 (n=11)	***≥ 25.0 (n=11)***	***≤ 35 (n=13)***	***> 35 (n=9)***	***< 0.80 (n=13)***	***≥ 0.80 (n=9)***	***≤ 0.0 (n=7)***	***> 0.0 (n=15)***
FLU MP1	8 ± 9	6 ± 5	7 ± 9	6 ± 4	7 ± 9	6 ± 4	6 ± 5	7 ± 8
FLU NP	7 ± 8	14 ± 19	12 ± 18	9 ± 49	8 ±9	15 ± 21	12 ± 10	10 ± 17
EBV EBNA1	17 ± 15	22 ± 20	21 ± 20	17 ± 13	17 ± 19	24 ± 15	34 ± 18	13 ± 13*
EBV BZLF1	26 ± 44	26 ± 36	31 ± 41	18 ± 38	27 ± 40	26 ± 40	25 ± 39	27 ± 41
VZV IE63	3 ± 3	6 ± 8	5 ± 8	4 ± 1	5 ± 8	4 ± 2	4 ± 3	5 ± 7
VZV gE	3 ± 5	6 ± 6	5 ± 7	4 ± 4	4 ± 6	6 ± 5	6 ± 5	5 ± 6

Total n=22. Data shown as means ± SD, SFUs/250,000 PBMCs. * p<0.05 between lifestyle groups.

BMI; body mass index. kg; kilograms. m; metres. W:H; waist to hip ratio. DEXA; dual energy X-ray. MP1; matrix protein1. NP; nucleoprotein. VZV; Varicella Zoster Virus. IE63; immediate-early protein 63. gE; glycoprotein E. EBV; Epstein Barr Virus. EBNA1; Epstein Barr nuclear antigen 1. BZLF1; BamHIZ leftward reading frame 1.

No significant differences were seen in CMV seropositive women in terms of step counts, on average, being over or under 10,000 steps per day (Table 19.). A significant difference was only observed in response to FLU NP peptide whereby 13 more T-lymphocytes responded in those who were less physically active (PAL below 1.70) versus those who were more physically active (PAL above 1.70) ($F(1,19) = 5.401$, $p=0.031$) (Table 19.) PAL only significantly correlated with T-lymphocyte response to one viral antigen, FLU NP, ($r=0.493$, $p=0.023$, $n=21$, $r_2=0.243$). No significant correlations were found between step count and response to any viral antigen.

Table 19. Differences in number of T-lymphocytes responding to viral stimulation in CMV seropositive individuals only, grouped by physical activity thresholds

	PAL		**Steps per day**	
	< 1.70	***≥ 1.70***	***< 10,000***	***≥ 10,000***
	(n=8)	***(n=7)***	***(n=6)***	***(n=9)***
FLU MP1	7 ± 4	7 ± 9	7 ± 5	7 ± 8
FLU NP	19 ± 21	6 ± 6*	20 ± 25	8 ± 7
EBV EBNA1	27 ± 19	17 ± 16	23 ± 22	20 ± 16
EBV BZLF1	29 ± 39	27 ± 42	37 ± 44	24 ± 40
VZV IE63	5 ± 2	4 ± 8	5 ± 2	5 ± 7
VZV gE	6 ± 5	4 ± 6	4 ± 5	5 ± 6

Total n=15. Data shown as means ± SD, SFUs/250,000 PBMCs. * $p<0.05$ between lifestyle groups.

PAL, physical activity level. MP1; matrix protein1. NP; nucleoprotein. VZV; Varicella Zoster Virus. IE63; immediate-early protein 63. gE; glycoprotein E. EBV; Epstein Barr Virus. EBNA1; Epstein Barr nuclear antigen 1. BZLF1; BamHIZ leftward reading frame 1.

As very few women scored non-normal scores in assessment of depression, anxiety and stress, categories were grouped by those who score 0 and those who scored above 0. No significant differences were seen in these groups in terms of T-lymphocyte response to viral antigens for depression, anxiety and stress ($p>0.05$) (Table 20.). No significant correlations were found between any psychological variables and T-lymphocyte response to tumour-associated antigens.

Table 20. Differences in number of T-lymphocytes responding to viral stimulation in CMV seropositive individuals only, grouped by psychological thresholds

	Depression		Anxiety		Stress	
	0 (n=11)	***> 0 (n=10)***	***0 (n=13)***	***> 0 (n=8)***	***0 (n=7)***	***> 0 (n=14)***
FLU MP1	7 ± 8	6 ± 5	5 ± 6	4 ± 3	5 ± 6	7 ± 8
FLU NP	6 ± 5	6 ± 6	12 ± 18	9 ± 9	12 ± 24	10 ± 8
EBV EBNA1	18 ± 18	17 ± 20	18 ± 20	23 ± 8	8 ± 11	24 ± 18
EBV BZLF1	23 ± 41	32 ± 39	28 ± 38	22 ± 4	49 ± 52	16 ± 30
VZV IE63	2 ± 3	7 ± 8	5 ± 8	4 ± 3	2 ± 2	5 ± 7
VZV gE	3 ± 3	7 ± 7	4 ± 6	6 ± 5	1 ± 1	6 ± 6

Total n=21. Data shown as means ± SD, SFUs/250,000 PBMCs. * p<0.05 between lifestyle groups.

MP1; matrix protein1. NP; nucleoprotein. VZV; Varicella Zoster Virus. IE63; immediate-early protein 63. gE; glycoprotein E. EBV; Epstein Barr Virus. EBNA1; Epstein Barr nuclear antigen 1. BZLF1; BamHIZ leftward reading frame 1.

4.3.7 Effect of lifestyle and psychological factors on T-Lymphocyte IFN-γ release in response to tumour-associated antigens among CMV+ individuals only

A significantly higher number of T-lymphocytes responded to tumour-associated antigen ERB ECD in CMV seropositive women over 42 years old (12 ± 8 SFUs/250,000 PBMCs) versus those younger (3 ± 2 SFUs/250,000 PBMCs), $F(1,9) = 5.889$, $p=0.038$. (Table 21). No other significant differences were seen between number of T-lymphocytes responding to tumour-associated antigens and the percentage of women responding to tumour-associated antigens when grouped by age ($p>0.05$). TARP elicited a significantly higher number of responding T-lymphocytes in those who were less fit (< 50_{th} percentile) versus those more fit with on average, 7 more responding cells per 250,000, $F(1,12) = 5.379$, $p=0.039$. This relationship was not seen in response to any other tumour-associated antigen (Table 21.). No significant differences were found in T-lymphocyte response to viral antigens when groups were split by CMV specific IgG ($p>0.05$).

Significant correlations were found between age and two tumour-associated antigens. Age was significantly correlated with magnitude of T-lymphocyte response to ERB ICD and

TARP (r=0.552, p=0.033, n=15, r_2=0.305 and r=0.704, p=0.007, n=13, r_2=0.496 respectively). Predicted cardiorespiratory fitness was also correlated with magnitude of T-lymphocyte response to TARP, this time negatively (r=−0.685, p=0.007, n=13, r_2=0.469) but also towards Survivin and MUC1 (r=−0.749, p=0.003, n=13, r_2=0.561 and r=−0.541, p=0.046, n=14, r_2=0.293 respectively).

Table 21. Differences in number of T-lymphocytes responding to tumour-associated antigen stimulation in CMV seropositive individuals only, grouped by lifestyle thresholds

	Age (years)		**Predicted $\dot{V}O_2$ max (ml.kg.min^{-1})**		**Systolic blood pressure (mmHg)**		**Systolic blood pressure (mmHg)**		**CMV specific IgG (IU/L)**	
	≤ 42 (n=10)	***> 42 (n=12)***	***≤ 50th percentile (n=9)***	***> 50th percentile (n=13)***	***< 120 (n=13)***	***≥120 (n=9)***	***< 80 (n=17)***	***≥ 80 (n=5)***	***> 12 (n=13)***	***≥ 12 (n=9)***
MamA SFUs	6 ± 5	4 ± 3	5 ± 3	4 ± 5	6 ± 5	3 ± 2	5 ± 5	4 ± 2	3 ± 3	7 ± 5
MamA % of responders	70	75	67	77	85	56	76	60	77	67
CEA SFUs	2 ± 1	8 ± 15	3 ± 2	8 ± 15	4 ± 3	10 ± 20	3 ± 3	14 ± 26	1 ± 2	11 ± 17
CEA % of responders	50	79	56	73	69	61	62	80	54	83
Cl6 SFUs	3 ± 2	4 ± 5	5 ± 5	3 ± 2	4 ± 4	3 ± 2	4 ± 4	2 ± 1	3 ± 2	5 ± 4
Cl6 % of responders	45	54	50	50	58	39	56	30	38	67
CycB1 SFUs	2 ± 2	11 ± 12	10 ± 12	4 ± 5	5 ± 6	9 ± 14	7 ± 10	3 ± 2	6 ± 11	8 ± 8
CycB1 % of responders	65	54	72	50	65	50	65	40	65	50
ERB ECD SFUs	3 ± 2	12 ± 8*	10 ± 10	7 ± 7	6 ± 6	11 ± 19	7 ± 8	10 ± 9	6 ± 8	10 ± 7
ERB ECD % of responders	45	50	44	50	50	44	50	40	42	56
ERB ICD SFUs	2 ± 1	10 ± 11	8 ± 10	5 ± 8	6 ± 7	8 ± 12	7 ± 10	3 ± 3	5 ± 10	8 ± 8
ERB ICD % of responders	65	67	89	50	65	67	68	60	65	67
MMP11 SFUs	1 ± 1	5 ± 5	5 ± 5	1 ± 0	3 ± 5	2 ± 1	3 ± 4	2 ± 1	1 ± 1	5 ± 5
MMP11 % of responders	40	29	50	23	38	28	35	30	27	44
TARP SFUs	1 ± 1	8 ± 8	9 ± 9	2 ± 2*	4 ± 5	8 ± 9	5 ± 7	6 ± 6	5 ± 8	6 ± 5
TARP % of responders	55	54	61	50	58	50	62	30	54	56
MUC1 SFUs	2 ± 2	9 ± 12	12 ± 13	3 ± 1	5 ± 5	12 ± 16	7 ± 11	5 ± 6	7 ± 12	6 ± 6
MUC1 % of responders	45	67	67	50	65	44	62	40	58	56
SUR SFUs	2 ± 1	10 ± 15	10 ± 15	3 ± 4	4 ± 4	11 ± 18	8 ± 13	5 ± 1	8 ± 16	5 ± 4
SUR % of responders	50	58	78	38	54	56	59	40	54	56

Total n=22. Data shown as means ± SD SFUs/250,000 PBMCs. * p<0.05 between lifestyle groups.

$\dot{V}O_2$ max; maximal oxygen consumption. mmHg; millimoles of mercury. MamA; mammaglobin-A. CEA; carcinoembryonic antigen. Cl6; claudin-6. CycB1; cyclin-B1. ERB ECD; receptor tyrosine-protein kinase erbB-2 extracellular domain. ERB ICD; receptor tyrosine-protein kinase erbB-2 intracellular domain. MMP11; stromelysin-3. TARP; TCRgamma alternate reading frame protein. MUC1; mucin-1. SUR; survivin. SFUs; spot forming units per 250,000 PBMCs. CMV; cytomegalovirus, IgG; Immunoglobulin G. IU; international units. L; litres

No significant differences were seen in number of T-lymphocytes responding to any of the tumour-associated antigens in those overweight (as determined by BMI, body fat percentage or W:H) and those of normal weight ($p>0.05$) (Table 22.). It was observed that in CMV seropositive individuals, there was a significantly higher number of women who responded to tumour-associated antigen SUR that were overweight as determined by BMI (82%) versus normal weight 27% (χ (1) = 6.600, p = .010). When classifying women by W:H, those with a higher W:H (≥ 0.80) had a significantly higher proportion of women who responded to CEA (100% versus 46%) (χ (1) = 7.108, p = 0.008). No other significant differences were found between groups split according to BMI, body fat percentage and W:H. Women who had a Z-score ≤ 0.0 had a significantly higher number of women who responded to ERB ECD with 79% of women responding compared to 33% of CMV seropositive women who had a Z-score over 0 (χ (1) = 5.238, p = .022) (Table 22.) No significant correlations were found between any body composition variables and T-lymphocyte response to tumour-associated antigens.

Table 22. Differences in number of T-lymphocytes responding to tumour-associated antigen stimulation and the % of women within each group that had a positive response to tumour-associated antigens in CMV seropositive individuals only, grouped by body compositional thresholds

	BMI (kg.m2)		**DEXA measured body fat (%)**		**W:H**		**Z-score**	
	< 25.0 (n=11)	***≥ 25.0 (n=11)***	***≤ 35 (n=13)***	***> 35 (n=9)***	***< 0.80 (n=13)***	***≥ 0.80 (n=9)***	***≤ 0.0 (n=7)***	***> 0.0 (n=15)***
MamA SFUs	4 ± 4	5 ± 5	5 ± 5	5 ± 4	4 ± 4	6 ± 5	6 ± 5	4 ± 3
MamA % of responders	64	82	77	67	69	78	86	67
CEA SFUs	10 ± 17	3 ± 2	8 ± 15	4 ± 3	3 ± 3	9 ± 16	12 ± 19	3 ± 2
CEA % of responders	68	64	73	56	46	100*	86	57
Cl6 SFUs	3 ± 2	5 ± 5	3 ± 2	7 ± 5	4 ± 3	4 ± 5	4 ± 3	4 ± 4
Cl6 % of responders	55	45	62	33	50	56	36	57
CycB1 SFUs	5 ± 6	8 ± 12	6 ± 11	9 ± 7	7 ± 10	9 ± 9	4 ± 6	9 ± 12
CycB1 % of responders	50	68	65	50	73	39	79	50
ERB ECD SFUs	9 ± 9	7 ± 8	7 ± 8	10 ± 8	6 ± 10	9 ± 7	7 ± 7	9 ± 9
ERB ECD % of responders	32	64*	58	33	35	67	79	33*
ERB ICD SFUs	7 ± 18	6 ± 10	6 ± 10	8 ± 8	6 ± 10	8 ± 8	6 ± 9	7 ± 10
ERB ICD % of responders	50	82	65	67	65	67	79	60
MMP11 SFUs	2 ± 1	4 ± 5	1 ± 1	6 ± 6	2 ± 1	6 ± 6	1 ± 0	4 ± 5
MMP11 % of responders	32	36	35	33	35	33	36	33
TARP SFUs	2 ± 2	8 ± 9	5 ± 7	7 ± 6	5 ± 6	6 ± 6	4 ± 4	6 ± 9
TARP % of responders	50	59	62	44	62	44	64	50
MUC1 SFUs	3 ± 2	9 ± 12	6 ± 11	8 ± 7	8 ± 12	6 ± 6	4 ± 3	10 ± 13
MUC1 % of responders	41	73	65	44	58	56	79	47
SUR SFUs	5 ± 5	8 ± 14	8 ± 16	6 ± 4	9 ± 15	5 ± 5	5 ± 4	9 ± 15
SUR % of responders	27	82*	54	56	54	56	72	47

Total n=22. Data shown as means ± SD SFUs/250,000 PBMCs. * p<0.05 between lifestyle groups.

BMI; body mass index. W:H; waist to hip ratio. kg; kilogram. m; metres. MamA; mammaglobin-A. CEA; carcinoembryonic antigen. Cl6; claudin-6. CycB1; cyclin-B1. ERB ECD; receptor tyrosine-protein kinase erbB-2 extracellular domain. ERB ICD; receptor tyrosine-protein kinase erbB-2 intracellular domain. MMP11; stromelysin-3. TARP; TCRgamma alternate reading frame protein. MUC1; mucin-1. SUR; survivin. SFUs; spot forming units per 250,000 PBMCs.

No significant differences were seen in CMV seropositive individuals who were grouped by PAL or step count and their T-lymphocyte response to tumour-associated antigen both in terms of magnitude and ability to produce a response on stimulation (Table 23.) ($p>0.05$). In those who were less active as determined by PAL, MamA and CEA were the most immunodominant antigens with 88% of women responding whilst in those more active ERB ICD was the most immunodominant. When activity was defined by step count per day this trend continued whereby MamA was the most immunodominant in those less active (100% responded) and ICD in those more active (Table 23.). The only significant correlation between a measure of physical activity and T-lymphocyte response to tumour-associated antigen was seen between step count and magnitude of T-lymphocyte response to MamA ($r=0.475$, $p=0.040$, $n=19$, $r_2=0.226$).

Table 23. Differences in number of T-lymphocytes responding to tumour-associated antigen stimulation and the % of women within each group that had a positive response to tumour-associated antigens in CMV seropositive individuals only, grouped by physical activity thresholds

	PAL		**Steps per day**	
	< 1.70 (n=8)	***≥ 1.70 (n=13)***	***< 10,000 (n=6)***	***≥ 10,000 (n=11)***
MamA SFUs	3 ± 2	6 ± 5	3 ± 2	5 ± 4
MamA % of responders	88	61	100	82
CEA SFUs	10 ± 18	3 ± 2	3 ± 3	4 ± 3
CEA % of responders	88	50	83	72
Cl6 SFUs	3 ± 3	5 ± 4	3 ± 4	7 ± 5
Cl6 % of responders	25	62	33	73
CycB1 SFUs	5 ± 7	9 ± 12	2 ± 2	9 ± 7
CycB1 % of responders	50	62	50	82
ERB ECD SFUs	7 ± 7	9 ± 9	3 ± 1	10 ± 8
ERB ECD % of responders	75	35	67	59
ERB ICD SFUs	6 ± 9	7 ± 10	2 ± 1	8 ± 8
ERB ICD % of responders	75	65	83	86
MMP11 SFUs	1	4 ± 4	1	6 ± 6
MMP11 % of responders	6	50	8	59
TARP SFUs	5 ± 4	6 ± 9	5 ± 4	7 ± 6
TARP % of responders	44	62	58	73
MUC1 SFUs	4 ± 3	11 ± 14	3 ± 4	8 ± 7
MUC1 % of responders	75	42	83	59
SUR SFUs	4 ± 4	10 ± 16	3 ± 2	6 ± 4
SUR % of responders	75	46	83	64

Total n=15. Data shown as means ± SD SFUs/250,000 PBMCs. * p<0.05 between lifestyle groups.

PAL; physical activity level. MamA; mammaglobin-A. CEA; carcinoembryonic antigen. Cl6; claudin-6. CycB1; cyclin-B1. ERB ECD; receptor tyrosine-protein kinase erbB-2 extracellular domain. ERB ICD; receptor tyrosine-protein kinase erbB-2 intracellular domain. MMP11; stromelysin-3. TARP; TCRgamma alternate reading frame protein. MUC1; mucin-1. SUR; survivin. SFUs; spot forming units per 250,000 PBMCs.

In CMV seropositive individuals who scored 0 versus those who scored over 0 in assessment of depression, those with higher levels of depression, on average, had significantly more TARP specific T-lymphocytes (11 ± 9 SFUs/250,000 PBMCs versus 2 ± 2 SFUs/250,000 PBMCs in those who scored 0) ($F(1,12) = 5.294$, $p=0.039$). Furthermore, the number of women responding to tumour-associated antigen Cl6 significantly differed between groups. In those who were more depression (scoring over 0) only 15% of women responded compared to 79% of women in the group that scored 0 ($X (1) = 8.824$, $p = 0.003$) (Table 24.). No other significant differences were seen between groups split in terms of levels of depression, anxiety and stress ($p>0.05$) (Table 24.). No significant correlations

were found between depression and anxiety and the response to tumour-associated antigens. Significant correlations were found between stress and magnitude of T-lymphocyte response to MamA and SUR ($r=0.452$, $p=0.045$, $n=19$, $r_2=0.204$ and $r=0.691$, $p=0.013$, $n=13$, $r_2=0.478$).

Table 24. Differences in number of T-lymphocytes responding to tumour-associated antigen stimulation and the % of women within each group that had a positive response to tumour-associated antigens in CMV seropositive individuals only, grouped by psychological thresholds

	Depression		Anxiety		Stress	
	0 (n=11)	***> 0 (n=10)***	***0 (n=13)***	***>0 (n=8)***	***0 (n=7)***	***>0 (n=14)***
MamA SFUs	5 ± 5	4 ± 2	4 ± 4	6 ± 5	2 ± 1	6 ± 5
MamA % of responders	67	80	79	63	71	79
CEA SFUs	3 ± 2	10 ± 18	3 ± 3	11 ± 20	3 ± 3	7 ± 15
CEA % of responders	63	70	61	75	57	75
Cl6 SFUs	3 ± 2	7 ± 9	3 ± 2	7 ± 5	4 ± 3	4 ± 4
Cl6 % of responders	79	15*	50	50	36	61
CycB1 SFUs	3 ± 2	12 ± 13	7 ± 11	6 ± 7	1 ± 0	8 ± 10
CycB1 % of responders	63	55	61	56	29	79
ERB ECD SFUs	3 ± 2	10 ± 9	8 ± 8	8 ± 7	3 ± 1	11 ± 9
ERB ECD % of responders	33	65	50	44	57	46
ERB ICD SFUs	3 ± 2	10 ± 12	8 ± 11	5 ± 3	2 ± 1	8 ± 10
ERB ICD % of responders	67	65	64	69	57	75
MMP11 SFUs	2 ± 1	7 ± 8	1 ± 1	5 ± 5	1 ±	3 ± 4
MMP11 % of responders	46	20	25	50	7	50
TARP SFUs	2 ± 2	11 ± 9*	5 ± 7	6 ± 8	3 ± 2	7 ± 8
TARP % of responders	58	50	64	38	57	57
MUC1 SFUs	3 ± 2	10 ± 13	7 ± 11	7 ± 9	3 ± 1	9 ± 12
MUC1 % of responders	50	65	71	31	57	61
SUR SFUs	2 ± 51	12 ± 16	8 ± 14	4 ± 4	1 ± 1	10 ± 14
SUR % of responders	50	60	64	38	57	57

Total n=21. Data shown as means ± SD SFUs/250,000 PBMCs. * p<0.05 between lifestyle groups.

MamA; mammaglobin-A. CEA; carcinoembryonic antigen. Cl6; claudin-6. Cyc1B; cyclin-B1. ERB ECD; receptor tyrosine-protein kinase erbB-2 extracellular domain. ERB ICD; receptor tyrosine-protein kinase erbB-2 intracellular domain. MMP11; stromelysin-3. TARP; TCRgamma alternate reading frame protein. MUC1; mucin-1. SUR; survivin. SFUs; spot forming units per 250,000 PBMCs.

4.4 DISCUSSION

The aim of this study was to identify differences in immune responses towards tumour-associated and viral antigens in CMV seropositive and CMV seronegative healthy women and identify differences in lifestyle and psychological variables between these groups. A further aim was to investigate if differences in the lifestyles of CMV seropositive women are related to T-lymphocyte response to tumour-associated and viral antigens. No significant differences were identified between lifestyles of CMV seropositive and seronegative individuals however, an increased level of stress in CMV seropositive women was observed. Resistin levels were beneficially significantly lower in seronegative women but no other differences in cell counts or circulating serum markers were found. No significant differences were found between CMV seropositive and seronegative women in terms of T-lymphocyte response to viral antigens. For each of the 10 tumour-associated antigens, a higher proportion of CMV seropositive women demonstrated a positive response. This was statistically significantly higher in 6/10 tumour-associated antigens. In terms of magnitude of T-lymphocyte response, a significant difference was found between CMV seropositive and seronegative women towards ERB ICD which was higher in CMV seropositive women. In CMV seropositive women only, CMV specific IgG levels correlated with age. Furthermore, when these women were grouped using lifestyle characteristics, characteristics of healthier lifestyles (lower body fat, increased fitness, decreased depression, younger, higher BMD) there were small associations towards a lower proportion of women responding to tumour-associated antigens and a lower magnitude of response towards tumour-associated and viral antigens when compared to those with less healthy lifestyles.

In the present study, T-lymphocyte IFN-γ secretion in response to tumour-associated antigens was significantly higher towards 1/10 tumour-associated antigen and significantly more individuals responded to 6/10 tumour-associated antigens out of those who were CMV seropositive compared to seronegative. Whilst not statistically significantly different, the proportion of responses continued to be higher in seropositive individuals towards the other 4 tumour-associated antigens. It is thought that higher responses to tumour-associated antigens may protect individuals from cancer as previous studies in breast cancer patients have shown improved prognosis in individuals who elicit higher immune responses to tumour-associated antigens (Tiriveedhi *et al.*, 2014; Muraro *et al.*, 2015a; Blixt *et al.*, 2011) and the ability of the immune system to detect and respond to tumour cells is an important cancer defence mechanism (Hanahan and Weinberg, 2011; Vigneron, 2015; Steer *et al.*, 2010). Our initial hypothesis stated that due to the acceleration of immunosenescence in

CMV seropositive individuals, and the importance of immune defence against cancer, CMV seropositive women would demonstrate reduced responses to tumour-associated antigens. The increased response to tumour-associated antigens in the current study may demonstrate CMV positive individuals possess pre-existing memory T-lymphocytes towards tumour-associated antigens. It may be that these individuals may have an ability to respond to tumour-associated antigens compared to those who are CMV seronegative who may indeed possess more naïve T-lymphocytes but less memory T-lymphocytes specific to tumour-associated antigens (perhaps due to no prior exposure), but due to the nature of the assay used this is not measured in the current study.

Interestingly, significant correlations were observed between responses to CMV peptide IE1, pp65 and CMV specific IgG, suggesting as humoral immunity increases, so too does cellular immunity. Evidence has demonstrated that individuals who are CMV seropositive have higher frequencies of CMV-specific IFN-γ producing memory T-lymphocytes compared to seronegative individuals (Lúcia *et al.*, 2014), however no further analysis was taken to look within the IgG levels of seropositive individuals. IE1 represents the immediate early phase of the virus life cycle (Zhu *et al.*, 1995), whereas pp65 represents the late phase (Hislop *et al.*, 2007). Previous studies have suggested that the primary target for CMV specific CD8+ T-lymphocytes is protein pp65 (Diamond *et al.*, 1997; Kern *et al.*, 2002; La Rosa *et al.*, 2001; Wills *et al.*, 1996), with increased IFN-γ release in response to this protein as assessed by flow-based CD107a/b degranulation/mobilization and intracellular cytokine assays (Lacey *et al.*, 2006). However, the findings of the present study suggest that as CMV-specific IgG increases, so too does T-lymphocyte response to both the early and late phase CMV peptides in ELISpot which helps support the belief that the overnight ELISpot predominantly measured memory T-lymphocyte responses, rather than naïve.

The present study also showed an increase in CMV specific IgG and IFN-γ secretion in response to CMV peptide IE1 with increasing age. It is likely that the increase in CMV specific IgG with increasing age is down to the cumulative effect of viral reactivations, with an increased number of viral reactivations with time, leading to inflammation and greater expansion of CMV specific cells (Chiu *et al.*, 2016; Söderberg-Nauclér, 2006). It has previously been observed that CMV IgG titers increase with age in healthy individuals (Parry *et al.*, 2016) (r=0.479 p=0.001) and patients (Yang *et al.*, 2018) (r=0.15, p=0.003), similarly to the results from the current study (r=0.646, p=0.01). In a study of 1,468 elderly individuals, those with CMV IgG antibody titres in the highest quartile had a 1.43 times higher risk of ACM compared with lower quartiles (Roberts *et al.*, 2010), even after adjusting

for a number of covariates such as age and biological sex. A nested-case control study demonstrated higher mean CMV specific IgG levels in individuals who went on to be breast cancer patients compared to controls (Richardson *et al.*, 2004; Cox *et al.*, 2010). This suggests lower IgG levels may be protective of breast cancer, potentially due to the increased naïve T-lymphocyte pool available for recognition of tumour-associated antigens. It is therefore not surprising, given the robust link between breast cancer risk and ageing (Benz, 2008), with risk rising exponentially until menopause more slowly thereafter, that in the present study a relationship is observed between increasing age and increasing IgG antibody titre. It is also possible that the higher IgG levels may be caused by late exposure to CMV (Richardson, 1997) or recent reactivation (Stowe *et al.*, 2007; Trzonkowski *et al.*, 2003).

Furthermore, research has demonstrated ageing is associated with the expansion of CMV specific CD8+ T-lymphocytes (Stowe *et al.*, 2007), therefore suggesting that the magnitude of CD8+ T-lymphocyte response to CMV antigens may be larger in aged individuals. In healthy individuals the frequency of CD8+ T-lymphocytes responding to CMV antigen pp65 has shown to increase with age, but not the frequency of T-lymphocytes responding to IE1 (Parry *et al.*, 2016). However, this study included healthy participants over 70 years old unlike the current study and when analysed further the number of T-lymphocytes recognising IE1 initially increased with age up to 50-60 years (the maximum age in the current study) and then began to decrease (Parry *et al.*, 2016). Therefore, reasons for this trend not to be observed in the current study may be due discrepancies may be due to the lower maximum age of participants in the current study. Other studies have demonstrated an increase in the number of CMV specific T-lymphocytes in peripheral blood with ageing (Khan *et al.*, 2002; Pourgheysari *et al.*, 2007). If T-lymphocyte response to CMV antigens also correlates with IgG levels, which in turn correlates with age, it can be hypothesised that a higher number of T-lymphocytes responding to CMV antigens represents an increased number of CMV specific memory T-lymphocytes. The T-lymphocytes may in turn take up most of the immune compartment, consequently leaving less ‘space’ for other T-lymphocytes. The increased number of IFN-γ secreting T-lymphocytes in response to CMV peptide IE1 with ageing also suggests that, due to the increased response of CMV specific cells, there is a reduction in the number of naïve T-lymphocytes. This may be detrimental to future immune challenges (Leng *et al.*, 2011), again aligning with the increased risk of breast cancer observed with ageing (Dixon, 2012; Ory *et al.*, 2014; Races, 2002; Singletary, 2003).

Regarding haematological measures such as cell counts and circulating markers in the blood, a significant difference was observed in circulating resistin in those who were CMV seropositive compared to those who were seronegative. Resistin is an adipokine and thus increases with increasing adiposity (Wijetunge *et al.*, 2019; Nieva-Vazquez *et al.*, 2014). Whilst no significant differences in body composition were observed in the current study there was a tendency for BMI and DEXA measured body fat % to be slightly higher in CMV seropositive individuals, which may explain the increase in resistin seen in this group. Resistin has also been linked with a roles in immune function (Cohen *et al.*, 2008) and pro-inflammatory cytokines (Reilly *et al.*, 2005; Bokarewa *et al.*, 2005; Silswal *et al.*, 2005). Whilst is has been shown that resistin can modulate dendritic cell function, impairing antigen uptake and therefore the proliferation and differentiation of T-lymphocytes (Jang *et al.*, 2009) while simultaneously upregulating Treg cells (Son *et al.*, 2010), in the current study little differences in T-lymphocyte responses to viral antigens were observed.

In terms of lifestyle measurements, differences in individuals who are CMV seropositive and CMV seronegative were overall absent in the current study. This may be explained by the lack of extreme values in the population; only 5/50 participants (10%) with a BMI in the obese category (>30kg.m$_2$), 2/50 participants (4%) with a predicted $\dot{V}O_2$ max within the 10$_{th}$ percentile based on age and only 4/50 (8%) of women with a DEXA assessed body fat percentage over 40%. Furthermore, it may be the case that there are very few lifestyle differences between CMV seropositive and CMV seronegative women, as little research has been done in this area to support this.

Stress was significantly higher in those who are CMV seropositive than those who were seronegative. Associations between stress and CMV IgG have previously been made, with stress promoting reactivation of CMV (Rector *et al.*, 2014), however it is less well known whether stress levels are affected by CMV serostatus. It must be considered that stress levels for both groups were still within 'normal' ranges, therefore it is difficult to draw any firm conclusions. Given the relationship between stress, ageing and immunity (Vitlic *et al.*, 2014; Graham *et al.*, 2006) and the likely acceleration of immunosenescence in CMV seropositive individuals (Pawelec and Derhovanessian, 2011), this finding may be particularly important. Psychological stress can exacerbate and speed up the detrimental effects of ageing on the immune function (Kiecolt-Glaser and Glaser, 2001; Sapolsky *et al.*, 1986) such as decreased function of T-lymphocytes (Bartrop *et al.*, 1977), immobilisation of lymphocytes (Domínguez-Gerpe and Rey-Méndez, 2001), worsened lymphocyte proliferation after mitogenic stimulation (Kiecolt-Glaser *et al.*, 1987) and lower CD4+/Treg

ratio (Pariante *et al.*, 1997) in part mediated by the action of glucocorticoids (Vitlic *et al.*, 2014; Sorrells and Sapolsky, 2007). Therefore, CMV seropositive women should be aware of, and take action to, minimise and reduce their personal stress to minimise any stress induced immune dysregulation which may accelerate immune ageing.

Interestingly, when looking at CMV seropositive women alone, all significant differences in response to viral and tumour-associated antigens followed the same trend; individuals with characteristics of unhealthier lifestyles (assessed through fitness, diet, body composition) demonstrated a higher number of T-lymphocyte responding cells to viral and tumour-associated antigens, or a higher proportion of women able to respond to tumour-associated antigens. This suggests that unhealthy lifestyles are may be associated with higher T-lymphocyte responsiveness to tumour-associated antigens, however from Chapter 3 in this thesis it is demonstrated that this is not necessarily the case. It has previously been predicted that cancer risk increases with excess weight and a sedentary lifestyle through various mechanisms (McTiernan, 2008). Perhaps individuals with 'unhealthier' lifestyles may have had increased prior exposure to cancer cells and tumour-associated antigens compared to their healthier counterparts. The reason they have remained free from diagnosis could be down to the ability of their immune system to detect and destroy these cells as determined by high T-lymphocyte responses. Conversely, in those who lead healthy lifestyles, it may be that there has been little or no exposure to tumour-associated antigens, therefore few or no memory T-lymphocytes reside within the body and thus smaller or absent memory T-lymphocyte responses exist. Further investigation is needed to confirm these findings but, if correct, lifestyle interventions can be developed to reduce exposure to tumour-associated antigens which may be monitored through enumerating the number of tumour-associated antigen specific memory T-lymphocytes.

As total PBMCs were used in the ELISpot assay of the current study we are unable to confirm whether the IFN-γ secreting cells are of naïve or memory phenotype. Due to the short incubation (~16h) of antigens with PBMCs and the large spot counts witnessed towards CMV peptides pp65 and IE1 (mean 149 and 122 spots per 250,000 PBMCs respectively) in CMV seropositive individuals, it is concluded that it is likely this response is a memory response. Future research should determine the phenotype of T-lymphocytes responding within the assay to confirm whether responses are memory or naïve driven. Further research should also use CD4+ depleted PBMC samples to asses CD8+ responses only towards both peptides and vice versa so that specific types of cell can be targeted via lifestyle interventions to improve cancer and viral immunosurveillance. To identify whether

differences do exist in lifestyle of CMV seropositive and negative individuals, future research should include women with more extreme lifestyle characteristics.

In conclusion, CMV seropositive individuals had consistently higher responses to tumour-associated antigens when compared with seronegative individuals. Within CMV seropositive individuals, higher responses to tumour-associated antigens were associated with less healthy lifestyle characteristics. It is speculated that whilst unhealthy lifestyles are a risk factor for cancer, in this population, strong anti-cancer T-lymphocyte responses are responsible for individuals remaining free from cancer diagnosis.

CHAPTER 5: Changes in lifestyle, leukocyte counts and T-lymphocyte IFN-γ release to viral and tumour-associated antigens in 6 neoadjuvant breast cancer patients

5.1 INTRODUCTION

Breast cancer is the most common female malignancy in the world and is the primary cause of death among women globally (Benson and Jatoi, 2012). Many treatment options are available, but since the 1980s, neoadjuvant chemotherapy has often been prescribed for locally advanced breast cancer patients (Mieog *et al.*, 2007). Whilst male breast cancer does exist, it only accounts for 390 cases diagnosed in the UK each year (CRUK, 2017).

Breast cancer treatment can be detrimental to multiple aspects of lifestyle and psychological function including cardiorespiratory fitness, physical activity levels, diet, body composition and levels of depression, anxiety and stress (Jones *et al.*, 2012; Dieli-Conwright and Orozco, 2015; Schmitz *et al.*, 2015), which in turn can increase the risk of comorbidities (Jones *et al.*, 2009). For example, it is reported that patients gain weight during chemotherapy (Demark-Wahnefried *et al.*, 2001; Vance *et al.*, 2011; Gadea *et al.*, 2012) with estimates of 35-96% women experiencing significant weight gain by ~1.4 - 6.2kg (Demark-Wahnefried *et al.*, 1997b; Demark-Wahnefried *et al.*, 1997a; Ingram and Brown, 2004; Freedman *et al.*, 2004; Harvie *et al.*, 2004; Campbell *et al.*, 2007; Makari-Judson *et al.*, 2007). These increases in body fat can have negative influences on health and disease prognosis irrespective of baseline BMI (Caan *et al.*, 2012b; Ghose *et al.*, 2015; Bradshaw *et al.*, 2012; Chan *et al.*, 2014; Playdon *et al.*, 2013; Rodríguez *et al.*, 2013; Goodwin, 2015b; Brenner *et al.*, 2016).

Higher levels of physical activity have been shown to improve survival outcomes in breast cancer patients with a range of diagnoses, with a 45% lower risk of death in women who increased activity after diagnosis compared to those who were inactive (Irwin *et al.*, 2008a; Chlebowski, 2013; Dieli-Conwright and Orozco, 2015; Courneya *et al.*, 2014). A randomised controlled trial in 242 breast cancer patients demonstrated eight-year disease-free survival was 82.7% in those who exercised for the duration of their chemotherapy (either aerobic exercise on a treadmill or ergometer three times a week at 60-80% $\dot{V}O_2$max or resistance exercise comprising of two sets of 8-12 repetitions of nine exercises at 60-70% 1 repetition max) compared to 75.6% for those who did not (Courneya *et al.*, 2014). More recent literature confirmed this and concluded that there was with a 44% exercise-associated risk reduction in ACM and a reduction in breast cancer death for tumours <2 cm (Ammitzbøll *et al.*, 2016; Jones *et al.*, 2016). Evidence recommends breast cancer patients should

undertake moderate-intensity aerobic exercise at least 3 times per week, for at least 30 minutes to ease the side effects of treatment and improve health and should 'avoid inactivity' (Campbell *et al.*, 2019a).

Murine studies suggest exercise can directly control cancer progression through effects on tumour growth rate, metastasis, metabolism, and immunogenicity through interplay with systemic factors which may reduce adverse events related to cancer and its treatment, and improve treatment efficacy (Hojman *et al.*, 2018). Whilst this relates to acute bouts of exercise, maintenance of fitness through exercise and physical activity throughout treatment may create cumulative protective bouts of acute exercise. The postulated benefits of exercise may provide a potential modifiable method to optimise treatment.

Measuring aspects of lifestyle in clinical settings poses a challenge to clinicians due to limited time and resource. The IPAQ questionnaire is a quick and simple tool to assess individual physical activity. However, a review concluded that correlations between the total physical activity level measured by IPAQ and objective standards had large ranges; with none reaching the minimal acceptable standard. It was concluded that IPAQ overestimated physical activity level by an average of 84% (Lee *et al.*, 2011a). In cancer patients undergoing chemotherapy, self-reported IPAQ reported significantly higher levels of moderate and vigorous physical activity when compared to Sensewear objectively measured activity by ~366% (Vassbakk-Brovold *et al.*, 2016) demonstrating that the IPAQ may be insufficient to assess physical activity in a variety of cancer patients.

The non-invasive bioelectrical impedance is often implemented to track body composition in cancer patients. Importantly measures of fat mass have been correlated with nutritional status, prognosis and survival in cancer patients (Crawford *et al.*, 2009; Halpern-Silveira *et al.*, 2010; Gupta *et al.*, 2008; Norman *et al.*, 2010; Grundmann *et al.*, 2015). Whilst DEXA is the current reference method for the assessment body composition, limitations such as safety, limited number of scans per year, cost, and technical expertise (Marra *et al.*, 2019) make bioelectrical impedance an appealing alternative. Thus, a comparison between the two methods should be made within the breast cancer population.

Beneficial effects of exercise and physical activity on cancer may be mediated through the immune system (McTiernan,2008). The immune system plays a pivotal role in breast cancer as demonstrated by the importance of tumour-infiltrating lymphocytes in treatment prognosis (Zgura *et al.*, 2018) whereby low numbers of T-lymphocytes within the tumour-

microenvironment are linked with poor effectiveness of chemotherapy and worsened survival. In cancer patients, T-lymphocyte response to various tumour-associated antigens have been linked to longer survival and improved prognosis (Cobbold *et al.*, 2013b; Muraro *et al.*, 2015a; Muraro *et al.*, 2011; Inokuma *et al.*, 2007b; Roscilli *et al.*, 2014b; Epel *et al.*, 2008; Stadler *et al.*, 2016; Kao *et al.*, 2001; Criscitiello, 2012; Galvis-Jimenez *et al.*, 2013), (Table 25). Leukaemia patients have demonstrated reduced or absent CD8+ T-lymphocyte IFN-γ response to phosphopeptides after ex vivo ELISpot compared to healthy controls (Cobbold *et al.*, 2013b) and in HER2 positive breast cancer patients undergoing neoadjuvant chemotherapy, IFN-γ CD8+ T-lymphocyte responses in those exhibiting a pCr were significantly higher in response to tumour-associated antigens MamA and Survivin. This evidence suggests that breast cancer patients demonstrating a pCr to treatment exhibit an increased functionality in immune response to tumour cells, thus priming the immune response may benefit disease prognosis.

Table 25. Previous literature evidencing the protective role of T-lymphocyte response to tumour-associated antigens

Author	**Cancer type**	**Tumour-associated antigen**
Kao et al 2001	Breast adenocarcinoma	Cyclin B1
Inokuma et al 2007	Breast cancer	CEA, HER-2, MAGE-A3
Epel et al 2008	Breast and prostate cancer	TARP
Muraro et al 2011	Locally advanced breast cancer	HER-2
Cobbold et al 2013	Leukaemia	61 tumour-specific phosphopeptides
Galvis-Jimenez et al 2013	Breast cancer	Mammaglobin
Roscilli et al 2014	Breast and prostate cancer	MMP11
Muraro et al 2015	Breast cancer	Survivin, MamA, HER-2

CEA; carcinoembryonic antigen. HER-2; Human epidermal growth receptor. MAGE-A3; Melanoma associated antigen 3, TARP; TCR gamma alternative reading frame protein. MMP11; Metalloproteinase matrix 11. MamA; Mammaglobin-A

Evidence suggests that some chemotherapy regimens further contribute to the generation of an anti-cancer T-lymphocyte activity (Kroemer *et al.*, 2013). Chemotherapy causes cell death which may result in dendritic cells processing the antigen from the dead cancer cells and presenting it to T-lymphocytes, which in turn recognise and respond, surveying the body for other cells presenting such antigen. This process is termed immunogenic cell death (Kroemer *et al.*, 2013; Green *et al.*, 2009). Anthracyclines (such as Doxorubicin) used in chemotherapy, have been shown to initiate this process by enhancing tumour-antigen proliferation of CD8+ T-lymphocytes in tumour draining lymph nodes and promoting tumour

infiltration of IFN-γ secreting CD8+ T-lymphocytes (Ghiringhelli *et al.*, 2009; Mattarollo *et al.*, 2011b). However, clinical responses to chemotherapy vary between individual patients, suggesting that the efficiency of anti-tumour $CD8^+$ T-lymphocytes induced by chemotherapy might vary from patient to patient.

Despite the immune system becoming a focus for mainstream cancer researchers, it has, until now, never been robustly investigated whether the relationships between cancer and lifestyle can be explained by interactions with aspects of immune function. Modifiable factors such as diet and exercise may be able to influence the magnitude and effectiveness of breast cancer treatment through anti-cancer immune mechanisms. The aim of this study is to examine if T-lymphocyte IFN-γ response to tumour-associated antigens in patients is related to both lifestyle factors and clinical response. It is hypothesised that stronger immune responses will be seen in patients with a higher predicted $\dot{V}O_2$ max, lower BMI and lower percentage body fat, and those that eat a healthier diet (less saturated fats and sugars). Furthermore, we hypothesise that in breast cancer patients, CD8+ T-lymphocyte responses to tumour-associated antigens are linked with improved treatment outcomes (e.g. greater reduction in tumour size and improved pathological response). A further aim of the study is to decipher whether the ability of CD8+ T-lymphocytes to recognise tumour-associated antigens is related with lifestyle factors such as fitness and body composition change from baseline, mid-chemotherapy and post-chemotherapy.

Whilst the previous introduction highlights the initial aims of the study, it was not feasible to answer the questions posed in the time frame of the current PhD. The feasibility of the research will be reported and discussed within the upcoming results and discussion sections. As the initial questions surrounding the relationships between T-lymphocyte IFN-γ response to tumour-associated antigens and lifestyle factors and/or clinical response could not be answered within the scope of the data collected, the focus of the current chapter will be on the individual changes in lifestyle factors, blood cell counts and T-lymphocyte responses to tumour-associated and viral antigens with reference to individual clinical outcomes. Furthermore, this chapter will act as a feasibility paper, commenting on the challenges faced in clinical studies whilst introducing some of the main themes.

5.2 METHODS

5.2.1 Participants and study design

Patient volunteers (41 ± 8 years, BMI 26.9 ± 4.1 kg.m$_2$, predicted $\dot{V}O_2$ max 29.8 ± 3.8 ml.kg.min^{-1} at baseline, Table 26.), were recruited following oncologist consultation to take part in a longitudinal study. Inclusion criteria were; female, aged between 25-69 years (range of age of patient volunteers in the current study was 30-49 (Table 26.)), had a biopsy-proven stage I-III, non-metastatic, invasive unilateral breast cancer with a planned treatment of neoadjuvant chemotherapy, patients had a WHO (World Health Organisation) performance status of 0-1 (as assessed by oncologist), adequate renal, liver and bone marrow function and were free from cardiovascular disease. Ethical approval was granted by a local NHS research ethics committee (reference: 15/SW/0004). Patients were informed verbally and in writing about the rationale, nature and demands of the study (and of their right to withdraw) before providing written informed consent. Patients subsequently completed a general health questionnaire, a physical activity readiness questionnaire, a sex specific cancer risk factor questionnaire, depression, anxiety and stress questionnaire (UNSW, 2014) and the IPAQ (Committee, 2016).

Table 26. Baseline characteristics of participants

Participant	Age (years)	Height (m)	Weight (kg)	BMI (kg.m$_2$)	Predicted $\dot{V}O_2$ max (ml.kg.min^{-1})	DEXA assessed body fat (%)
1	49	1.62	89.0	33.9	24.69	46.5
2	30	1.72	72.7	24.6	28.56	35.5
3	49	1.61	63.8	24.7	28.50	37.9
4	44	1.64	81.4	30.2	33.73	41.5
5	40	1.78	76.0	23.9	25.90	24.2
6	34	1.58	61.0	24.3	33.50	34.4

BMI; body mass index. $\dot{V}O_2$ Oxygen consumption. DEXA; dual energy x-ray absorptiometry. kg; kilograms. ml; millilitre. min; minute

5.2.2 Treatment

Ultra-sound, mammogram, tumour biopsy and histopathology were used to confirm tumour diagnosis. All patients underwent neoadjuvant chemotherapy via intravenous infusion with two schedules: 500mg/m_2 5-fluorouracil, 75mg/m_2 epirubicin and 500mg/m_2 cyclophosphamide followed by three cycles of Docetaxel 100mg/m_2. HER2 positive patients received Docetaxel in combination with Trastuzumab. Participants underwent 6 cycles of chemotherapy, once every 3 weeks, over a period of approximately 18 weeks, unless there were complications with side-effects or response to treatment was low.

5.2.3 Baseline and Follow-up Procedures

At baseline (after diagnosis, prior to first chemotherapy cycle (time point 1: diagnosis)) and follow-up (>3 weeks from final chemotherapy cycle, time point 3: post-chemotherapy) patients arrived at the laboratory between 07:00-11:00 following an overnight fast and after refraining from exercise, alcohol and caffeine in the previous 24 hours. Patients rested for 15 minutes in the supine position, during which heart rate and blood pressure were measured using an automated sphygmomanometer (Bosch and Sohn, Germany). Following this period of rest, a blood sample was collected by venepuncture from an antecubital vein. Approximately 40ml of blood was collected into a sterile syringe containing sodium heparin (2.0 international units/ml) for isolation of PBMCs. A further 10ml of blood was collected into a syringe free from anti-coagulant and aliquoted immediately into a 5ml EDTA vacutainer tube (Becton Dickinson, U.S), for preparation of plasma and a 5ml plain vacutainer tube (Becton Dickinson, U.S), for preparation of serum. At mid-chemotherapy (after 2 cycles of chemotherapy, immediately before the third cycle of chemotherapy: time point 2) a blood sample only was collected without any prior controls put in place.

Assessment of body composition, cardiorespiratory fitness, physical activity and habitual diet occurred at time point 1: diagnosis, and time point 3: post-chemotherapy. Methodology mirrored methodology in chapters 3 and 4 of the current thesis. To avoid repetition, methodology is not repeated within this chapter. As platelet to lymphocyte ratio and neutrophil to lymphocyte ratio have been used in previous clinical literature as prognostic markers in breast cancer (Cuello-Lopez *et al.*, 2018; Guo *et al.*, 2019), these were also calculated in the current study.

5.2.4 Clinical and pathological response

Clinical responses after 2 cycles of chemotherapy were defined using radiological imaging and physical examination. Pathological responses after surgery were defined by histopathology alone to check whether any cancerous cells remained. Tumour size was determined using radiological imaging or measurement on removal post-surgery.

Biochemical and immunological procedures were conducted in line with methodology of chapters 4 and 5. PBMCs were isolated, and the ELISpot assay was run on fresh cells. Due to the reduced number of samples no ELISAs were run on samples at this stage, therefore all data is presented regarding T-lymphocyte response to all antigens.

5.2.5 Statistical analysis

A purely descriptive approach was used on data when the full data set was not available (<6 data points per time point or variable). Where the full data set was available (n=6) non-parametric tests were used on data due to low sample size as previously recommended (Öztuna *et al.*, 2006). Wilcoxon signed ranks tests were used on all pre- and post- samples. Friedman's test for non-parametric data was used to compare data with three time points. As Friedman's test for non-parametric data is notoriously low powered and the robustness of ANOVA when data is non-normally distributed, ANOVAs were also conducted on data with 3 time points (Appendix 3) (Zimmerman and Zumbo, 1993). Statistical significance was accepted at $p < 0.05$. Standardised effect sizes (Cohen's d) were calculated for variables and CIs were reported. Bland Altman plots were employed to investigate agreement measurement techniques. The limits of agreement between different methods were defined as mean ± 1.96SD of the difference between the methods (95% CI). Statistical analyses were conducted using SPSS version 22. Data is shown throughout using individual data point shapes (defined in Table 28). Individuals with a pCr after surgery are shown in red. Individuals with a pPr after surgery are shown in blue. For the purposes of this thesis, cell counts reported in the results section are blood cell counts collected and analysed at RUH (Royal United Hospital) Bath and therefore it is unknown whether patients were in controlled conditions (fasted, 24h without alcohol, exercise and caffeine). The reason for this decision was to offer a complete data set for these values despite some cell count data points being missing due to unforeseen difficulty in collecting controlled samples at the University of Bath (discussed in results and discussion section of the current chapter).

5.3 RESULTS

5.3.1 Feasibility

The study was conducted in 1 centre (RUH, Bath). Recruitment for the current study took place between March 2018 and March 2019. During this time, 18 patients were diagnosed with breast cancer and scheduled neoadjuvant chemotherapy for treatment of disease (Figure 13.). Of these 18 patients, 6 (33%) were enrolled in the study and 4 patients (67% of all recruited) were retained throughout the study period (Figure 13). Reasons for not taking part included overbooking of clinics which led to a short time period between initial consultation and beginning of chemotherapy ~2 days, lack of time to take part, anxiety of diagnosis, priority to preserve fertility before onset of chemotherapy, lack of response to phone calls, changes in surgical techniques and competing clinical trials. Of those eligible, recruitment rate was predicted to be ~ 13 patients per year (~1 per month), ~20% enrolment. However, recruitment rate was much lower at 6 patients per year a higher enrolment (33%) (Table 27). Missing data is shown in Figure 1 Feasibility of recruitment and study design3.

Table 27. Actual recruitment compared to predicted recruitment

	Cumulative predicted number diagnosed	Cumulative actual number diagnosed	Cumulative predicted number recruited	Cumulative actual number recruited	Predicted recruitment rate (%)	Actual recruitment rate (%)
March 2018	4	3	1	2	20	67
April 2018	8	4	2	2	20	50
May 2018	12	5	3	3	20	60
June 2018	16	5	4	3	20	60
July 2018	20	5	5	4	20	80
August 2018	24	6	6	4	20	67
September 2018	28	6	7	5	20	83
October 2018	32	8	8	5	20	63
November 2018	36	10	9	5	20	50
December 2018	40	12	10	5	20	42
January 2019	44	13	11	6	20	46
February 2019	48	18	12	6	20	33

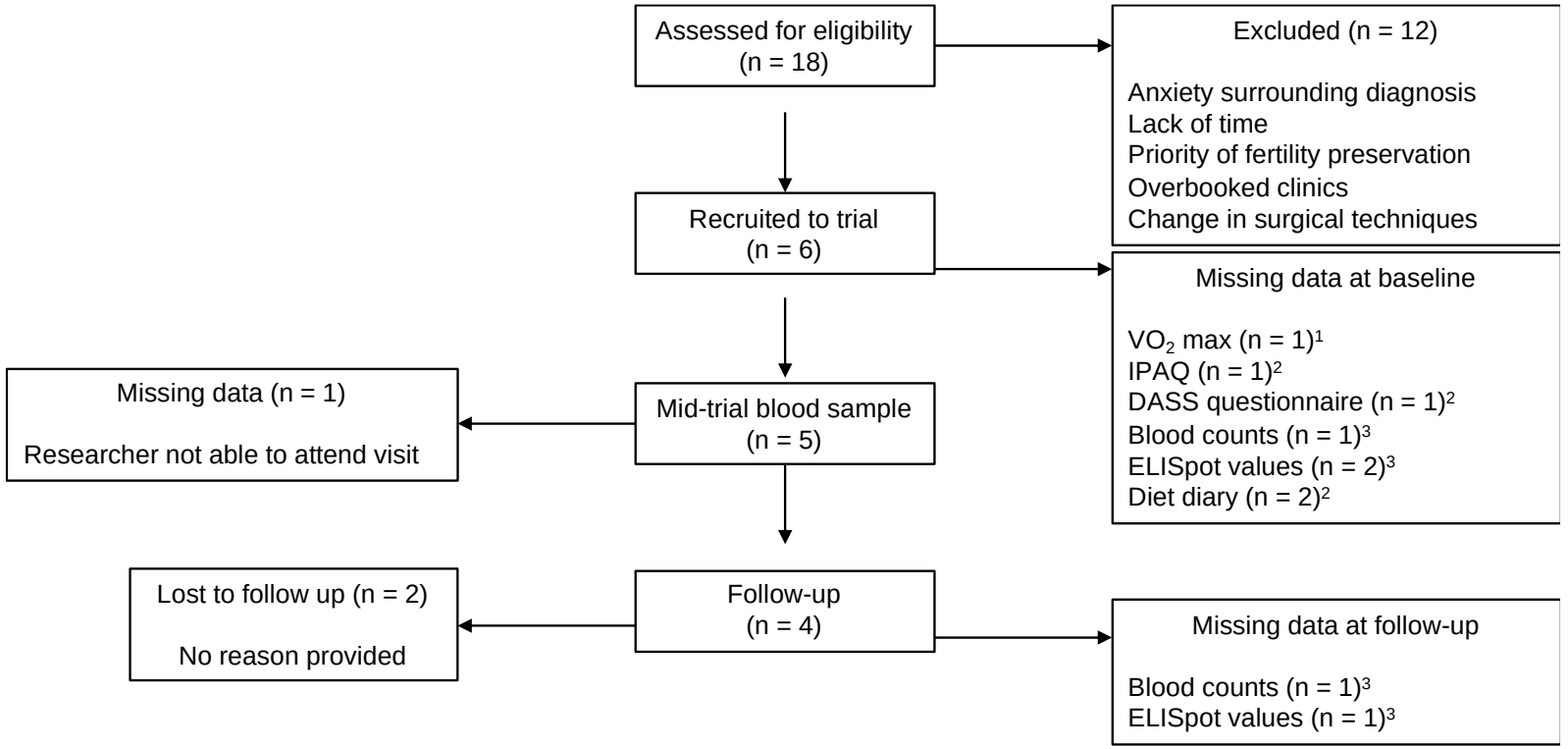


Figure 13. Feasibility of recruitment and study design. 1 Prediction equation provided inaccurate and unrealistic values, maybe due to inaccurate heart rate readings 2 Questionnaires were misplaced by participant 3 Not enough blood collected to carry out analysis or unsuccessful venepuncture

Blood counts represent samples collected at the University of Bath. No missing blood count values for RUH Bath collected samples.

IPAQ; International physical activity questionnaire. DASS; depression anxiety and stress questionnaire. $\dot{V}O_2$ max; Maximal oxygen uptake. ELISpot; Enzyme-linked immune absorbent spot.

5.3.2 Clinical Parameters

Grade, size, type and histochemistry of tumours, alongside treatment and response to treatment are shown in Table 28.

Table 28. Clinical parameters of each individual participant

Participant	Shape	Clinical baseline nodes	Age (years)	Grade	ER +ve	HER2 +ve	Chemotherapy regimen	Clinical response after 2 cycles of chemo-therapy	Pathological Response	Tumour size at baseline (mm)	Tumour size reduction (mm)	Tumour size reduction (%)
1		N1	49	3	−	−	FEC-T	*PR*	*PR*	26	26	99%
2		N1	30	3	−	−	FEC-T	*PR*	*CR*	20	15	75%
3		N0	49	3	−	−	FEC-T	*PR*	*CR*	20	20	100%
4		N1	44	3	−	+	FEC-TPH	*PR*	*PR*	n/a	n/a	n/a
5		N0	40	2	−	+	FEC-TPH	*SD*	*PR*	51	45	88%
6		N0	34	3	−	−	FEC-T	*CR*	*PR*	16	15	91%

N=6. *N; nodes involved. +; positive. −; negative. ER; oestrogen receptor +ve. HER2; human epidermal growth factor receptor +ve. PR; partial response. CR; complete response. SD; stable disease. mm; millimetres. Pathological clinical response as a complete response shown in red (pCr). Pathological clinical response as partial response shown in blue (pPr). n/a; Tumour sizes not available as participant diagnosed with inflammatory breast cancer*. *FEC-T; 5-fluorouracil, epirubicin, cyclophosphamide and docetaxel. FEC-TH*; *5-fluorouracil, epirubicin, cyclophosphamide, docetaxel, trastuzumab and pertuzumab.*

5.3.2 Lifestyle: Physical activity levels

Changes in physical activity at diagnosis versus post-chemotherapy are shown in Figure 14. Physical activity measured via IPAQ stayed relatively consistent in patients who had a pPr for all intensities. In the patient 3, who had a pCr, total MET min per week decreased by 5826 MET min per week, (81%) post-chemotherapy. Amount of time spent undertaking sedentary activities, as measured by Sensewear, decreased post chemotherapy compared to diagnosis in all participants. Reversely, time spent doing light activities increased in all patients, regardless of pathological response. Time spent undertaking moderate activities stayed consistent for patient 3 who had a pCr (average of 147 minutes per day pre- and post-chemotherapy) but increased in patient 5 from 46 minutes per day pre-chemotherapy to 91 minutes per day post-chemotherapy and decreased slightly in patient 1 by 16 minutes. Time spent undertaking vigorous activities rose dramatically post-chemotherapy in patient 3 by 62% (pCr), dropped slightly in patient 1 by 9 MET min per day (pPr) and stayed similar in patient 5 (pPr). The mean differences and limits of agreements between IPAQ and Sensewear from the Bland-Altman plots for physical activity were 28 ± 154, 172 ± 233 and 23 ± 62 minutes per day for light, moderate and vigorous activity per day respectively (Figure 15.).

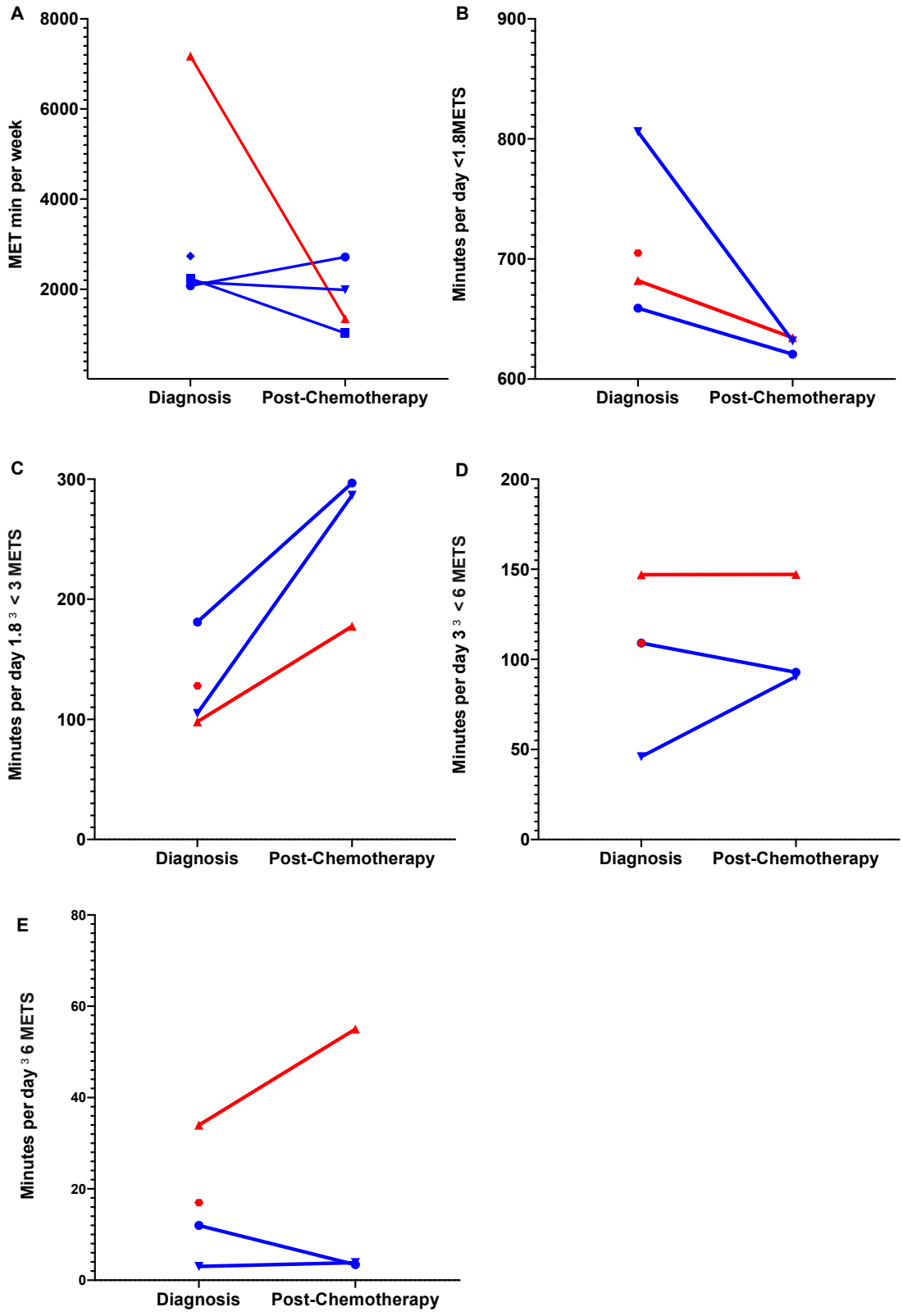
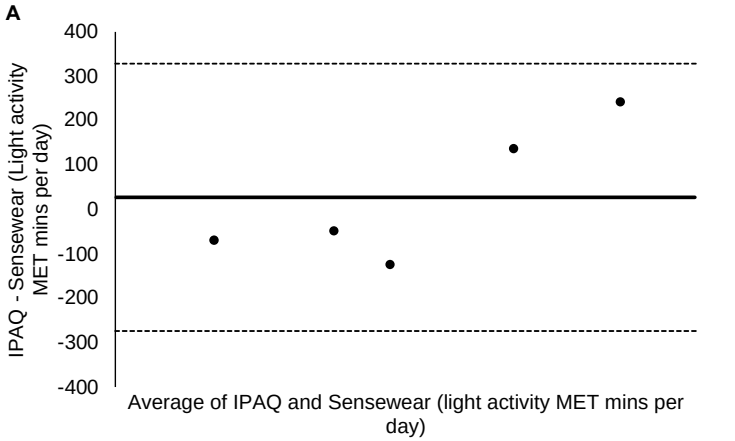
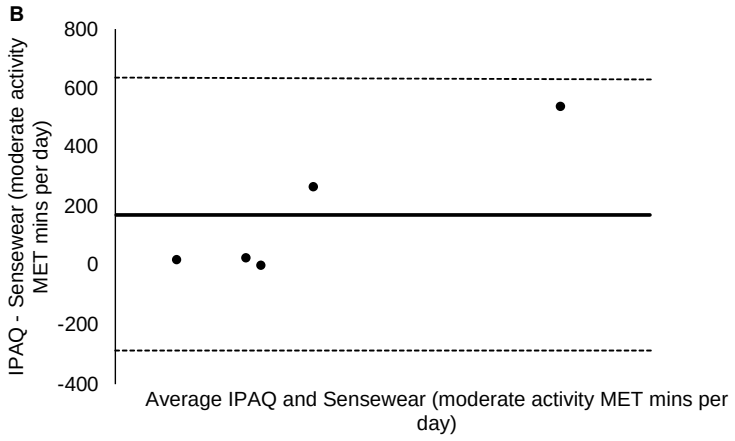
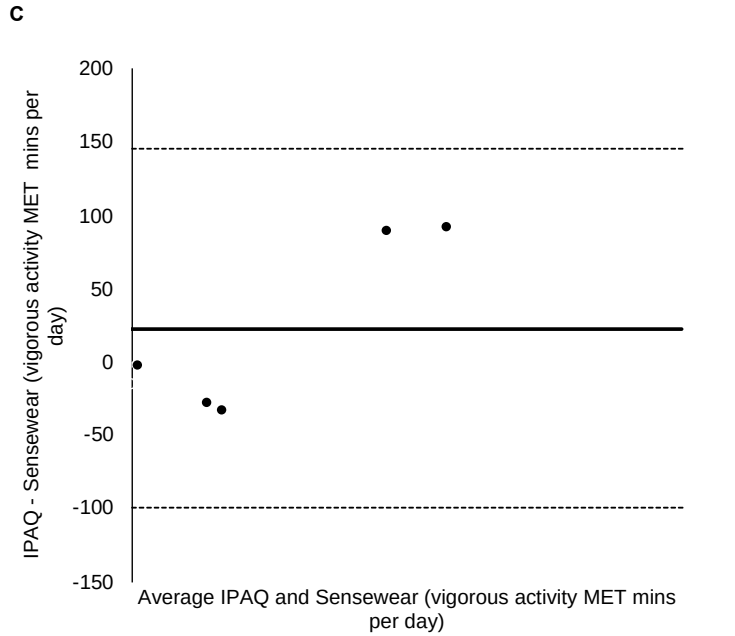


Figure 14. Physical activity levels pre vs post chemotherapy. A. IPAQ measured MET minutes per week. B. Sensewear measured sedentary time. C. Sensewear measured light activity D. Sensewear measured moderate activity time. E. Sensewear measured vigorous activity time. Lines and shapes represent individual participants. Total n=6. *MET; metabolic equivalent. Min; minutes.*







2673

2674 Figure 15. Bland-Altman plot between two measures of physical activity; Self-reported IPAQ
2675 and Sensewear armband for A. Light activity per day, B. Moderate activity per day and C.
2676 Vigorous activity per day.
2677 *IPAQ; international physical activity questionnaire. MET; metabolic equivalent.*

5.3.3 Lifestyle: Cardiorespiratory fitness and blood pressure

Predicted $\dot{V}O_2$ max dropped consistently from pre- versus post-chemotherapy by 53% and 58% in patients 1 and 4 respectively (both of whom had pPr) and 52% in the patient 3 (pCr). Both systolic and diastolic blood pressure dropped post-chemotherapy compared to diagnosis in 2 patients (3 and 5). In patient 4, systolic and diastolic blood pressure increased throughout chemotherapy, however this stayed within normal ranges. In patient 1, diastolic pressure dropped from 99 mmHg to 76 mmHg, but systolic blood pressure stayed slightly elevated increasing from 123 mmHg to 124 mmHg (Figure 16).

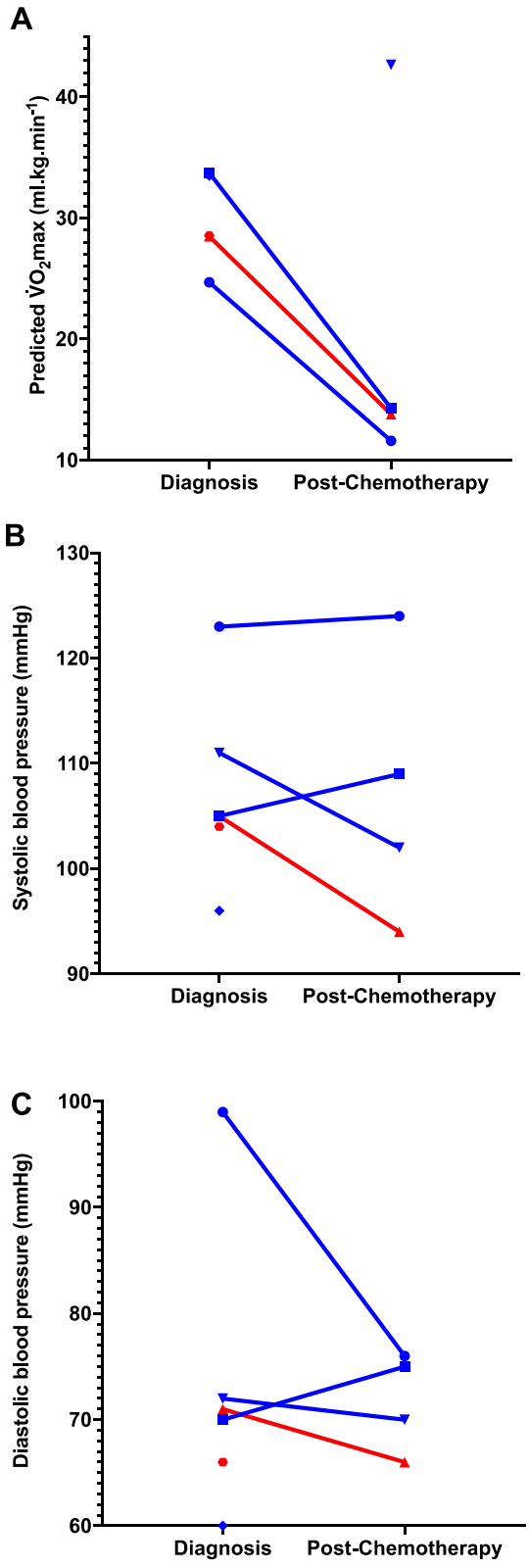


Figure 16. Physiological changes pre- vs post- chemotherapy. A. Predicted $\dot{V}O_2$ max. B. Systolic blood pressure. C. Diastolic blood pressure. Individual lines and shapes represent individual participants. Total n=6

$\dot{V}O_2$; *oxygen uptake. ml; millilitre. Min; minute. mmHg; millimetres of mercury.*

5.3.4 Lifestyle: Body composition

Body composition remained relatively unchanged for all participants. In patients 1, 4 and 5, who all had a pPr, BMI decreased on average by 1.3 $kg.m_2$ post-chemotherapy. Body fat percentage decreased in patients 1 and 4 alongside an increase or no change in lean mass. In patient 5, an increased body fat percentage post-chemotherapy was observed by 0.5%. Following a similar trend, W:H decreased in patients 1 and 4 yet stayed within the increased risk of metabolic complications category. W:H in patient 5 increased from <0.80 (no risk) to >0.80 (increased metabolic risk) (ACSM, 2013), however this participant was very lean as shown by very little fat mass (24.2 and 24.7% body fat pre- and post-chemotherapy respectively). BMI increased in patient 3 who had a pCr, however this is explained by the increase in lean mass and decrease in body fat percentage and body fat mass in this patient. For this patient W:H ratio also increased from 0.75 to 0.78, yet both values fell within the 'no increased risk of metabolic syndrome' category (ACSM, 2013). BMD decreased in all participants post-chemotherapy compared to diagnosis by 2.3% in patient 3 (pCr) and by 2.3%, 0.4% and 1.4% in patients 1, 4 and 5 respectively (pPr). Lean mass decreased in two patients (4 and 5, pPr). Patient 3, who had a pCr, had an increase in lean mass by 3kg post-chemotherapy (Figure 17). The mean differences and limits of agreements between bioelectrical impedance and DEXA from the Bland-Altman plots for body fat percentage were −1.5 ± 5.6% (Figure 18.).

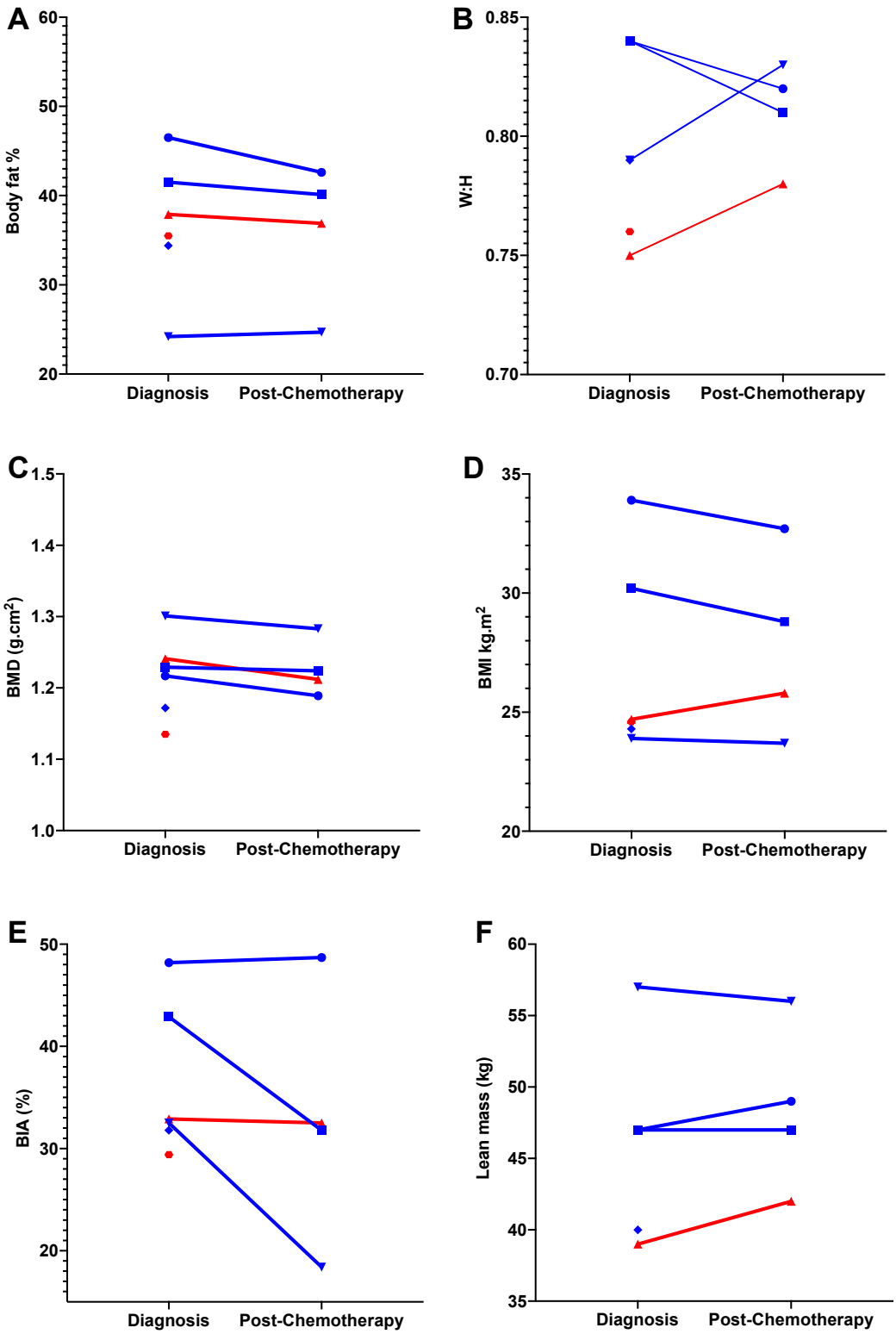


Figure 17. Body compositional changes pre- vs post- chemotherapy. A. Body fat percentage (DEXA measured). B. Waist to hip ratio. C. Bone mineral density. D. Body mass index. E. Bioelectrical impedance measured body fat percentage. F. Lean mass. Individual lines and shapes represent individual participants. Total n=6

W:H; waist to hip ratio. BMD; bone mineral density. BMI; body mass index. kg; kilograms. m; metres. BIA; bioelectrical impedance. DEXA; dual energy x-ray absorptiometry.

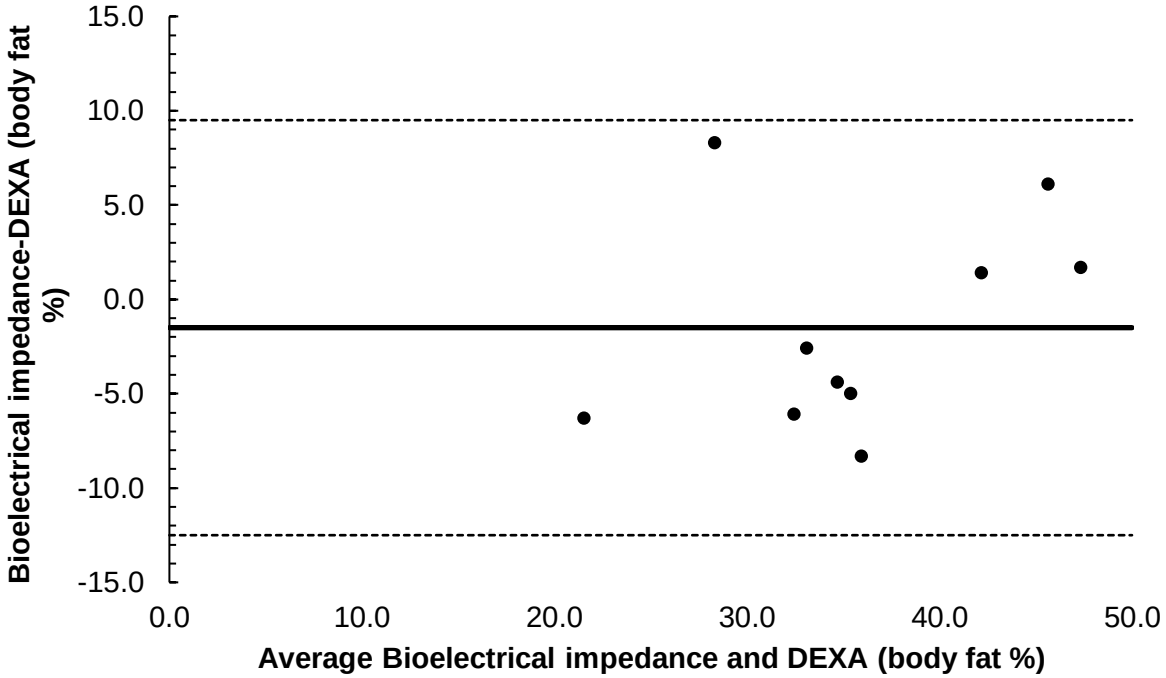


Figure 18. Bland-Altman plot between two measures of body fat percentage; bioelectrical impedance and DEXA. Data shown for individuals at all time points.

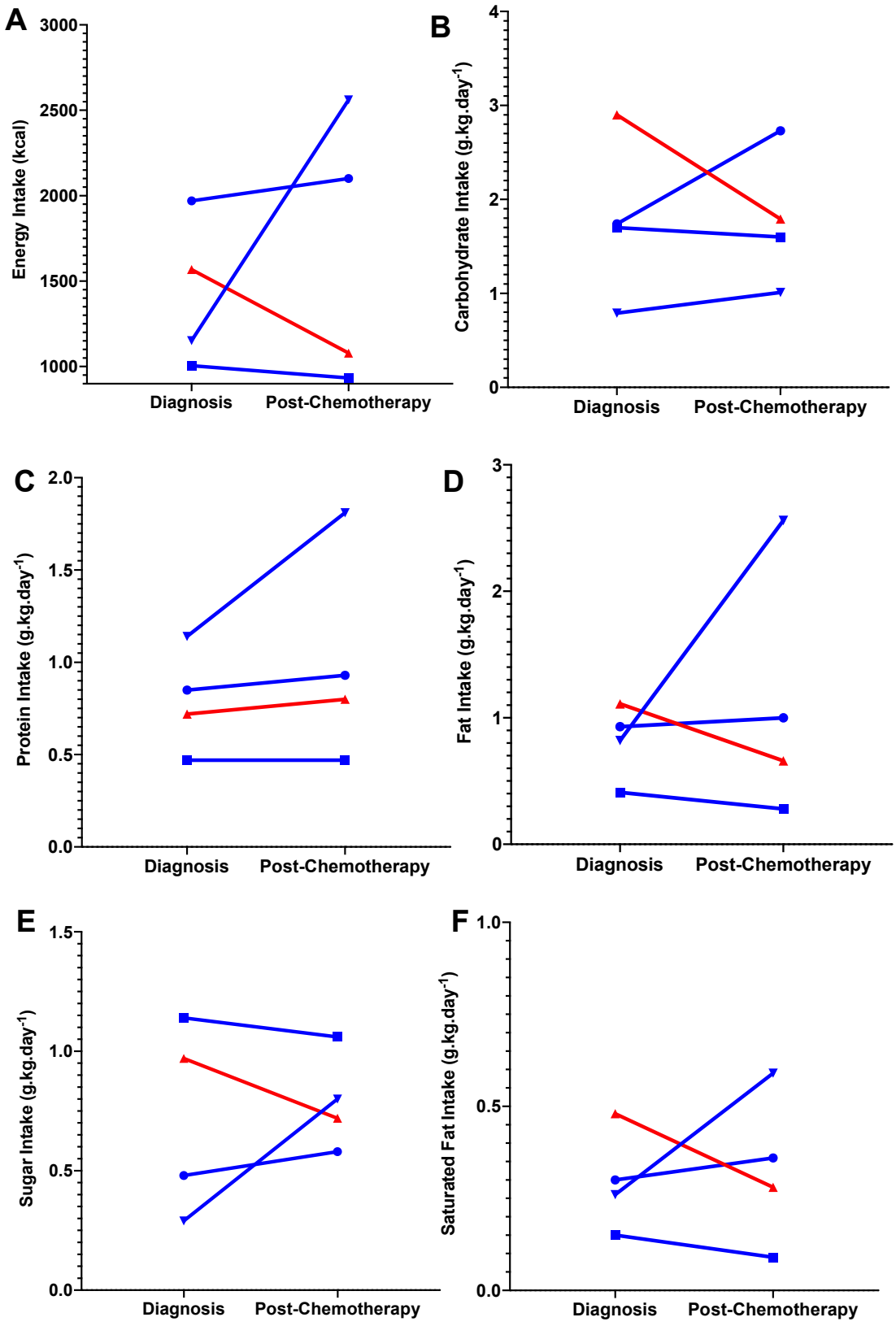
BIA; bioelectrical impedance. DEXA; dual energy x-ray absorptiometry.

5.3.5 Lifestyle: Nutritional intake

Energy intake increased pre- versus post-chemotherapy in 2 patients with a pPr (patients 1 and 5) by 131 and 1409 kcal respectively (Figure 19A). Patient 4 with pPr reported a decrease in energy intake. Patient 3 (pCr) decreased energy intake by 489 kcal post-chemotherapy. In patient 3 (pCr), intake of macronutrients decreased for carbohydrate, fat, sugar and saturated fat but increased slightly in terms of protein intake from 0.72 g.kg.day^{-1} to 0.80g.kg.day^{-1}. This trend was also observed in patient 4 who showed a slight decrease in all macronutrient intakes and a small increase in protein intake. In line with the increases in energy intake in patients 1 and 5, intake of all macronutrients increased post-chemotherapy. Interestingly, carbohydrate intake was below recommended values for all participants at all time points (<3g.kg.day^{-1}). In patients 5 (pPr), protein intake was above recommended values (0.8g.kg.day^{-1}) both pre- and post-chemotherapy whereas all other patients' protein intake was below this value. In terms of fat intake, patients 3 (pCr) and 1 (pPr) were within recommended range (0.5-1.5g.kg.day^{-1}) at both time points, patient 5 (pPr) was within range pre-chemotherapy but above this level post-chemotherapy

(2.56g.kg.day^{-1}) and patient 4 (pPr) had fat intakes below recommended values at both timepoints (Figure 19).

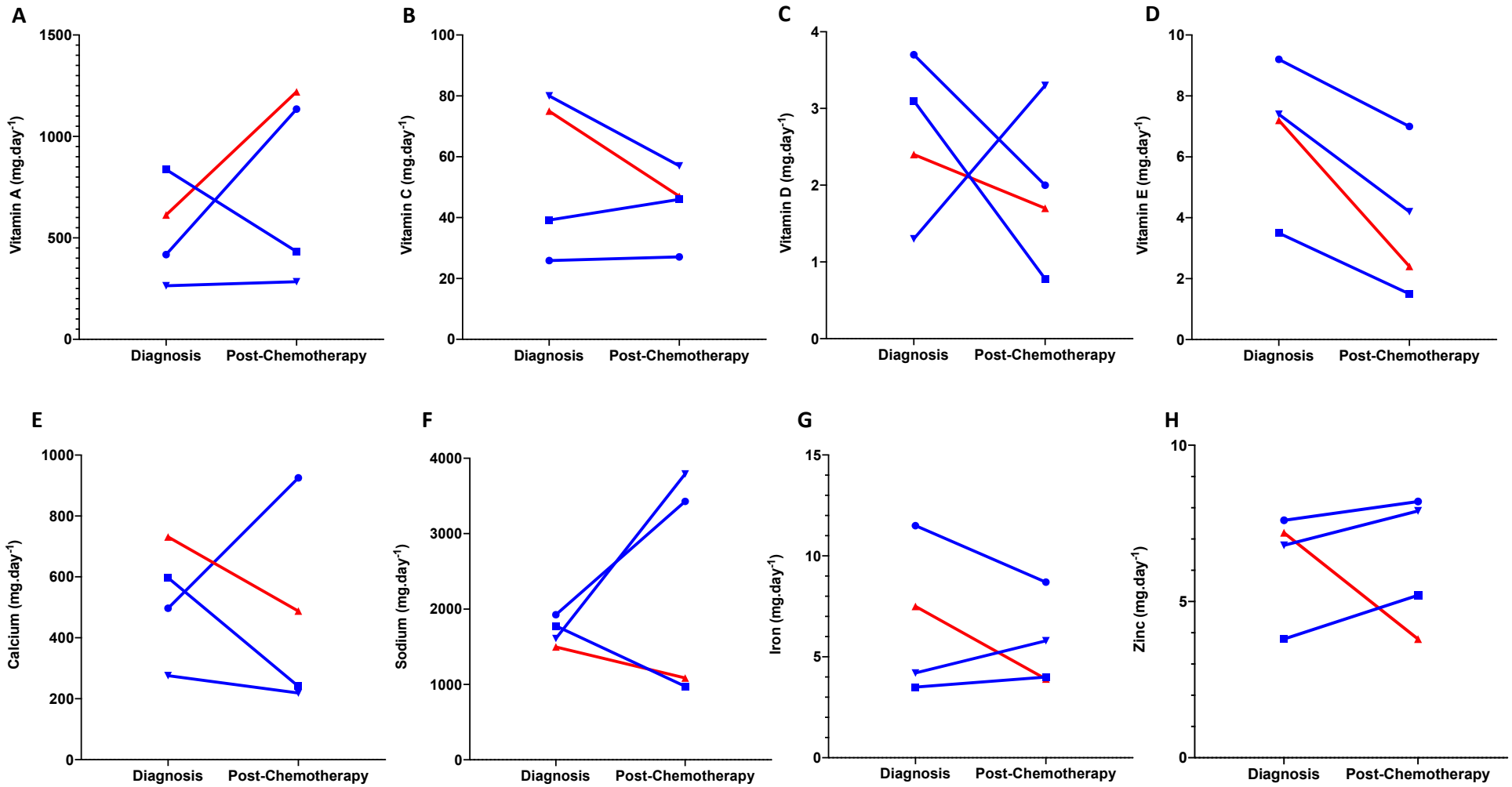
In terms of micronutrient intake, mixed changes were observed between patients (Figure 20.). In patient 3 (pCr) intake of vitamins C, D and E all decreased post chemotherapy whereas intake of vitamin A increased by 607mg.day^{-1} post-chemotherapy. Intake of calcium, sodium, iron and zinc all decreased post chemotherapy in this patient. In patient 1 (pPr), increases in vitamin A, C, calcium, sodium and zinc were observed post-chemotherapy whilst a decrease in vitamin D, E and iron was apparent. Patient 4 (pPr) reported a decrease in vitamin A, D and E intake but an increase in vitamin C intake of 5.8mg.day^{-1} post-chemotherapy. Whilst this patient reported decreases in calcium and sodium intake, increases in iron (by 0.5mg.day^{-1}) and zinc (1.4mg.day^{-1}) were reported. This trend in calcium, sodium, iron and zinc intake was mirrored in patient 5 (pPr). In this patient vitamin A intake remained stable, vitamin D intake increased by 2mg.day^{-1}, and both vitamin C and vitamin E intake decreased. Intake of vitamin A was within normal ranges (300mg.day^{-1}) at both timepoints for all patients except patient 5 whose intake fell slightly below this pre- (264mg.day^{-1}) and post-chemotherapy (284mg.day^{-1}). Pre-chemotherapy, only patients 3 (pCr) and 5 (pPr) had vitamin C intakes above daily recommendations (75mg.day^{-1}) but these fell below this threshold post-chemotherapy. Vitamin D and E intake (15 and 14mg.day^{-1} respectively) were below recommended levels for all patients at all timepoints.



2767

2768 Figure 19. Nutritional changes pre vs post chemotherapy. Individual lines and shapes
2769 represent individual participants. A. Energy Intake. B. Carbohydrate intake. C. Protein

intake. D. Fat intake. E. Sugar intake. F. Saturated fat intake. Total n=6. *g; grams. kcal; kilocalories. kg; kilograms*



2772

2773 Figure 20. Micronutrient changes pre vs post chemotherapy. Individual lines and shapes represent individual participants. A. Vitamin A. B. Vitamin

2774 C. C. Vitamin D. D. Vitamin E. E. Calcium. F. Sodium. G. Iron. F. Zinc Total n=6. m*g; milligrams.*

5.3.6 Psychological measures

Scores of depression, as measured by DASS, varied between individual participants. Patients 1 with pPr reported a doubling in score for depression post-chemotherapy whereas patient 5 who also had pPr observed a 6-point decrease in depression post-chemotherapy (Figure 21A). Both patients 3 (pCr) and patient 4 (pPr) recorded scores of 0 for depression pre- and post-chemotherapy. Patient 6 (pPr) had a diagnosis depression score of 8. All scores for depression were within normal ranges at all time points. In terms of anxiety patients 1 and 5 had decreases in anxiety post-chemotherapy. Patient 1 (pPr) had an 'extremely severe' anxiety score at diagnosis which halved post-chemotherapy to 'moderate'. Patient 5 (pPr) had a 'mild' anxiety score at diagnosis and this dropped within 'normal' ranges post-chemotherapy. Patients 3 and 4 with pCr and pPr respectively, reported increases in anxiety post-chemotherapy, although ranges were always within 'normal' ranges (Figure 21B). In terms of stress, all patients with pPr had a higher level of stress at diagnosis than patient 3 (pCr). In patients with pPr the levels of stress dropped post-chemotherapy. In patient 1 stress levels dropped by 4 points (both within 'normal' ranges), in patient 4 dropped by 4 points (again both within normal ranges) and patient 5 dropped dramatically by 18 points from a 'severe' score to a 'mild' score. Patient 3 (pCr) saw a slight increase in stress post-chemotherapy but this was still within 'normal' ranges. Patient 6 had a stress score at diagnosis within 'normal' range (Figure 21C).

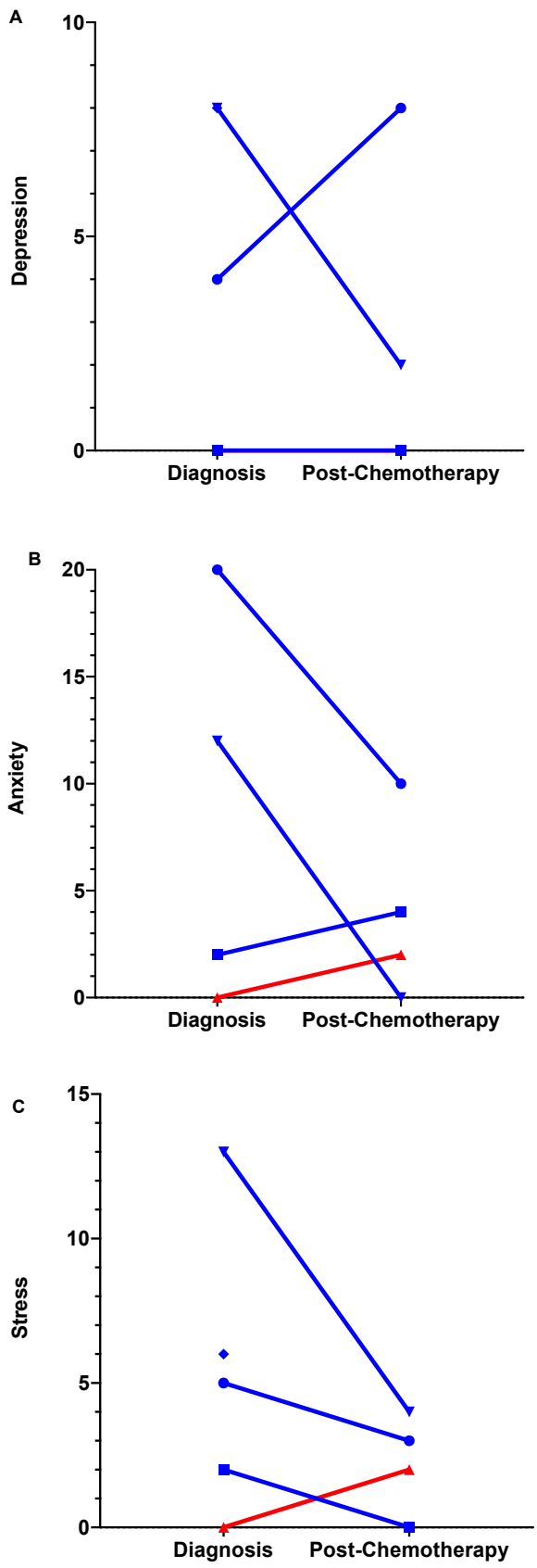


Figure 21. Changes in psychological measures pre- vs post-chemotherapy. Individual lines and shapes represent individual participants. A. Depression B. Anxiety. C. Stress. Total n=5.

5.3.7 Blood cell counts

Timing of venepuncture used for cell counts at each time point are shown in Table 29. On average the blood sample taken at time point 1 was 8 ± 7 days before the first chemotherapy cycle. The second blood sample was 7 ± 2 days before the next chemotherapy infusion. Finally, the timing on the third blood sample was on average 30 ± 20 days since the final chemotherapy infusion.

No significant differences were seen in cells counts and time points ($P>0.05$) other than for haemoglobin levels (Figure 22). However according to normal cell counts (Dean and Dean, 2005), red blood cell count dropped below normal values in patient 4 at mid-chemotherapy (3.34×10^{12}/L) and further decreased post-chemotherapy to 3.23×10^{12}/L. Patient 5 observed a decreased red blood cell count out of normal ranges post-chemotherapy to 3.46×10^{12}/L. All patients with pPr saw an average decrease in red blood cell count of 0.50×10^{12}/L at mid-chemotherapy versus diagnosis. This decrease continued post-chemotherapy in 3 out of 4 patients with pPr (patients 1, 4 and 6). In patient 5 (pPr), red blood cell count increased from mid-chemotherapy to post-chemotherapy by 0.09×10^{12}/L. In patient 2 (pCr) red blood cell count stayed the same at diagnosis and mid-chemotherapy (4.14×10^{12}/L) and increased to 4.19×10^{12} post-chemotherapy. In patient 3 (pCr), red blood cell count decreased mid chemotherapy but subsequently increased higher than pre-chemotherapy to 4.65×10^{12}/L post-chemotherapy. Patients who had pCr (patients 2 and 3) had consistently higher red blood cell count post-chemotherapy versus those with pPr (average cell count $4.42 \pm 0.33 \times 10^{12}$/L and $3.67 \pm 0.23 \times 10^{12}$/L respectively) (Figure 22A).

Total leukocyte count was within normal clinical ranges ($4\text{-}11 \times 10^{9}$/L) for all patients at all time points. Leukocyte count varied amongst individuals. Half of patients with pPr (patients 4 and 5) had a decrease in total leukocyte count mid-chemotherapy versus pre-chemotherapy from 6.7×10^{9}/L to 5.0×10^{9}/L (patient 4) and 7.4×10^{9}/L to 5.7×10^{9}/L (patient 5). For patient 4, total leukocyte count increased to 6.1×10^{9}/L post-chemotherapy whilst patient 5 saw a further decrease to 4.2×10^{9}/L. The other patients with pPr (patient 1 and 6) saw an increase mid-chemotherapy of 0.7 and 0.6×10^{9}/L total leukocyte count respectively and subsequent decrease post-chemotherapy by 2.6×10^{9}/L and 0.2×10^{9}/L. Patient 2 with pCr had a WBC count of 5.7×10^{9}/L at diagnosis and mid-chemotherapy which then increased to 6.4×10^{9}/L post-chemotherapy. In patient 3 (pCr) total leukocyte count decreased throughout by 1.7×10^{9}/L at mid-chemotherapy and again by 1.2×10^{9}/L post-chemotherapy (Figure 22B).

Haemoglobin level followed a similar pattern as RBC counts in all individuals. A significant decrease in haemoglobin was seen between diagnosis (Median= 129g/L) and mid-chemotherapy (Mdn = 115g/L) ($T = 20.000$, $p = 0.046$) and at diagnosis (Mdn = 129g/L) and post-chemotherapy (Mdn = 118g/L) ($T = 0.000$, $p = 0.043$), (Figure 22C).

Platelet count varied for all individuals at all time points. Patient 2 (pCr) demonstrated no change in platelets counts at diagnosis and mid-chemotherapy (185 x 10^9/L). Patient 3 (pCr) had a 39% increase in platelet count mid-chemotherapy versus pre-chemotherapy but this decreased by 11% between mid-chemotherapy and post-chemotherapy. Patients 1 and 4 (pPr) had an increase of 73 and 54 x 10^9/L respectively mid-chemotherapy with a subsequent decrease to values similar to baseline post-chemotherapy. Patients 5 and 6 with pPr observed a decrease of 99 and 51 x 10^9/L platelets at mid-chemotherapy respectively. In patient 5, this decrease mid-chemotherapy was to 148 x 10^9/L which is below normal range (Dean and Dean, 2005). Patient 5 observed a recovery in platelet cell counts through an increase of 113 x 10^9/L post-chemotherapy whilst patient 6 observed a further decrease to 285 x 10^9/L when diagnosis count was 376 x 10^9/L and mid-chemotherapy 325 x 10^9/L (Figure 22D).

Lymphocyte count dropped in 3 patients with pPr (patients 1, 4 and 5) by 0.1, 0.9 and 0.5 x 10^9/L for timepoints respectively. For patient 5, mid-chemotherapy lymphocyte count was below normal thresholds (1.5-3.5 x 10^9/L) at 0.9 x 10^9/L. Patient 1 and 4 continued to have decreased lymphocyte counts post-chemotherapy (further drop of 0.5 and 0.4 x 10^9/L respectively to make lymphocytes counts of 1 and 0.9 x 10^9/L respectively, below normal range) whereas lymphocyte counts in patient 5 increased by 0.1 x 10^9/L (but was still below normal range). Patient 6 (pPr) demonstrated an increased lymphocyte count mid-chemotherapy (2.1 x 10^9/L versus 1.7 x 10^9/L at diagnosis) which subsequently dropped to 1.9 x 10^9/L post-chemotherapy. In patient 2 (pCr) lymphocyte count stayed relatively similar throughout however mid-chemotherapy, count was 1.4 x 10^9/L (below normal thresholds) but this increased to 1.5 x 10^9/L post-chemotherapy. In patient 3 lymphocyte count increased consistently as time went on from 1.2 x 10^9/L, which was below normal thresholds pre-chemotherapy, 1.5 x 10^9/L mid-chemotherapy and 2.0 x 10^9/L post-chemotherapy (Figure 22E).

Neutrophil counts stayed relatively similar across diagnosis, mid-chemotherapy and post-chemotherapy in patient 6 (pPr) and patient 2 (pCr) only fluctuating by a maximum of 0.3 x

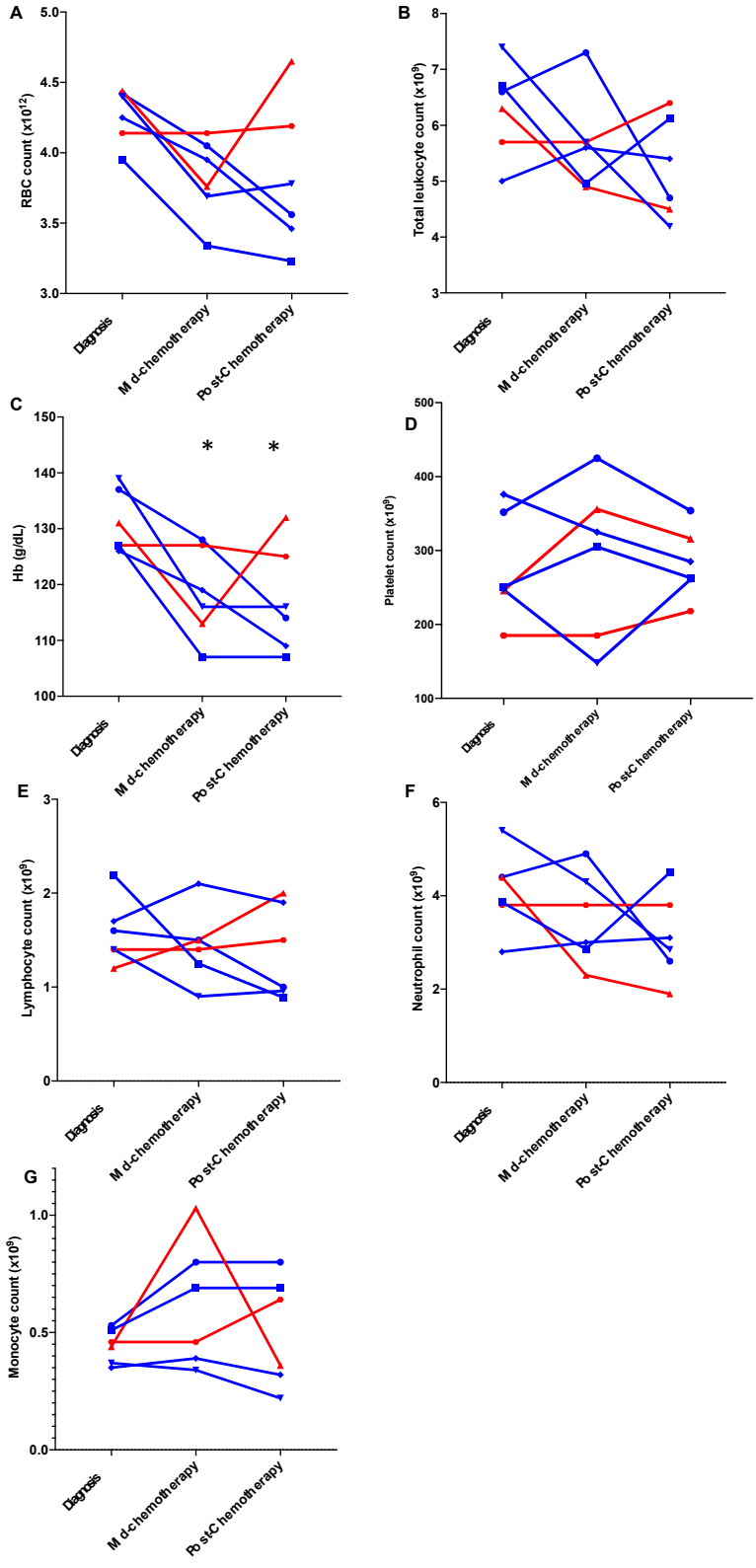
10^9/L. In patients 4 and 5 (pPr), neutrophil counts decreased mid-chemotherapy by 1.0 and 1.1 x 10^9/L respectively. In patient 4 neutrophil counts increased post chemotherapy by 1.6 x 10^9/L which was higher than at diagnosis but in patient 5 neutrophil counts continued to decrease, this time by a further 1.5 x 10^9/L. In patient 1 (pPr) neutrophil counts increased from 4.4 x 10^9/L at diagnosis to 4.9 x 10^9/L mid-chemotherapy but then decreased to 2.6 x 10^9/L (47% decrease from mid-chemotherapy) post-chemotherapy. Patient 3 (pCr) demonstrated a decrease in neutrophil counts mid chemotherapy by 2.1 x 10^9/L mid-chemotherapy to 2.3 x 10^9/L below normal levels (2.5-7.5 x 10^9/L) and then again by 0.4 x 10^9/L to 1.9 x 10^9/L post-chemotherapy (Figure 22F).

Monocyte counts increased for 4 patients (1, 3, 4 and 6) mid-chemotherapy compared with pre-chemotherapy. In patient 3 (pCr), monocyte count rose to 1.03 x 10^9/L, above normal ranges (0.2-0.8 x 10^9/L) but this dropped below baseline post-chemotherapy. In patient 1 and 4 (both pPr), after a 0.27 and 0.18 x 10^9/L increases in monocyte count respectively mid-chemotherapy no further change was observed post-chemotherapy. In patient 6 (pPr) after a slight increase in monocyte count mid-chemotherapy, post-chemotherapy count dropped below baseline. In patient 2 (pCr), no change in monocyte count was observed mid-chemotherapy (0.46 x 10^9/L) but a slightly increase of 0.18 x 10^9/L was observed post-chemotherapy. In patient 5 (pPr) a decrease was observed in monocyte count mid-chemotherapy compared to diagnosis and a further decrease was seen post-chemotherapy thus total monocyte reduction was 0.15 x 10^9/L (Figure 22G).

Table 29. Timing of blood samples with reference to chemotherapy infusions

Participant	Shape	Time point 1: Diagnosis sample (Days before start of chemotherapy)	Time point 2: Mid-chemotherapy sample (days before chemotherapy infusion 3)	Time point 3: Post-chemotherapy sample (days since final chemotherapy infusion)
1		3	7	50
2		1	2	60
3		17	1	12
4		16	1	19
5		6	5	26
6		5	4	15

N=6. Pathological clinical response as a complete response shown in red (pCr). Pathological clinical response as partial response shown in blue (pPr).



2896

2897 Figure 22. Individual changes in blood cell counts over time; pre-chemotherapy, mid chemotherapy
2898 (after 2 cycles of chemotherapy, before the third cycle) and post-chemotherapy (at least 10 days
2899 after final chemotherapy infusion). A. RBC count B. Total leukocyte counts C. Hb level D. Platelet
2900 count E. Lymphocyte count F. Neutrophil count G. Monocyte count. Data show as individual
2901 responses. Individual lines and shapes represent individual participants. * Significantly difference to
2902 pre-chemotherapy. *RBC; red blood cell, Hb; haemoglobin.*

5.3.8 Cell count ratios

PLR increased in 2/4 with pPr (patient 1 and patient 4). In the other two patients with pPr (patient 5 and 6) PLR decreased at the mid-chemotherapy time point. In patient 5 PLR then increased to above baseline whereas in patient 6 PLR continue to decrease. In patients with pCr (patients 2 and 3), PLR increased mid chemotherapy and in patient 2 increased again post-chemotherapy, all be it very slightly and in patient 6 PLR decreased to below baseline (Figure 23A).

NLR varied between individuals. Patients 1 and 5 (pPr) followed similar trends, increasing NLR from diagnosis to mid-chemotherapy and then decreasing back to bassline at post-chemotherapy. Patient 4 (pPr) also increased NLR at mid-chemotherapy but continued to increase further post-chemotherapy whilst patient 5 (pPr) had a relatively constant NLR throughout treatment. NLR also remained relatively unchanged in patient 3 (pCr) but decreased from 3.7 to 1.5 and then to 1.0 from diagnosis, mid-chemotherapy and post-chemotherapy in patient 2 (Figure 23B).

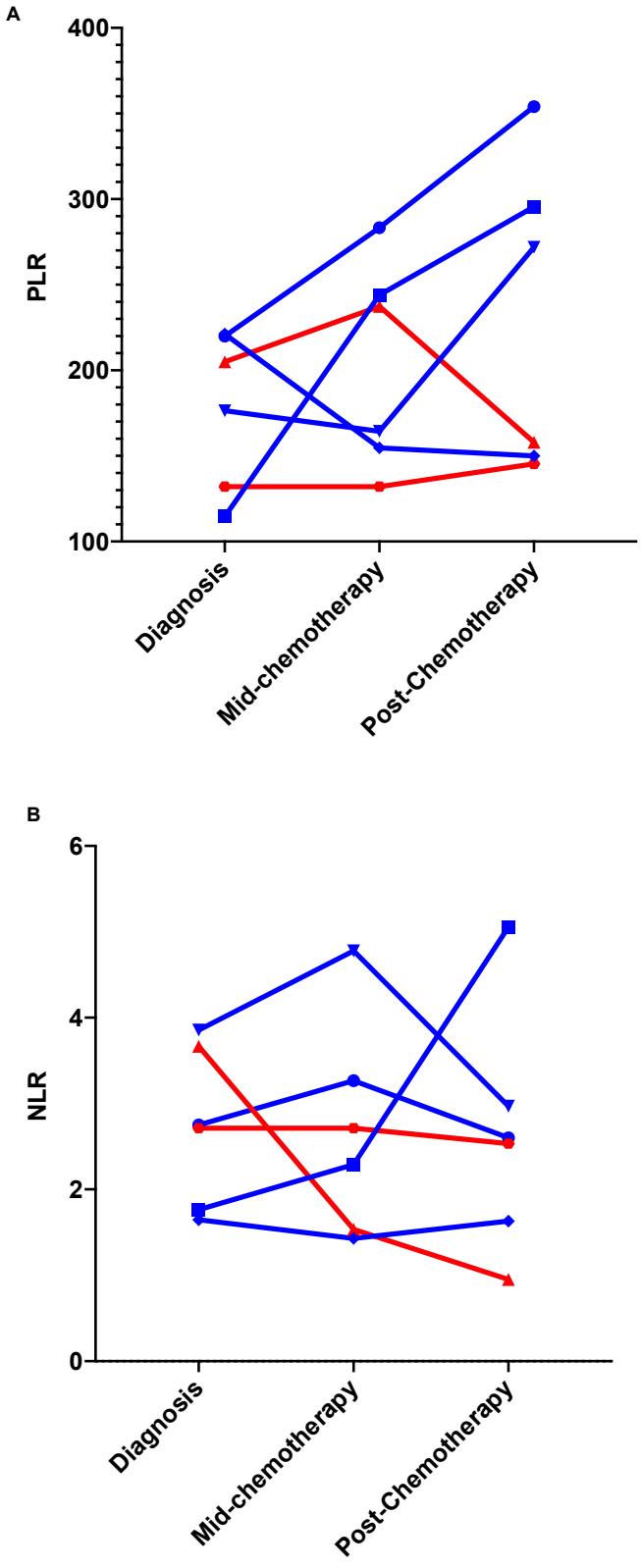


Figure 23. Individual changes in A. PLR and B. NLR over time; pre-chemotherapy, mid chemotherapy (after 2 cycles of chemotherapy, before the third cycle) and post-chemotherapy (at least 10 days after final chemotherapy infusion).

PLR; platelet to lymphocyte ratio. NLR; neutrophil to lymphocyte ratio

5.3.9 T-lymphocyte response to viral antigens

Responding number of IFN-γ secreting T-lymphocytes in response to FLU peptides MP1 and NP increased in all patients where venepuncture was successful and enough PBMCs counted and used in the ELISpot assay from pre-to mid- to post-chemotherapy (Figure 24.).

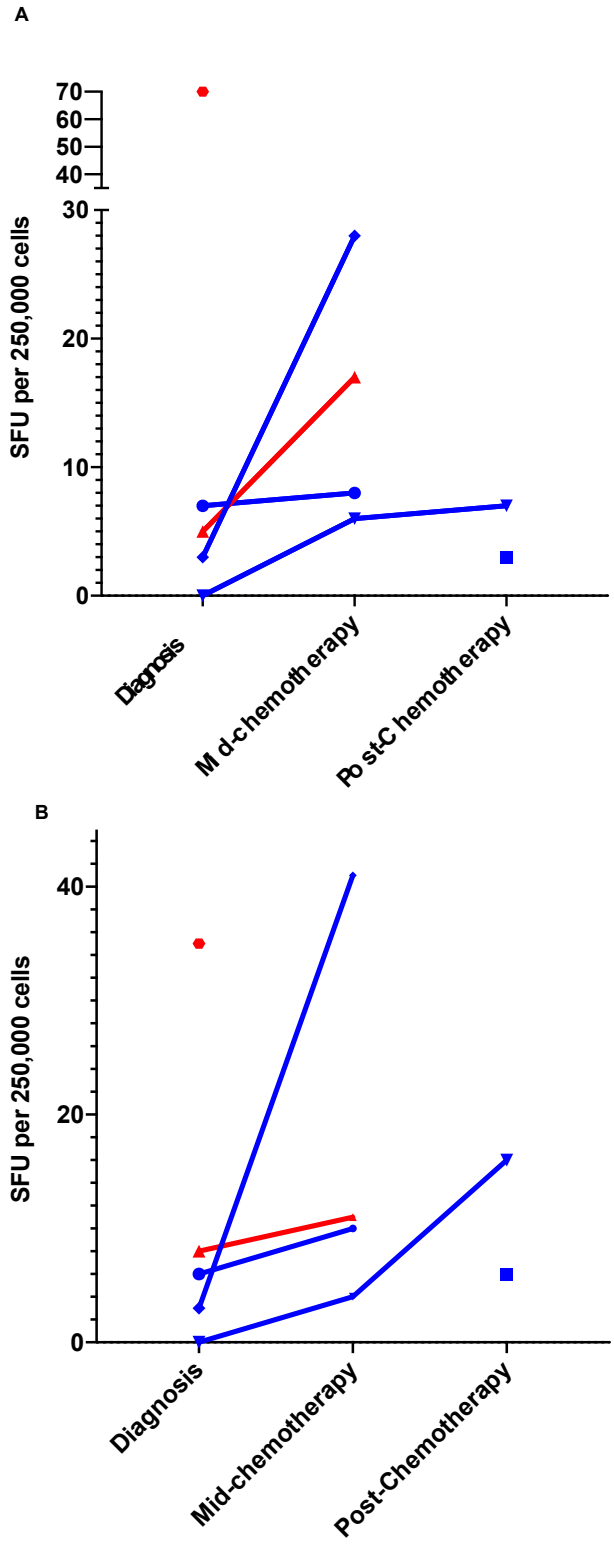


Figure 24. The relevant magnitude of specific T-lymphocytes compared against A. FLU MP1 B. FLU NP at diagnosis (before start of chemotherapy), mid-chemotherapy (before the third infusion of chemotherapy) and post-chemotherapy (minimum of 5-weeks after the final chemotherapy infusion, before surgery). Data show as individual responses. Individual lines and shapes represent individual participants. *SFU, spot forming units. MP1, matrix protein1. NP, nucleoprotein.*

In response to EBV peptides, patient 6 (pPr) had an increased number of SFUs/250,000 cells for both BZLF1 and EBNA1 at the mid-chemotherapy time point compared to diagnosis (2 vs 21 SFUs and 14 vs 57 SFUs for EBNA1 and BZLF1 respectively). However, patient 5 (pPr) had no increase in SFU from diagnosis and mid-chemotherapy for EBNA1 (0 SFU at both time points) but observed an increase in SFUs in response to BZLF1 (2 vs 8 SFUs at diagnosis and mid-chemotherapy respectively). In this patient (patient 5), when comparing the mid-chemotherapy time point versus the post-chemotherapy time point SFUs increased in response to both EBV peptides (0 to 14 and 8 to 33 for EBNA1 and BZLF1 respectively). The final patient with pPr, patient 1, demonstrated a decrease in T-lymphocyte response to both EBV peptides when comparing pre- to mid-chemotherapy by 1 spot and 3 spots for EBNA1 and BZLF1 respectively. In patient 3 (pCr), SFU doubled in response to both EBV peptides from diagnosis to mid-chemotherapy from 3 to 6 and 2 to 4 spots for EBNA1 and BZLF1 respectively (See Figure 25).

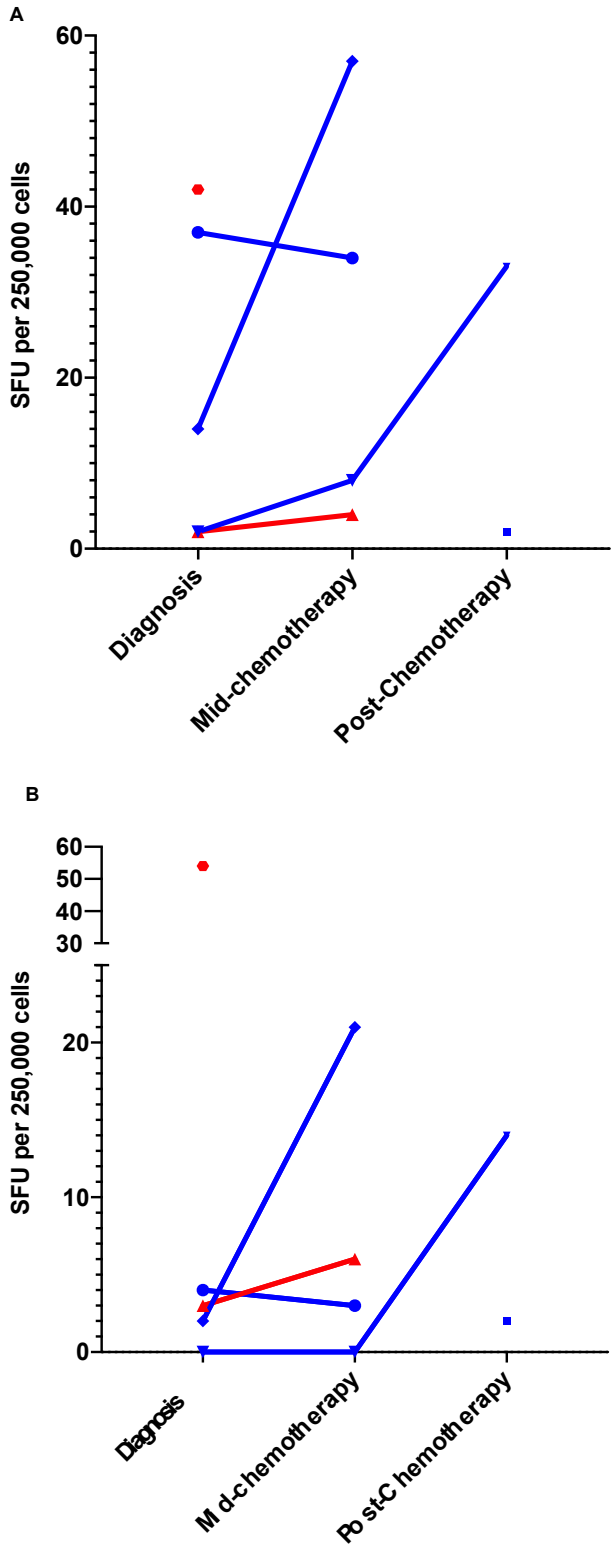


Figure 25. The relevant magnitude of specific T-lymphocytes compared against A. EBV BZLF1 B. EBV EBNA1 at pre-chemotherapy, mid-chemotherapy (before the third infusion of chemotherapy) and post-chemotherapy (minimum of 5-weeks after the final chemotherapy infusion, before surgery). Data show as individual responses. Individual lines and shapes represent individual participants. *SFU, spot forming units.*

SFUs in response to CMV peptide pp65 was higher at baseline for both pCr patients (patients 2 and 3) versus pPr patients (patients 1, 4, 5 and 6). Patients 1 and 6 (pPr) observed SFUs increase from pre- to mid-chemotherapy in response to both CMV peptides, pp65 and IE1. In patient 5 (pPr), SFUs decreased to 0 at the mid-chemotherapy time point and then increased to 1 SFU and 13 SFUs for pp65 and IE1 peptides respectively post-chemotherapy. In patient 3 (pCr) SFUs decreased from 243 to 176 in response to pp65 at diagnosis versus mid-chemotherapy and increased from 22 to 61 SFUs in response to IE1 (See Figure 26).

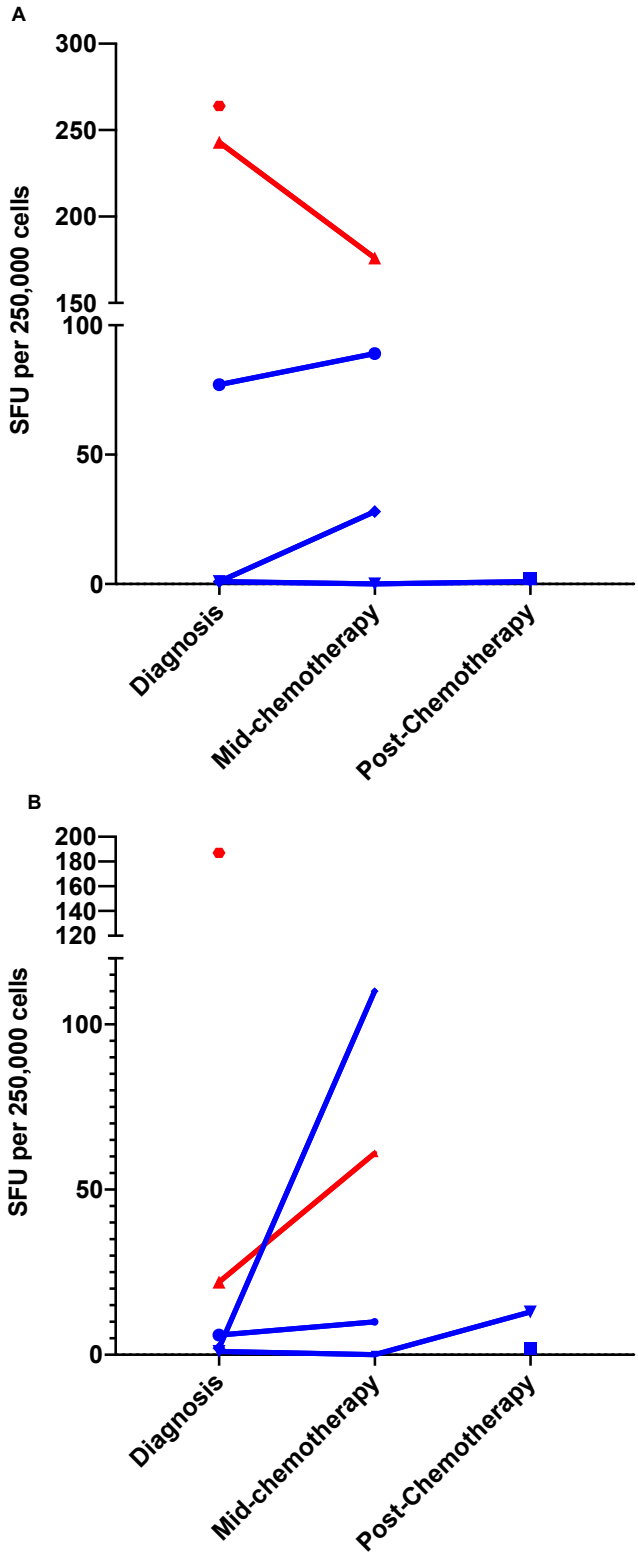


Figure 26. The relevant magnitude of specific T-lymphocytes compared against A. CMV pp65 B. CMV IE1 at pre-chemotherapy, mid-chemotherapy (before the third infusion of chemotherapy) and post-chemotherapy (minimum of 5-weeks after the final chemotherapy infusion, before surgery). Data show as individual responses. Individual lines and shapes represent individual participants. *SFU, spot forming units.*

Finally, patients 1, 5 and 6 (pPr) had consistently higher SFUs in response to VZV peptide IE63 mid-chemotherapy when compared to diagnosis. Patient 5 observed a further increase in SFUs post-chemotherapy compared to mid-chemotherapy (2 vs 14 SFUs). Patients 1 and 6 (pPr) observed higher numbers of SFUs at the mid-chemotherapy time point compared to diagnosis in response to VZV peptide gE. Patient 5 (pPr) had 1 SFU at diagnosis, 0 SFU at mid-chemotherapy and 4 SFUs post-chemotherapy. In patient 3 (pCr) there was 1 more SFU in response to IE63 and 3 more SFUs in response to gE when comparing diagnosis and mid-chemotherapy (Figure 27).

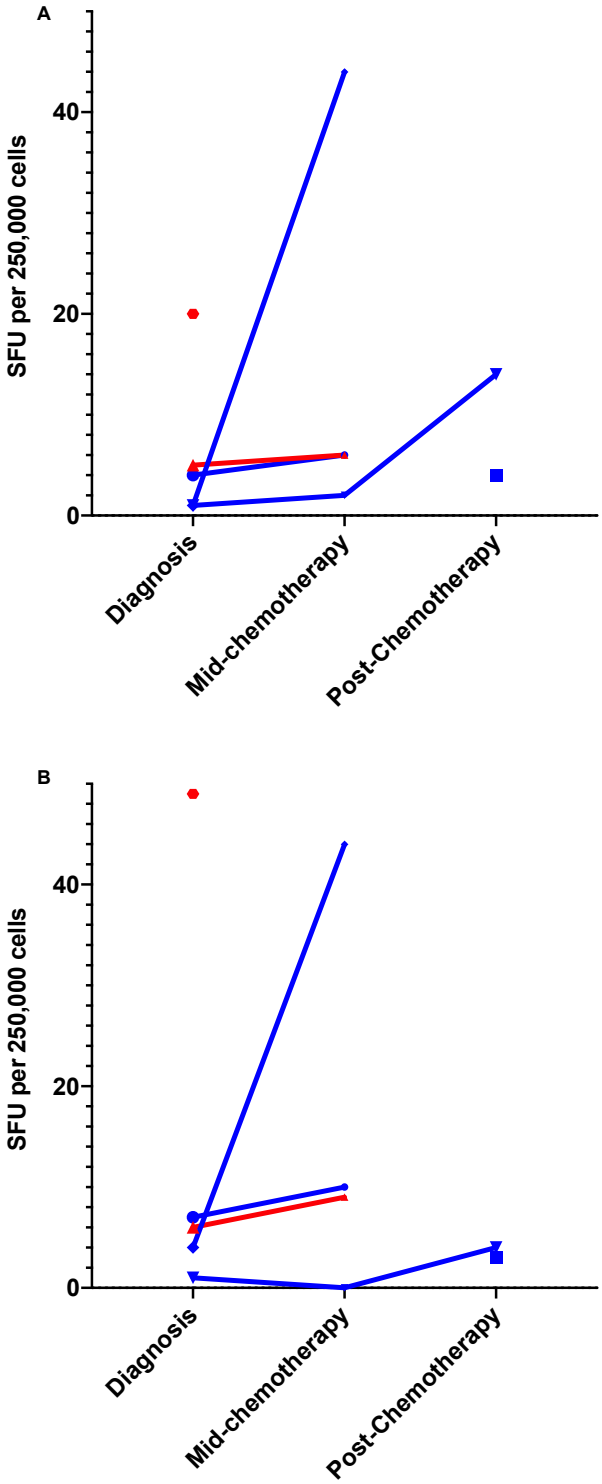


Figure 27. The relevant magnitude of specific T-lymphocytes compared against A. VZV IE63 B. VZV gE pre-chemotherapy, mid-chemotherapy (before the third infusion of chemotherapy) and post-chemotherapy (minimum of 5-weeks after the final chemotherapy infusion, before surgery). Data show as individual responses. Individual lines and shapes represent individual participants. *SFU, spot forming units.*

Patient 3 (pCr) had an increasing SFU for all virus peptides other than towards CMV peptide pp65 at mid-chemotherapy compared to diagnosis. Of the patients who had a pPr, patient 1 had increasing SFUs towards Flu, CMV and VZV peptides but not to EBV peptides, patient 5 had increasing SFUs to Flu peptides, BZLF1 and IE63, a lower SFU at mid-chemotherapy for gE and no chance in response to EBNA, pp65 or IE1 (Figures 11-14). Patient 6 had higher SFUs in response to all peptides at mid-chemotherapy versus pre-chemotherapy. When comparing mid-chemotherapy to post-chemotherapy, patient 5 (pPr), had a higher SFU post-chemotherapy for Flu, EBV, VZV and CMV peptide IE1 and no change in response to CMV peptide pp65 (Figures 24-27).

5.3.10 T-lymphocyte response to tumour-associated antigens

In terms of response to tumour-associated antigens, individual responses varied. In patient 3 (pCr) SFUs increased in response to all tumour-associated antigens at the mid-chemotherapy time point compared to pre-chemotherapy, apart from response towards MamA which stayed the same (7 SFUs per 250,000 PBMCs at each time point). On average the increase in SFUs was 14 spots. The largest increase was seen in response to tumour-associated antigen survivin with an increase of 39 SFUs mid-chemotherapy compared to diagnosis. Patient 6 consistently had an increasing SFU at the mid-chemotherapy time point versus pre-chemotherapy (average of 13 SFUs and 1 SFU respectively, with an average change of 12 SFUs). The highest increase was seen in response to ERB ECD with an increase in 43 SFUs at mid-chemotherapy whilst the lowest increased was in response to TARP, with an increase in only 1 SFU. On the other hand, patient 1 had a reduced response towards 6/10 tumour-associated antigens mid-chemotherapy versus pre-chemotherapy, no change in 2/10 (Cl6 and MMP11) and an increased response in 2/10 (CEA and TARP). Finally, patient 5 had data available at all time points. Response was generally low at all time points; average of 1 SFU at diagnosis, 2 SFUs at mid-chemotherapy and 3 SFUs post-chemotherapy. When comparing diagnosis to post-chemotherapy SFUs increased for all tumour-associated antigens, other than survivin (Figure 28). Patients with pCr had higher baseline SFUs compared to patients who had a pPr towards all tumour-associated antigens.

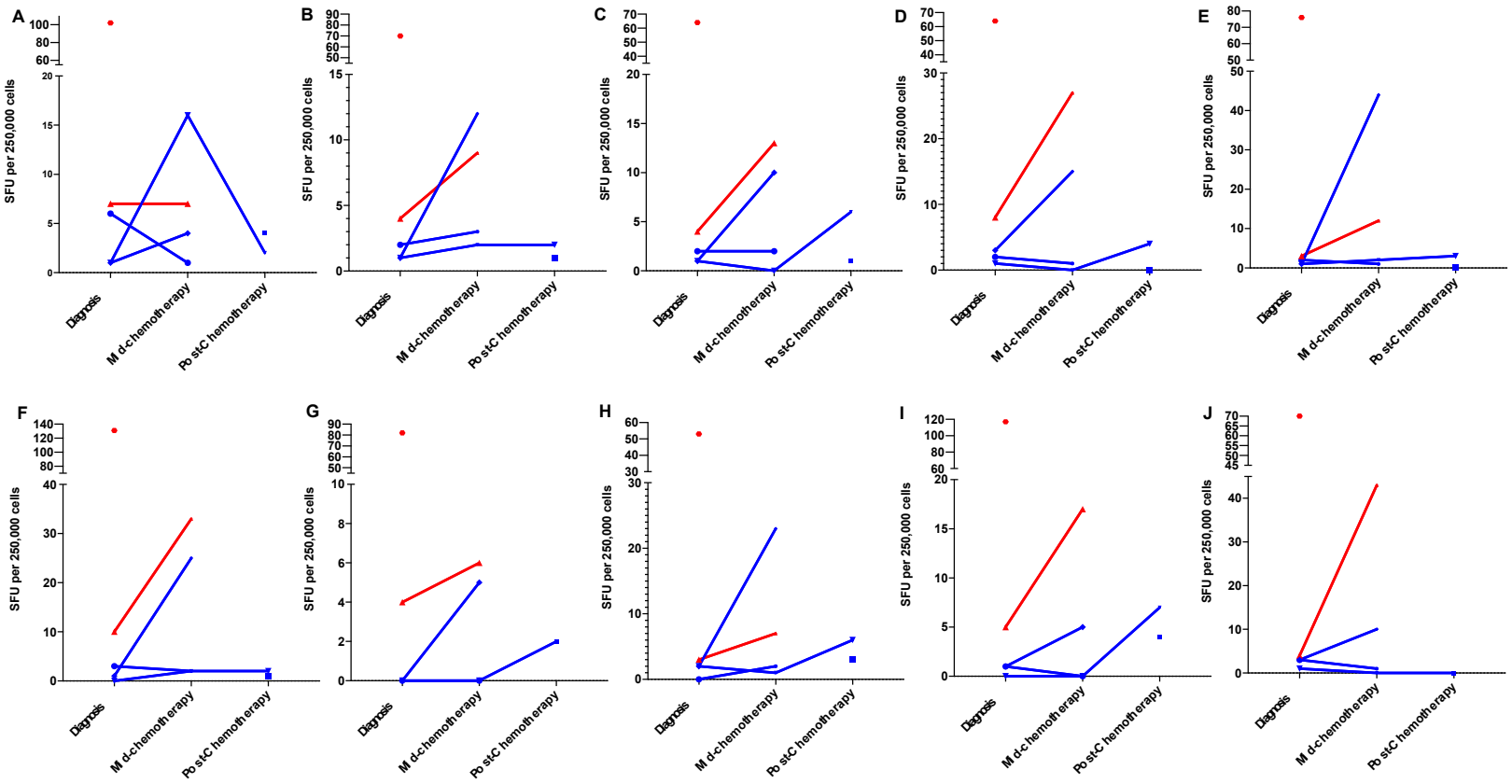


Figure 28. The relevant magnitude of specific T-lymphocytes compared against tumour-associated antigens with example ELISpot wells at pre-chemotherapy, mid-chemotherapy (before the third infusion of chemotherapy) and post-chemotherapy (minimum of 5-weeks after the final chemotherapy infusion, before surgery). Data show as individual responses. Individual lines and shapes represent individual participants. A. MamA B. CEA C. Cl6 D. CycB1 E. ERB ECD F. ERB ICD G. MMP1 H. TARP I. MUC1 J. SUR *SFU, spot forming units.*

DISCUSSION

The aim of this study was to examine if anti-cancer T-lymphocyte immunity in breast cancer patients is related to lifestyle or psychological factors and pathological clinical response to treatment. A further aim was to decipher whether the ability of CD8+ T-lymphocytes to release IFN-γ in response to tumour-associated antigens and lifestyle factors change from pre-, mid- and post-chemotherapy. As previously mentioned, due to feasibility issues which will be discussed further in this section, the original aims of the study could not be met. The main findings of the current study are; recruitment into a neoadjuvant study is likely to be ~33%, with less than 1 patient per month being recruited from 1 NHS site (depending on this size of the clinic), with an expected loss of contact in around 1/3 of patients enrolled. Both predicted $\dot{V}O_2$ max and BMD decline (average 15.7 ± 3.3 ml.kg.min^{-1} and 0.020 ± 0.010 g.cm$_2$) with neoadjuvant treatment. Higher red blood cell count (average 0.1 ± 1.8 x 10$_9$L) and haemaglobin levels (average 4 ± 56 g/dL) may be suggestive of a pCr. Finally, T-lymphocyte release of IFN-γ in response to viral and tumour-associated antigens tends to increase through treatment, and a higher T-lymphocyte response to tumour-associated antigens was observed in a patient with pCr.

Of those eligible, recruitment rate was predicted to be 12 patients per year (1 per month) with a 20% enrolment. Recruitment rate in the current study was higher (33%), however the number of eligible patients was lower than expected (a total of 6 over 1 year). Previous studies have experienced difficulties with recruitment of neoadjuvant chemotherapy breast cancer patients. The NEOCENT study, conducted in 12 UK centres over a period of 28 months, recruited 38 patients. As recruitment in the current study was only conducted in a single UK centre over 12 months, recruiting 6 women demonstrated improved recruitment per site compared to the NEOCENT study (Palmieri *et al.*, 2014). The NEOCENT study also conducted recruitment in 1 site in South Korea where uptake was higher (42 patients over 28 months), suggesting the UK healthcare system may be a limiting factor towards recruiting patients (Palmieri *et al.*, 2014).

Reasons for eligible patients not taking part in this study included a lack of time, psychological stressors and prioritisation of fertility (as chemotherapy can often cause immediate or premature infertility (Ries *et al.*, 2003; Odo and Potter, 2009; Soliman and Agresta, 2008; Lee *et al.*, 2006)). Actual recruitment may have been lower than predicted recruitment due to changes in preferred treatment options for breast cancer patients. Neoadjuvant chemotherapy for treatment of breast cancer has recently been questioned (Vaidya *et al.*, 2018), particularly in ER positive patients (Colleoni *et al.*, 2004; Berry, 2006),

reducing the prescription of neoadjuvant chemotherapy. Lack of time available between consultation (whereby potentially eligible patients are approach regarding the study) and onset of neoadjuvant chemotherapy (~1-5 days) may have also limited recruitment of patients with adherence criteria (24h without caffeine, alcohol and exercise).

Furthermore, ~50% of cancer patients suffer from anxiety and depression, both of which are particularly high prior to onset of chemotherapy (Knobf, 2011; Lim *et al.*, 2011). This is supported by results of the current study that show that even patients that did take part had clinically high levels of anxiety and stress at diagnosis. Consultants in the present study recorded anecdotally that patients have so much to take on board during consultations that they can't contemplate another thing such as participation in a research study. As consultants only approached patients who, in their professional opinion, are suitable for participation, this may also contribute to the lower than expected recruitment. To increase recruitment, multiple centres should be added, and identification and contact to eligible patients should occur where possible before consultation.

Retention of patients from diagnosis to post-surgery was another challenge in the current study as 1/3 patients were lost at follow up, despite numerous strategies employed including telephone and email reminders, investigator face to face meetings, flexibility in appointment times and collecting multiple contact details. It has been suggested that combining data collection with routine hospital visits and being flexible on appointment location may improve retention, thus rather than using a separate site for data collection, collecting data at the hospital itself may benefit (Kearney *et al.*, 2017; Zweben *et al.*, 2009). Other strategies include the use of gifts or payment on completion of the study and a study website (Bower *et al.*, 2014) which were not employed in the current study.

In the current study predicted $\dot{V}O_2$ max consistently decreased in all patients post-chemotherapy. This agrees with previous results demonstrating $\dot{V}O_2$ max is lower in breast cancer patients than healthy controls (Peel *et al.*, 2014a; Dolan *et al.*, 2010; Jones *et al.*, 2012; Jones *et al.*, 2007b; Jones *et al.*, 2007a). Furthermore, a recent review of 21 studies demonstrated mean $\dot{V}O_2$ max was 10% lower (2.4 ml.kg.min^{-1}) post-treatment (Jones *et al.*, 2011), following the same trend in the current study. Interestingly a decline of 3.5 ml.kg.min^{-1} has been associated with 18% increased risk of cardiovascular mortality. As the results of the current study show a much larger decrease (Myers *et al.*, 2002; Barlow *et al.*, 2012) the implications this may have on cardiovascular risk is paramount.

Breast cancer treatments such as chemotherapy (particularly anthracyclines such as epirubicin) negatively impact cardiorespiratory fitness through intercalation between DNA base pairs, inhibition of DNA topoisomerase II with subsequent blocking of replication and transcription, and the generation of iron-mediated oxygen free radicals that damage DNA, proteins and cell membranes (Carvalho *et al.*, 2014). These toxic effects can lead to endothelial injury, endothelial dysfunction, vascular remodelling and alterations in autonomic tone, which may have negative implications for heart rate reserve and cardiorespiratory function (Kenk *et al.*, 2010; Nousiainen *et al.*, 2001; Jones *et al.*, 2007a; Jones *et al.*, 2007b). The addition of adjuvant Trastuzumab for HER2+ patients may further increase clinical and subclinical cardiotoxicity rates (Piccart-Gebhart *et al.*, 2005; Romond *et al.*, 2005). Due to the low sample size in the current study we are unable to draw conclusions surrounding differences between HER2 positive and negative patients.

The observed decline in fitness is slightly surprising given a trend for a reduction in sedentary time (<1.8 METS) and an increase in light activity. However, pre- post- measures were only available in 3/6 patients and were made at least 3 weeks after the final chemotherapy infusion. Therefore, data may not be representative of physical activity levels throughout chemotherapy which may be responsible for fitness. The IPAQ is a quick and simple way for clinicians to measure physical activity. However, the results of the current study, and previous literature suggest that this subjective, self-reported measure of physical activity may not be accurate when compared against objective measures (Lee *et al.*, 2011b). Greater variability was seen in moderate and vigorous activity levels, also in agreement with previous research (Lee *et al.*, 2011b). In this study we found that on average IPAQ overestimated physical activity by 23, 176 and 144% for light, moderate and vigorous activity respectively. A separate study that compared IPAQ to Sensewear measured physical activity in cancer patients reported 366% higher moderate and vigorous physical activity compared to Sensewear, confirming the over estimation when using IPAQ to measure physical activity (Vassbakk-Brovold *et al.*, 2016). We conclude that whilst use of IPAQ may be easier to implement than objectively measured physical activity, results should be treated with caution as evidence suggests that patients over-estimate their activity, particularly at moderate and vigorous levels.

Whilst body composition in terms of body fat measured by bioelectrical impedance and DEXA did not differ over treatment, the two measures correlate well and were in agreement. This is particularly encouraging given the disadvantages and challenges of using DEXA, such as cost and exposure to radiation. This is in agreement with other literature that reports

bioelectrical impedance estimates of body fat show good absolute agreement with DEXA (Thomson *et al.*, 2007). Taken collectively, the results suggest bioelectrical impedance can be used as an alternative to DEXA to measure body fat percentage in this population.

BMD declined post-chemotherapy for all 4 participants whom it was measured in, on average by 23%. Previous research using the same DEXA scan technique assessed BMD in 492 breast cancer patients who had previously had neoadjuvant or adjuvant chemotherapy (Tang Axelsen *et al.*, 2018; Greep *et al.*, 2003). 1 year following treatment results showed patients had a significant loss of BMD in the hip and lumbar spine (Tang Axelsen *et al.*, 2018).

Chemotherapy is known to induce bone loss (Bjarnason *et al.*, 2008; Chen *et al.*, 2015) which may cause osteoporosis, a skeletal disorder of weakened bone strength in combination with an increased risk of fracture (Hernlund *et al.*, 2013). Literature suggests that the highest extent of bone loss takes place within the first six months of chemotherapy onset (Cameron *et al.*, 2010; Hershman *et al.*, 2010). In the current study the post-chemotherapy BMD was measured 5-6 months after the first chemotherapy infusion, supporting this conclusion. Literature states a bone loss of 10% is expected to increase the risk of fractures by a factor two to three (Marshall *et al.*, 1996) and the bone loss observed in the current study was over 10% in all patients, demonstrating an increased risk of osteoporosis which should be considered and monitored and/or treated by clinicians.

In pre-menopausal women a likely mechanism for BMD loss is premature ovarian dysfunction (Greep *et al.*, 2003; Cameron *et al.*, 2010; Hadji, 2009; Kanis *et al.*, 1999; Coleman *et al.*, 2013; Hadji *et al.*, 2007) which occurs in the majority of premenopausal women undergoing chemotherapy. This leads to a decreased systemic oestrogen level which in turn increases bone loss (Vehmanen *et al.*, 2006; Riggs *et al.*, 2002). Other mechanisms may surround the use of chemotherapy with bone-wasting agents (such as doxorubicin and cyclophosphamide), a lack of physical activity (Reichman and Green, 1994; Reyno *et al.*, 1993; Rodríguez-Rodríguez *et al.*, 2005; Shapiro *et al.*, 2001) (demonstrated by the reduced cardiorespiratory fitness in the current study) and muscle wasting. Notably, glucocorticoids were administered to alleviate side effects within a ~2-day window around each cycle of chemotherapy. It has been suggested that the bone loss may be associated with this supportive care medication (Weinstein, 2011). Other factors such as habitual alcohol intake, prior hormone replacement therapy and level of vitamins within the blood

may also affect bone turnover (Feskanich *et al.*, 1999a; Karlsson, 2004; Watts *et al.*, 1995b; Feskanich *et al.*, 1999b).

In the current study mid-chemotherapy and post-chemotherapy Hb levels differed from pre-chemotherapy. Higher post-chemotherapy Hb and red blood cell count were observed in patients who had pCr. Decreased Hb and red blood cell count mid chemotherapy, in patients with pCr levels were restored post-chemotherapy whereas in patients with pPr, Hb levels tended to drop further. Hb levels have been reported to be related with treatment outcomes and survival in cancer, independent of tumour type (Caro *et al.*, 2001). Up to 40% of all cancer patients are anaemic (Tas *et al.*, 2002; Knight *et al.*, 2004) and studies have shown that low Hb at various time points around treatment is associated with worse local relapse-free survival in patients with primary breast cancer (Boehm *et al.*, 2007). In 157 T2-4, N0-1 M0, breast cancer patients, those with Hb levels over 12.5g/dl at diagnosis were more likely to respond to chemotherapy treatment (Bottini *et al.*, 2003). Another study reported that initial Hb levels ~10 g/dl were significantly associated with worse 10-year survival compared with patients with Hb level of 12-14 g/ dL (Lee *et al.*, 2017a). All patients in the current study had Hb levels >12.5g/dl pre-chemotherapy which may explain why all patients responded to treatment (either completely or partially). Patients who had pCr continued to have Hb levels post-treatment >12.5g/dl whilst those with pPr had post-chemotherapy Hb levels <12g/dL. Local relapse-free survival was not measured in the current study.

It has been noted that most Hb levels decline in the first 6 months after treatment initiation (Lee *et al.*, 2017a). This trend is seen in the current study in pPr patients and more interestingly in all patients at the mid-chemotherapy time point which is just over 2 months from the onset of treatment, suggesting these declines may happen more rapidly than initially thought. Causes of anaemia in breast cancer patients include the effects of chemotherapy and radiation-induced myelosuppression, bleeding, marrow infiltration by cancer invasion, suppression of production of the essential erythroid-cell growth factor erythropoietin, nutritional deficiencies, and cytokine-mediated anaemia (Lee *et al.*, 2017a). Anaemia can causes tissue hypoxia, which is tumour promoting through activation of genes in addition to erythropoietin that facilitate angiogenesis, glucose metabolism and cell proliferation, enhancing the resistance of tumour cells to radiotherapy or antimetabolites, as well as selecting for p53-defective cells (Muz *et al.*, 2015; Lee *et al.*, 2017a). Higher Hb levels during treatment might be the beneficial as it may suggest an increased blood flow and drug delivery to the tumour. However, research is inconsistent as pre-treatment levels of Hb in neoadjuvant chemotherapy breast cancer patients have been shown not to affect

clinical response, and correction of anaemia beyond what is clinically necessary may not improve outcomes (Beresford *et al.*, 2006). This agrees with the current study that showed no relationship between Hb levels at diagnosis and treatment outcome, likely because of similarities in patient population and tumour types.

No trends could be seen in cell count at pre-, mid- and post-chemotherapy regarding response to treatment. Previous literature has highlighted the importance of total leukocyte counts, specifically NLR. NLR at baseline has been shown to correlate with patient outcomes in breast cancer, whereby an elevated NLR correlates with poor treatment outcomes and relapse-free survival (Azab *et al.*, 2012). Previously, mean NLR has been reported as 2.18 in breast cancer patients with 44% of patients classified as having a low NLR (NLR <1.7) (Chae *et al.*, 2018; Wei *et al.*, 2016a). The mean NLR in the current study was 2.73 with only 1 patient (patient 6) having an NLR at diagnosis <1.7 (17%). Inconsistencies may surround study population as the current study included both HER2 positive and negative breast cancer patients and the low sample size.

Neutrophils may potentially exert pro-tumour activity (Coussens and Werb, 2002; DeNardo and Coussens, 2007; Kuang *et al.*, 2011; De Larco *et al.*, 2004; Rodriguez *et al.*, 2009; Müller *et al.*, 2009). In the current study all absolute neutrophil counts were below 5.5 x 10^9/L at all time points which may explain why no relationship with clinical response was observed (Hong *et al.*, 2019). Higher absolute lymphocyte counts have a relationship with improved disease-free survival and pCr (Denkert *et al.*, 2015a; Slamon *et al.*, 2011; Luen *et al.*, 2017; Inoue *et al.*, 2018; Li *et al.*, 2016; Savas *et al.*, 2016; Avci *et al.*, 2015; Castaneda *et al.*, 2016; García-Teijido *et al.*, 2016; Jung *et al.*, 2016b; Salgado *et al.*, 2015; Tung and Winer, 2015; Salgado *et al.*, 2014). The T-lymphocyte response is an important component of immunosurveillance and controls the progression in cancer (Shankaran *et al.*, 2001a). Anti-tumour T-lymphocyte responses, tumour-infiltrating lymphocytes and the trafficking of T-lymphocytes towards tumour have previously improved the killing of tumour cells, increased chemotherapy responsiveness and improved treatment outcome (Mahmoud *et al.*, 2011; Loi *et al.*, 2013; Gooden *et al.*, 2011; Denkert *et al.*, 2010; West *et al.*, 2011).

PLR, has been proposed as a predictive factor for response to breast cancer treatment and disease-free survival (Xu *et al.*, 2017; Asano *et al.*, 2016; Rafee *et al.*, 2016) and is often elevated in malignant disease (Levin and Conley, 1964). In a study with 288 breast cancer patients treated with adjuvant chemotherapy, pCr was significantly high in the low baseline

PLR group (defined as <150), suggesting that patients with low PLR have higher anti-tumour activity, better prognosis and better response to chemotherapy. In the current study 2 patients had PLR <150, one had pPr and the other had pCr (Cuello-López *et al.*, 2018). Furthermore, the estimated overall survival of patients with thrombocytosis was 71 months versus 99.5 months in patients without thrombocytosis (>400 x 10^9/L) (Taucher *et al.*, 2003) perhaps as circulating tumour cells use platelets to 'hide' from immune detection and destruction and as an intermediary, helping them to attach to endothelial cells at the destination sites of metastases and may promote of vascular integrity, promoting tumour progression (Ho-Tin-Noe *et al.*, 2011; Gay and Felding-Habermann, 2011). No patients in the current study had platelet counts >400 x 10^9/L at pre-chemotherapy and only 1 patient (patient 1, pPr) had a platelet count >400 x 10^9/L at any time point (mid-chemotherapy). Results are inconsistent as studies have shown a PLR >292 x 10^9/L or >215 x 10^9/L does not correlate with clinicopathologic variables (Krenn-Pilko *et al.*, 2014; Koh *et al.*, 2015). No patients in the current study had a diagnosis PLR >292 x 10^9/L but 2 patients had PLR >215 x 10^9/L, both of which had pPr.

In the current study, the number of T-lymphocytes responding to tumour-associated and viral antigens demonstrated a trend increasing from pre-, mid- and post-chemotherapy. The number of responding T-lymphocytes was higher at diagnosis in patients who demonstrated a pCr. Previously literature has detected T-lymphocyte IFN-γ secretion in response to CEA, HER2 and MAGE-A3 (Melanoma associated antigen 3) in breast cancer patients (Inokuma *et al.*, 2007a). The current study demonstrates patients can elicit T-lymphocyte responses towards 10 different tumour-associated antigens.

It has previously been demonstrated that neoadjuvant chemotherapy HER2 positive breast cancer patients with pCr had an increased number of HER2 specific CD8+ T-lymphocytes able to secrete IFN-γ in response to antigens, versus patients who only underwent pPr (Muraro *et al.*, 2011). Furthermore, during chemotherapy, women with pCr still had detectable HER2 specific T-lymphocyte responses whereas these were absent in patients with pPr. In the current study, the patient with a higher number of responding cells at baseline had pCr, aligning with these findings. It has also been demonstrated that an increased number of CD8 T-lymphocytes, as measured by 12 day in vitro culture and subsequent cytokine staining, is linked with improved 5 year survival (100% versus 38% in those who did not possess HER2 responsive T-lymphocytes) (Bailur *et al.*, 2015). A limitation of the current study is that immune cells were taken from the peripheral blood and it is unknown whether the cells of the periphery represent the cells at the site of the tumour.

Future research should focus on comparing the cells, and function of cells, from all sites to determine which site is most robustly linked with clinical outcomes.

Drugs commonly used in cancer chemotherapy can augment the antitumor effects of immunotherapeutic modalities, supporting findings from the current study, that because of chemotherapy, immune response to viral and tumour-associated antigens increases (Medler *et al.*, 2015; Opzoomer *et al.*, 2019). Chemotherapy induces tumour apoptosis, providing an abundance of tumour-associated antigens and danger signals which can be presented to T-lymphocytes and elicit a heightened immune response (Obeid *et al.*, 2007; Kepp *et al.*, 2009; Haynes *et al.*, 2008; Nowak *et al.*, 2003; Jackaman *et al.*, 2012) . This has been demonstrated in murine models where 5-fluorouracil treatment and doxorubicin (drugs used within the current chemotherapy regimen) induced caspase-dependent apoptosis enhanced CD8+ lysis of tumour cells (Tanaka *et al.*, 2002; Casares *et al.*, 2005a).

Interestingly it has previously been shown that the pattern of T-lymphocyte cytokines produced in response to tumour-associated antigens in breast cancer patients was significantly different from that produced in response to CMV or influenza in the same patients (Inokuma *et al.*, 2007b). Specifically, there was lower proportion of IFN-γ producing cells responding to tumour-associated antigens compared with CMV or influenza antigens. These results were mirrored in the current study where, overall, a higher number of IFN-γ producing cells were present in response to viral antigens

Further research should expand this current data set using multi-centre sites so that more robust conclusions can be made on lifestyle measures and the clinical value of haematological cell counts with consideration of tumour grade, type, and baseline characteristics (Lee *et al.*, 2017a). Future research should also follow patients over time to assess local relapse-free survival to form associations between diagnosis, during and post-treatment blood measures. Within the current study the proportion of phenotypes of the cells within each ELISpot well is unknown. Previous literature shows that T-lymphocytes responding to tumour-associated antigens are almost completely CD28+ CD45RA−, whereas those responding to CMV are broadly distributed amongst phenotypes with a high proportion of terminal effector cells (Inokuma *et al.*, 2007b). On top of this further research needs to be done to demonstrate whether peripheral blood is representative of the blood within the tumour itself, as it may be the case that lymphocytes are trafficking towards the tumour and therefore numbers in the periphery fall. Quantification of positive T-lymphocyte

responses towards tumour-associated antigen should also be made using the viral ELISA technique used within chapters 3 and 4 of this thesis.

The current study is limited in use due to the low sample size, however it sets the tone for potential discoveries surrounding lifestyle, immune function and treatment outcomes in breast cancer patients receiving neoadjuvant chemotherapy and potential markers of treatment outcome. It also acts as a pilot study, highlighting difficulties researchers may encounter when working with this population. Future research should include a greater number of patients so that conclusions can be drawn, and differences identified between patients exhibiting different tumour characteristics (e.g. HER2 +, ER+) and to follow these patients after treatment to monitor disease-free survival.

As more than 80% of breast cancer patients are expected to live over 10 years, it is important to focus research on the potentially harmful effects of treatment such as decreases in BMD and increased risk of osteoporosis. Further studies should take multiple measurements of BMD over time, record fracture incidents and evaluate the use of exercise interventions and prophylactic zoledronic acid to negate the effects of chemotherapy on BMD.

In conclusion, from this small data set, cardiorespiratory fitness and BMD detrimentally decline after chemotherapy, potentially impacting future health of patients. Furthermore, the elevated Hb and T-lymphocyte response to tumour-associated antigens observed in a patient that had pCr may demonstrate a potential association between these variables.

CHAPTER 6: Comparing a remotely monitored physical activity intervention to partly supervised exercise in breast cancer survivors: a randomised, controlled non-inferiority trial

6.1 INTRODUCTION

Breast cancer is the most common cancer in women in the UK with >55,000 cases diagnosed per year and around 78% of women diagnosed surviving for 10 or more years (Cancer Research UK, 2018). As a result, there are many women currently living with the negative lifestyle related consequences of a previous breast cancer diagnosis and its treatment, such as decreases in cardiovascular fitness and increases in body mass (Klassen *et al.*, 2014; Watts *et al.*, 1995a). These lifestyle changes can contribute to a reduction in quality of life and increase the prevalence of other conditions associated with less healthy lifestyles, such as metabolic syndrome and type II diabetes (Peel *et al.*, 2014b; Pedersen *et al.*, 2017; Irwin *et al.*, 2005a; Nissen *et al.*, 2011; Kim *et al.*, 2013; Goodwin, 2015a; Demark-Wahnefried *et al.*, 2000). Furthermore, cardiovascular disease is the most prevalent comorbidity among individuals who have undergone and finished breast cancer treatment (Reeves *et al.*, 2016; Mehta *et al.*, 2018).

Observational research has shown positive effects of physical activity on survival and disease recurrence in breast cancer survivors (Ogunleye and Holmes, 2009; Maliniak *et al.*, 2018; McTiernan, 2018). Randomised controlled trials demonstrate that exercise and physical activity has beneficial effects on cardiorespiratory fitness, body composition and fatigue in healthy populations (Lin *et al.*, 2015; Kendall and Fairman, 2014), breast cancer patients and breast cancer survivors (Galvão and Newton, 2005; McNeely *et al.*, 2006; Kessels *et al.*, 2018). For this reason, physical activity and exercise have become important for breast cancer survivors following cancer treatment (Wolin *et al.*, 2012; McTiernan *et al.*, 2019).

Clinicians often advise patients to change their lifestyle habits, by increasing physical activity and adopting a healthy diet. However, this approach is often ineffective as it involves high cost resources that are not within individual's budget and requires individuals to live near facilities such as gyms, which is difficult for non-urban women or women of low socioeconomic status (Haines *et al.*, 2010). Whilst it has been shown prescribed, supervised exercise lasting 8-weeks to 6 months can improve aspects of health, such as $\dot{V}O_2$max and body mass, this indoor approach to exercise may not be as enjoyable and sustainable (due to lack of facilities or costs) as other approaches towards exercise in this

population (Plante *et al.*, 2007). A self-administered questionnaire administered to 307 cancer survivors, including breast cancer survivors, concluded the majority of cancer survivors prefer unsupervised exercise (Jones and Courneya, 2002). In addition, in women following breast cancer treatment, 85% expressed interest in receiving remotely delivered exercise counselling, 80% expressed interest in participating in a remotely delivered exercise intervention and 68% expressed interest in using an exercise 'app' or website (Phillips *et al.*, 2017). Patients also reported that the most helpful technology-supported intervention components would be a physical activity tracker and personalised feedback (Phillips *et al.*, 2017). Such technological support, in the form of an activity tracking wristwatch, is a promising strategy that could increase exercise and physical activity levels (McCue *et al.*, 2010). Measuring heart rate, through a wrist worn device promotes increased motivation through providing immediate feedback to wearers (Ryan *et al.*, 2008; Standage and Ryan, 2011). Benefits similar to those for supervised interventions, such as increases in functional fitness tests like the 6-minute walk , decrease in body mass and increases in forced vital capacity, have been reported in other individuals that have low cardiorespiratory fitness such as cystic fibrosis patients, stroke survivors and obese individuals that have undertaken remotely monitored exercise (Moorcroft *et al.*, 2004; Olney *et al.*, 2006; Nicolai *et al.*, 2009).

Non-inferiority trials have been previously been used in clinical settings to test whether one experimental treatment (which may have advantages over standard treatment) is not unacceptably less efficacious than an active control treatment that is already in use (Hahn, 2012). An advantage of non-inferiority trials is that there is no need for a control or placebo group, which if implemented, can be deem unethical. Non-inferiority trials are beginning to make their way into exercise intervention research, with research examining the non-inferiority of home-based exercise versus cognitive behavioural therapy in breast cancer survivors (Mercier *et al.*, 2018), exercise interventions in patients with coronary artery disease (Serón *et al.*, 2019) and telerehabilitation versus physiotherapy following hip replacements and coronary heart disease whereby telerehabilitation was deemed non-inferior to standard care in improving fitness and body composition (Nelson *et al.*, 2020; Maddison *et al.*, 2019).

The primary objective of this randomised-controlled study is to evaluate whether 8-weeks of a remotely monitored physical activity intervention with the use of a physical activity tracking wristwatch, produces changes to cardiorespiratory fitness that are not meaningfully inferior to changes in cardiorespiratory fitness in response to a partly supervised exercise

programme in female breast cancer survivors (aged 35-69 years). Additionally, we will determine the influence of both the partly supervised exercise intervention and the remotely monitored intervention on broad aspects of physiology; including body composition, physical functioning, psychological measures, and biomarkers of health including markers of inflammation.

We hypothesise that an 8-week remotely monitored physical activity intervention will result in non-inferior improvements to cardiorespiratory fitness, quality of life, functional fitness, body composition, biomarkers of health, energy intake and immune function compared to 8-weeks of a partly supervised, exercise intervention.

6.2 METHODS

6.2.1 Participants and study design

This two-armed randomised (1:1) controlled trial with participants undertaking an 8-week technology-enabled, remotely monitored physical activity intervention or a partly supervised, exercise intervention was granted ethical approval by the NHS research ethics committee (reference: 18/WA/0314).

37 participant volunteers were recruited following appointments at the RUH Bath and 30 participants completed the exercise interventions (57 ± 6 years, 25.3 ± 3.3 BMI $kg.m_2$, $\dot{V}O_2max$ 28.9 ± 6.1 $ml.kg.min_{-1}$ at baseline.). All participants were female breast cancer survivors who had ended treatment >2 months prior but no longer than 2 years ago, aged 35-69 years, and had no contraindications to exercise or already be participating in 150 minutes of moderate and vigorous, structured exercise accumulated across at least 7 days a week as determined by the IPAQ. Patients were informed verbally and in writing about the rationale, nature and demands of the study (and of their right to withdraw) before providing written informed consent. Patients subsequently completed a general health questionnaire, a physical activity readiness questionnaire and a sex specific cancer risk factor questionnaire.

No significant differences were observed between the partly supervised and remotely monitored exercise intervention groups in terms of time since breast cancer diagnosis or time since surgery when enrolling in the trial (Table 30.) ((F(1,28)=0.380, p=0.542 and F(1,28)=2.372, p=0.125 for time since diagnosis and surgery respectively). No significant differences were observed when assessing the number of women who were ER and HER2 positive (methodology described in Chapter 5) within each group or the number of women previously treated with chemotherapy for their breast cancer diagnosis (X_2=0.216, p=0.642, X_2=0.574, p=0.580 and X_2=0.159, p=0.690 for ER, HER2 and chemotherapy respectively).

Table 30. Clinical information on breast cancer survivors

	Prescribed, partly supervised group (n=15)	**Remote, advisory group (n=15)**
Time since diagnosis (months)	12 ± 4	14 ± 8
Number ER positive (%)	11/15 (73%)	10/15 (67%)
Number HER2 positive (%)	1/15 (7%)	2/15 (13%)
Time since surgery (months)	10 ± 4	12 ± 6
Number treated with chemotherapy (%)	5/15 (33%)	4/15 (27%)

Data shown as means ± SD.

ER; oestrogen receptor, HER2, Human epidermal growth factor receptor

6.2.2 Sample size

The current study was powered to detect the non-inferiority of an 8-week remotely monitored exercise intervention versus a partly supervised exercise intervention on improvement in cardiorespiratory fitness. It has previously been reported that supervised exercise significantly increased $\dot{V}O_2$max by 4.4 ml.kg.min^{-1} with a standard deviation of 3.5 ml.kg.min^{-1} (Shinkai *et al.*, 1994a). Participants in the previous study were overweight, middle-aged women (mean ± SD; age 53 ± 5.9 years, BMI 27.2 ± 2.0 kg.m^2) (Shinkai *et al.*, 1994a), matching the demographic of participants to be recruited in the current study. The exercise training intervention in the previous study consisted of 12-weeks of aerobic exercise consisting of walking, jogging, cycling and swimming 3-4 times per week for 45-60 minutes per session at 50-60% $\dot{V}O_2$max. This intervention is similar to that in the present study therefore these values were used to inform our sample size using the website; Sealed Envelope (https://www.sealedenvelope.com/). The non-inferiority limit, d, (the largest difference in $\dot{V}O_2$max that is clinically meaningful) was chosen as 3 ml.kg.min^{-1} because, based on previous literature and judgement by researchers in this project, this represents a value whereby any value above this could be deemed a meaningful change. With 80% power and one-sided α=0.05, a total sample size of 34 people (17 people per group) need to be recruited to identify less than a 3 ml.kg.min^{-1} difference in mean cardiorespiratory fitness between the two treatment groups.

6.2.3 Study procedures

The study protocol and time frame are mapped out in Figure 29. Participants attended three laboratory visits pre-, mid- (4-weeks) and post- (8-weeks) the 8-week exercise interventions. The same experimental procedures were completed at baseline and follow up. The mid-intervention visit included all procedures other than blood sampling and DEXA scanning. Participants reported to the laboratory on all three occasions after a 10-hour overnight fast and after refraining from exercise, alcohol and caffeine in the prior 24h.

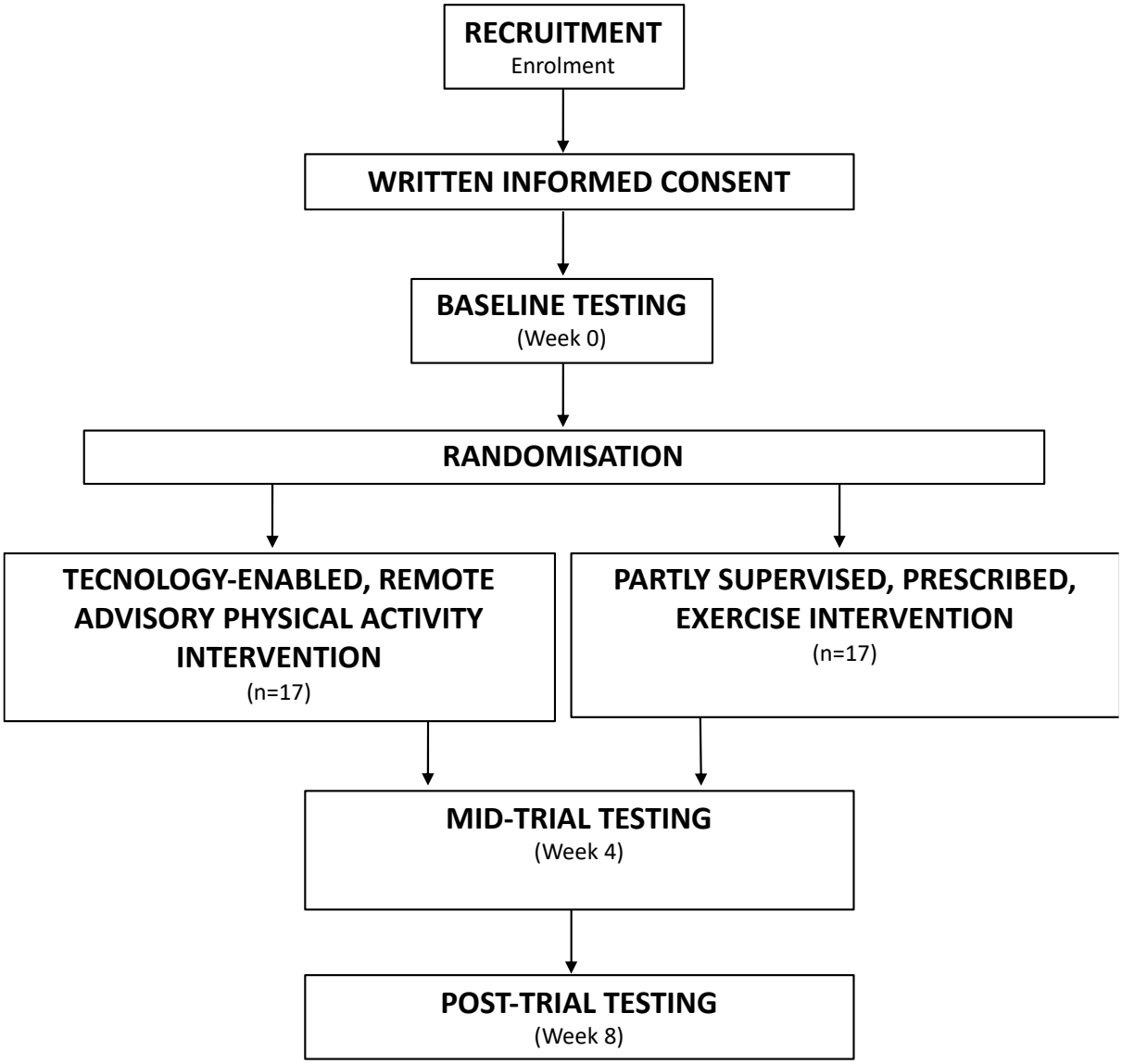


Figure 29. Flow diagram showing the study timeline of the non-inferiority trial in breast cancer survivors

Assessment of body composition, habitual diet, depression, anxiety and stress, and blood sampling were in line with methodology explained in Chapter 2, thus for conciseness, are not repeated here.

Assessment of cardiorespiratory fitness through maximal exercise, enjoyment of physical activity and exercise testing was in line with methodology explained in Chapter 2.

6.2.3.1 Assessment of physical function

Participants undertook the 6-minute walk test whereby participants were asked to walk as far as possible in 6-minutes by repeating shuttles between two cones placed 7m apart at a self-selected pace. Participants were permitted to stop to rest, slow down or speed up at any point during the test and were informed at the end of each minute how long there was left to go, but no further encouragement or feedback was provided. Participants then performed as many sit-to-stands (seated at the front of the chair, to rise until they reach full knee extension, and sit back from a chair) as possible in 30 seconds with arms folded across the chest to assess general lower extremity endurance. Participants undertook the 8ft get up and go test, whereby the participant rose from a seated position, walked 8ft and then returned to the seated position.

6.2.3.2 Medical History

Medical notes were provided to the research team with anonymised information about tumour stage, ER and HER2 status, in addition to carriers of known mutations for breast cancer risk genes (e.g., BRCA1 and BRCA2). Tumour biopsy and histopathology were used to confirm tumour diagnosis.

6.2.3.3 Biochemical analysis

Plasma glucose, triglycerides, CRP, NEFA, glycerol and cholesterol concentrations were determined using an automated analyser (Daytona, Randox Laboratories) according to manufacturer's instructions. ESR was measured according to manufacturer's guidelines (Guest Scientific, Switzerland).

6.2.4 Randomisation

After baseline laboratory testing participants were randomly allocated to one of the two intervention groups, allocation ratio 1:1. Randomisation was performed by an independent researcher using a computer-generated randomisation list. Randomisation was stratified for previous chemotherapy treatment (yes/no) and BMI (normal vs overweight, thresholds above and below 25kg.m2).

6.2.5 Interventions

6.2.5.1 Technology-enabled, remotely monitored exercise intervention

Participants allocated to the remotely monitored intervention were provided with a Polar A370 battery powered, silicone fitness tracker (Polar Electro, Kempele, Finland) that recorded continuous heart rate via photoplethysmography. The fitness tracker, with built in accelerometer, was placed on the participants chosen arm according to manufacturer's instructions. A guidance booklet and demonstration were given to the participant on how to use the monitor. The researcher asked the participant to undertake a set duration of physical activity per week whereby the duration and frequency of each individual session was decided by the participant. The only stipulation was that each individual training session lasted a minimum of 10 minutes. Participants were asked to record exercise using the pre-determined 'training' setting built into the Polar A370 device which provided instant feedback to the participant on heart rate. The goal was to achieve a minimum total duration of moderate activity per week starting at 105 minutes and finishing at 150 minutes per week, increasing by 15 minutes every 2 weeks, at a minimum heart rate starting at 55% HRmax (as recorded during the cardiorespiratory exercise test) and finishing at 70% HRmax, increasing by 5% every 2 weeks (Table 31) and updated with the most recent HRmax measured. The research team monitored individual training electronically at a minimum of once per week and had a weekly 30-minute telephone call to provide feedback about how the participant's activity compares to the goal set, motivation and setting the physical activity goal for the upcoming week. Daily steps and calorie burn were also estimated by the fitness tracker.

6.2.5.2 Partly supervised exercise intervention

Participants randomised to this group undertook 2 supervised exercise sessions per week at the University of Bath including treadmill and cycle ergometer exercise and one home-based exercise session per week of the participant's choice. The supervised exercise sessions progressed by extending the duration exercising on the treadmill by 5 minutes every 2 weeks whereby the final duration on the treadmill was 35 minutes long and total duration mapped onto recommended guidelines of 150 minutes per week (Table 31.). Intensity progressed throughout the 8-weeks on both the treadmill and the cycle ergometer by 5% every 2-weeks, whereby exercise during the final week was at 70% $\dot{V}O_2$max, updated in line with the most recent $\dot{V}O_2$max measured (Table 31.). The unsupervised exercise session consisted of exercise of the participant's choice that increased in duration by 5 minutes every 2-weeks so that the final home-based session lasted for a duration of 50 minutes (Table 31.). To set exercise intensity on the treadmill, absolute $\dot{V}O_2$ (ml.kg.min^{-1}) was plotted against work rate (either speed (kph) or gradient (%) to calculate the speed and/or gradient that would elicit the desired percentage $\dot{V}O_2$max. To set exercise intensity of the cycle ergometer, ACSM metabolic calculations were used to estimate work rate required to elicit a desired percentage $\dot{V}O_2$max as measured by a treadmill exercise test (ACSM, 2013). To monitor the intensity of exercise in the supervised exercise sessions, expired gas was collected into Douglas bags to determine actual $\dot{V}O_2$. If actual $\dot{V}O_2$ did not match prescribed $\dot{V}O_2$ then gradient, speed or wattage were altered accordingly. To monitor the intensity of exercise and determine compliance in the unsupervised exercise session, participants wore a Wahoo heart rate monitor (Wahoo Fitness, Atlanta, Georgia, USA) and chest strap, which provided no immediate feedback during their exercise sessions. Compliance was recorded by assessing the average heart rate and duration of exercise from the Wahoo heart rate monitor on a weekly basis.

Table 31. Descriptions of the partly supervised, prescribed exercise and the remotely monitored exercise interventions

	Partly supervised, prescribed intervention						Remotely monitored intervention	
	Supervised session			Unsupervised session				
Week	Intensity	Duration per session	Duration per week	Intensity	Duration	Total duration per week	Intensity	Duration per week
1	55% $\dot{V}O_2$max	35 minutes	70 minutes	55% HRmax	35 minutes	105 minutes	55% HRmax	105 minutes
2	55% $\dot{V}O_2$max	35 minutes	70 minutes	55% HRmax	35 minutes	105 minutes	55% HRmax	105 minutes
3	60% $\dot{V}O_2$max	40 minutes	80 minutes	60% HRmax	40 minutes	120 minutes	60% HRmax	120 minutes
4	60% $\dot{V}O_2$max	40 minutes	80 minutes	60% HRmax	40 minutes	120 minutes	60% HRmax	120 minutes
5	65% $\dot{V}O_2$max	45 minutes	90 minutes	65% HRmax	45 minutes	135 minutes	65% HRmax	135 minutes
6	65% $\dot{V}O_2$max	45 minutes	90 minutes	65% HRmax	45 minutes	135 minutes	65% HRmax	135 minutes
7	70% $\dot{V}O_2$max	50 minutes	100 minutes	70% HRmax	50 minutes	150 minutes	70% HRmax	150 minutes
8	70% $\dot{V}O_2$max	50 minutes	100 minutes	70% HRmax	50 minutes	150 minutes	70% HRmax	150 minutes

$\dot{V}O_2$; oxygen consumption. HR max; maximum heart rate.

6.2.6 Adherence

Adherence in the remotely monitored group was determined by meeting both the total duration of exercise set and having an average heart rate above the heart rate aim as assessed by average heart rate of each exercise session undertaken per week. Adherence in the partly supervised group was determined by attending and completing all supervised sessions and being over the set duration and heart rate during the unsupervised session.

6.2.7 Statistical analysis

Repeated measures and one-way ANOVAs were employed to test differences in continuous data pre- and post-intervention within and between groups respectively due to the robustness of normality. Wilcoxon signed ranks and Mann-Whitney U tests were employed to test differences in categorical data pre- and post-intervention within and between groups respectively. Changes in key outcome variables within and between trials were analysed with repeated measures ANOVA with appropriate post-hoc tests adjusted for multiple comparisons to examine changes over time in variables measured over 3 time points. Standardised effect sizes (Cohen's *d*) were calculated for variables (Lakens, 2013).

To determine non-inferiority, a point estimate method was used whereby the fraction of the effect estimate that was considered clinically significant was determined based on clinical judgement and expert opinion after consulting the literature. This margin (Δ) was considered by the authors as the largest clinically acceptable difference between the test (partly supervised group) and the active comparator (remotely monitored group) whereby the active comparator can be deemed non-inferior. For example, for cardiorespiratory fitness, Δ represents the highest difference between groups that would be deemed non-inferior. Based previous literature and author judgement (Shinkai *et al.*, 1994) Δ would be set at 3 ml.kg.min^{-1}. The margin altered in accordance with the outcome variable. 95% CI between the difference of treatments were calculated by firstly calculating the variance of the difference between groups $\sqrt{\frac{SD(PPS)}{n(PPS)} + \frac{SD(RM)}{n(RM)}}$, whereby *SD(PPS)* represents the standard deviation of the change within the partly supervised group, *SD(RM)* represents the standard deviation of the change within the remotely monitored group, n(PPS) represents the number of participants in the partly supervised group and n(RM) represents the number of people in the remotely monitored group. The variance was then multiplied by the Z-score (1.96) and subsequently added or subtracted from the average difference in the means between the partly supervised group and the remotely monitored group to calculate the upper and

lower CIs. The following criteria were defined based on where the upper and lower limits; 1) Lie above/below (whatever was deemed worse) 0 and Δ the remote, advisory group is non-inferior to the prescribed, partly supervised group, 2) Lie above/below (whatever was deemed worse) Δ but cross 0 the remote, advisory group is non-inferior, 3) lie above/below 0 (whatever was deemed better) but includes Δ the results are inconclusive and 4) lie above/below (whatever was deemed better) 0 and Δ the remote, advisory group is inferior (Le Henanff *et al.*, 2006). Statistical analyses were conducted using SPSS version 22. Figures were produced using GraphPad Prism 8. Statistical significance was accepted at $p<0.05$.

6.3 RESULTS

6.3.1 Changes in physical activity and exercise levels

No differences in IPAQ self-reported physical activity and exercise were observed between groups at baseline ($p>0.05$). No significant differences in self-reported exercise and activity were seen in either group pre- versus post-intervention. Vigorous activity pre- and post-intervention in partly supervised group and remotely monitored group was 17 ± 30 to 44 ± 58 ($p=0.051$) and 14 ± 26 to 37 ± 80 ($p=0.333$) minutes per week respectively. Moderate activity pre- and post- intervention the in the partly supervised group was 47 ± 76 to 30 ± 63 minutes per week ($p=0.539$) whilst in the remotely monitored group was 14 ± 37 to 18 ± 28 minutes per week ($p=0.710$) (Table 32.). In total, an average of 74 minutes per week of exercise was reported in the partly supervised group despite participants being prescribed 150 minutes per week.

Table 32. Differences between self-reported physical activity and exercise in prescribed, supervised group and remote advisory groups and changes pre- and post-exercise intervention

	Partly Supervised group (n=15)			Remotely monitored group (n=15)			
	Pre	***Post***	***Main effect of time (one-way ANOVA)***	***Pre***	***Post***	***Main effect of time (one-way ANOVA)***	***Interaction effect (two-way ANOVA)***
Minutes of moderate exercise per week	47 ± 76	30 ± 63	F(1,14)=0.397, p=0.539	14 ± 37	18 ± 28	F(1,14=0.144, p=0.710	F(1,28)=0.592, p=0.473
Minutes of vigorous exercise per week	17 ± 30	44 ± 58	F(1,14)=1.643, p=0.051	14 ± 26	37 ± 80	F(1,14)=1.005, p=0.333	F(1,28)=0.030, p=0.864
Light activity (MET.min^{-1} per week)	1899 ± 1657	1374 ± 1426	F(1,14)=1.823, p=0.198	1734 ± 2053	768 ± 529	F(1,14)=3.734, p=0.074	F(1,28)=0.485, p=0.492
Moderate activity (MET.min^{-1} per week)	1936 ± 1774	2676 ± 4077	F(1,14)=0.600, p=0.451	1267 ± 1757	642 ± 838	F(1,14)=1.455, p=0.248	F(1,28)=1.578, p=0.219
Vigorous activity (MET.min^{-1} per week)	476 ± 805	864 ± 1301	F(1,14)=1.643, p=0.221	607 ± 1269	712 ± 984	F(1,14)=0.054, p=0.819	F(1,28)=0.270, p=0.670
Total activity (MET.min^{-1} per week)	4311 ± 3101	4914 ± 5716	F(1,14)=0.296, p=0595	3608 ± 3120	2122 ± 1570	F(1,14)=2.863, p=0.113	F(1,28)=2.180, p=0.151

* significant difference from baseline, p<0.05. Data shown as means ± SD

min; minutes. MET; metabolic equivalent.

Table 33 shows the differences in the duration and intensity of exercise interventions. During weeks 1, 3 and 7, the remotely monitored group undertook significantly more minutes of exercise per week as assessed by the Polar wristwatch ($p<0.05$). This trend was seen consistently throughout each week. However, the standard deviations in the remotely monitored group are much larger (94-128 minutes) than those in the partly supervised group (20-43 minutes). No significant differences were seen in average heart rates recorded during the home-based exercise session in the partly supervised group and the remotely monitored group. Interestingly, this objectively measured physical activity data goes against self-reported data presented in Table 32. When comparing each intervention across time, no main effect of time was observed on intensity of exercise ($F(3,26)=1.749$, $p=0.184$) or duration of exercise ($F(7,98)=1.573$, $p=0.152$) in the remotely monitored group. However pairwise comparisons between each week revealed a significantly higher duration of exercise in week 7 when compared to week 2 ($p=0.045$), week 5 ($p=0.024$) and week 6 ($p=0.009$). Pairwise comparisons also revealed a significantly higher average HR of exercise in week 7 compared to week 2 ($p=0.019$) and week 4 ($p=0.044$) and in week 8 compared to week 2 ($p=0.028$) and week 3 ($p=0.013$). In the partly supervised group a significant effect of time was observed on duration of exercise ($F(7,98)=5.348$, $p=0.000$, $\eta^2=0.276$). Pairwise comparisons revealed significant differences between week 1 and weeks 4 ($p=0.001$), 5 ($p=0.000$), 6 ($p=0.003$), 7 ($p=0.000$) and 8 ($p=0.021$), week 2 and weeks 5 ($p=0.005$), 6 ($p=0.019$), 7 ($p=0.003$) and 8 ($p=0.037$) and week 3 and weeks 4 ($p=0.026$), 5 ($p=0.002$), 6 ($p=0.003$), 7 ($p=0.001$) and 8 ($p=0.018$). No significant main effect of time was seen on intensity of the unsupervised exercise in this group ($F(3,26)=1.749$, $p=0.184$ but pairwise comparisons revealed a significantly higher HR was achieved in week 5 and 7 versus week 2 ($p=0.040$ and $p=0.027$ respectively). Week 3 produced, on average, the highest number of minutes of exercise with 226 ± 129 minutes recorded, whilst week 6 was the lowest (146 ± 96 minutes) (Table 33).

Table 33. Comparison of exercise interventions in breast cancer survivors

	Partly supervised group (n=15)		Remotely monitored group (n=15)		ANOVA statistics (differences between groups)	
	Total Minutes of exercise	**Intensity of home-based exercise (HR, bpm)**	**Minutes of exercise**	**Intensity of exercise (HR, bpm)**	**Minutes of exercise**	**Intensity of exercise (HR, bpm)**
Week 1	104 ± 25	112 ± 16	169 ± 103	114 ± 15	F(1,29)=5.670, p=0.024*	F(1,24)=0.091, p=0.766
Week 2	110 ± 28	116 ± 18	166 ± 106	113 ± 11	F(1,29)=3.915, p=0.057	F(1,24)=0.329, p=0.572
Week 3	111 ± 21	119 ± 13	226 ± 129	114 ± 12	F(1,29)=32.312, p=0.000*	F(1,26)=0.869, p=0.360
Week 4	133 ± 32	116 ± 14	176 ± 116	112 ± 12	F(1,29)=1.937, p=0.175	F(1,26)=0.649, p=0.428
Week 5	142 ± 30	118 ± 10	157 ± 102	115 ± 13	F(1,29)=0.302, p=0.587	F(125)=0.361, p=0.553
Week 6	135 ± 20	118 ± 10	146 ± 96	115 ± 12	F(1,29)=0.200, p=0.658	F(1,26)=0.423, p=0.521
Week 7	141 ± 22	118 ± 8	209 ± 94	119 ± 13	F(1,29)=7.513, p=0.010*	F(1,27)=0.059, p=0.811
Week 8	138 ± 43	117 ± 13	179 ± 128	119 ± 10	F(1,29)=1.397, p=0.247	F(1,25)=-.791, p=0.382

Data shown as means ± SD. Heart rate data recorded using Polar A370 fitness tracker in the remote, advisory group and wahoo heart rate monitor in the prescribed, partly supervised group.*significantly different from prescribed, partly supervised group

HR; heart rate. bpm; beats per minute

No significant differences were seen in steps per days ($F(3,45)=0.407$, $p=0.762$) or energy expenditure per day ($F(2,16)=0.502$, $p=0.560$) over the 8 weeks in the remotely monitored group at any time point ($p>0.05$) (Table 34). The highest average step count per day was seen during week 7 (12775 ± 4991 steps per day). Energy expenditure per day stayed relatively stable throughout the 8 weeks. The highest average energy expenditure per day was observed during week 4 (2201 ± 320 kcal per day) (Table 34.).

Table 34. Changes in physical activity level in the remote advisory exercise intervention as measured by Polar A370

	Steps per day	**Energy expenditure (kcal per day)**
Week 1	11768 ± 2314	2148 ± 358
Week 2	11675 ± 4212	2135 ± 306
Week 3	12005 ± 3838	2192 ± 355
Week 4	13060 ± 4355	2201 ± 320
Week 5	12439 ± 4497	2049 ± 592
Week 6	11504 ± 4616	2124 ± 380
Week 7	12775 ± 4991	2220 ± 210
Week 8	12322 ± 4311	2157 ± 289

Data shown as means ± SD. Data recorded using Polar A370 fitness tracker

Kcal; kilocalorie

6.3.2 Influence of adherence and enjoyment

Adherence was significantly higher, on average, in those in the partly supervised group whereby participants successfully completed 87 ± 7% of all exercise session (21/24 exercise sessions) ($F(1,28)=9.923$, $p=0.004$, effect size 0.26, small). In the remotely monitored group, participants only successfully completed 64 ± 25% of all exercise. Furthermore, enjoyment was significantly higher in the partly supervised group (by 14 arbitrary units), $U=51.000$, $p=0.010$, effect size 0.47 (moderate) (Table 35). When introduced as covariates no changes were seen towards main outcome measures $\dot{V}O_2max$ and body fat percentage (data not shown).

Table 35. Differences between adherence and enjoyment in partly supervised group and remotely monitored groups

	Prescribed, partly supervised group (n=15)	**Remote, advisory group (n=15)**
Adherence (%)	87 ± 7	65 ± 26*
Enjoyment (PACES arbitrary units)	94 ± 14	80 ± 13*

* Significantly different from partly supervised $p<0.05$

PACES; physical activity enjoyment scale

6.3.3 Changes in characteristics post-interventions

No significant differences were seen between groups in terms of age and blood pressure at baseline ($p>0.05$). At baseline, those in the prescribed, partially supervised group on average had hypertensive blood pressure (135 ± 22 mmHg) which was significantly reduced post-intervention to 126 ± 22 mmHg ($F(1,14)=12.965$, $p=0.003$, $\eta p^2 =0.23$) (Table 36.). Those in the remotely monitored group had elevated systolic blood pressure at baseline 125 ± 21 mmHg which was 121 ± 19 mmHg post-intervention (Table 36).

Table 36. Differences between characteristics in partly supervised group and remotely monitored groups and changes pre- and post-exercise intervention

	Partly supervised group (n=15)			Remotely monitored group (n=15)			
	Pre	***Post***	***Main effect of time (one-way ANOVA)***	***Pre***	***Post***	***Main effect of time (one-way ANOVA)***	***Interaction effect (two-way ANOVA)***
Age (years)	58 ± 7	58 ± 7	F(1,14)=3.500, p=0.082	56 ± 6	56 ± 6	F(1,14)=0.189, p=0.670	F(1,28)=0.509, p=0.481
Systolic blood pressure (mmHg)	135 ± 22	126 ± 22*	F(1,14)=12.965, p=0.003	125 ± 21	121 ± 19	F(1,14)=2.382, p=0.145	F(1,28)=1.341, p=0.257
Diastolic blood pressure (mmHg)	80 ± 8	79 ± 10	F(1,14)=0.841, p=0.375	80 ± 11	79 ± 13	F(1,14)=0.665, p=0.428	F(1,28)=0.042, p=0.840

* significant difference from baseline, p<0.05. Data shown as means ± SD

mmHg; millimetres of mercury. ANOVA; analysis of variance.

No significant differences were seen in body fat percentage pre- versus post-intervention in either groups (Figure 30.) No significant differences were seen between groups at baseline in terms of body composition alongside no significant changes in body composition observed in either group ($p>0.05$) (Table 37.). On average, those in the partly supervised group were in the overweight BMI category pre- and post-intervention compared to those in the remotely monitored category who had a normal BMI.

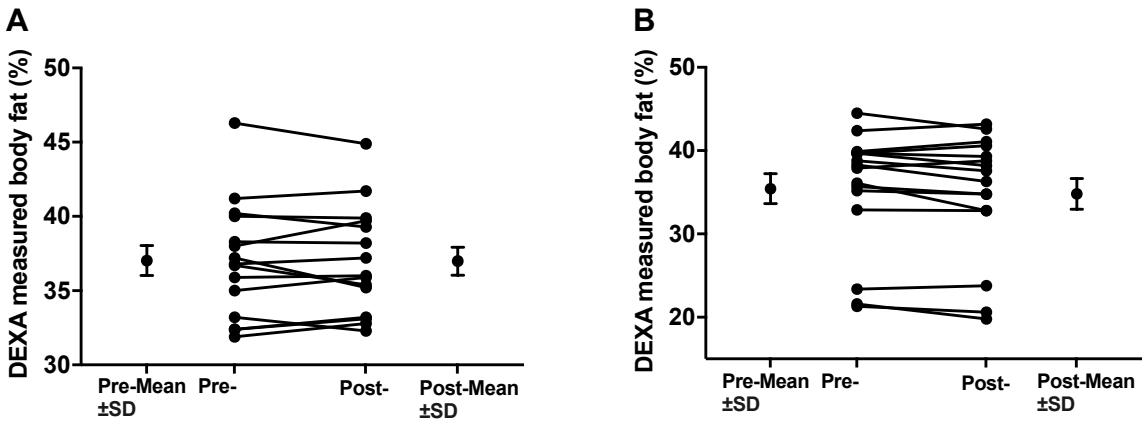


Figure 30. Changes in DEXA measured body fat percentage pre- and post-exercise interventions in A. Partly supervised group n=15 and B. Remotely monitored group n=15. Data shown as means ± SD and individual changes

Table 37. Differences between body composition in partly supervised group and remotely monitored groups and changes pre- and post-exercise intervention

	Partly supervised group (n=15)			Remotely monitored group (n=15)			
	Pre	***Post***	***Main effect of time (one-way ANOVA)***	***Pre***	***Post***	***Main effect of time (one-way ANOVA)***	***Interaction effect (two-way ANOVA)***
BMI (kg.m$_2$)	25.8 ± 2.9	25.9 ± 3.2	F(1,14)=0.274, p=0.609	24.4 ± 3.4	24.4 ± 3.5	F(1,14)=0.073, p=0.791	F(1,28)=0.086, p=0.772
W:H	0.81 ± 0.06	0.79 ± 0.05	F(1,14)=1.628, p=0.223	0.75 ± 0.16	0.79 ± 0.04	F(1,14)=0.876, p=0.365	F(1,28)=1.521, p=0.228
DEXA measured body fat (%)	37.0 ± 3.9	37.0 ± 3.6	F(1,14)=0.171, p=0.685	35.2 ± 7.3	34.6 ± 7.6	F(1,14)=2.827, p=0.115	F(1,28)=2.252, p=0.145
Fat mass (kg)	24.7 ± 4.8	25.0 ± 4.8	F(1,14)=0.819, p=0.381	23.1 ± 7.2	22.8 ± 7.5	F(1,14)=0.914, p=0.355	F(1,28)=1.726, p=0.200
Lean mass (kg)	41.7 ± 4.3	41.9 ± 4.3	F(1,14)=0.461, p=0.508	42.2 ± 3.8	41.6 ± 3.8	F(1,14)=2.689, p=0.123	F(1,28)=0.205, p=0.654
BMD (g.cm$_2$)	1.128 ± 0.106	1.112 ± 0.100	F(1,14)=1.948, p=0.185	1.098 ± 0.078	1.096 ± 0.085	F(1,14)=0.078, p=0.784	F(1,28)=1.054, p=0.313
T-score	0.2 ± 1.3	0.2 ± 1.2	F(1,14)=1.592, p=0.650	−0.1 ± 1.0	−0.1 ± 0.9	F(1,14)=0.093, p=0.765	F(1,28)=1.468, p=0.236
Z-score	0.4 ± 1.1	0.3 ± 0.9	F(1,14)=0.215, p=0.650	0.2 ± 0.7	0.1 ± 0.7	F(1,14)=0.133, p=0.718	F(1,28)=0.133, p=0.718

Data shown as means ± SD

kg; kilogram. m; metres. W:H; waist to hip ratio. DEXA; dual energy x-ray absorptiometry. g; grams. cm; centimetres. ANOVA; analysis of variance.

No significant differences were seen in $\dot{V}O_2$max between groups or pre- and post-intervention in either group. In participants in the partly supervised group, 9/15 participants had an improvement in $\dot{V}O_2$max. On average, in this group $\dot{V}O_2$max improved by 0.3 ml.kg.min^{-1} (Figure 31.). In the remotely monitored group 3 participants had an improvement in $\dot{V}O_2$max and 2 participants had no change. Improvements were seen in both groups in all functional tests (Table 38.). Those in the partly supervised group improved 6-minute walk by 43m (F(1,14)=20.106, p=0.001, ηp^2 =0.35, large effect) and sit to stand by 3 (F(1,14)=7.166, p=0.018, ηp^2 =0.12, medium effect). In the remotely monitored group, no significant differences were observed in 6-minute walk distance or get up and go but a significant improvement in sit to stand score was observed (F(1,14)=6.364, p=0.024, ηp^2 =0.10, medium effect) (Table 38.).

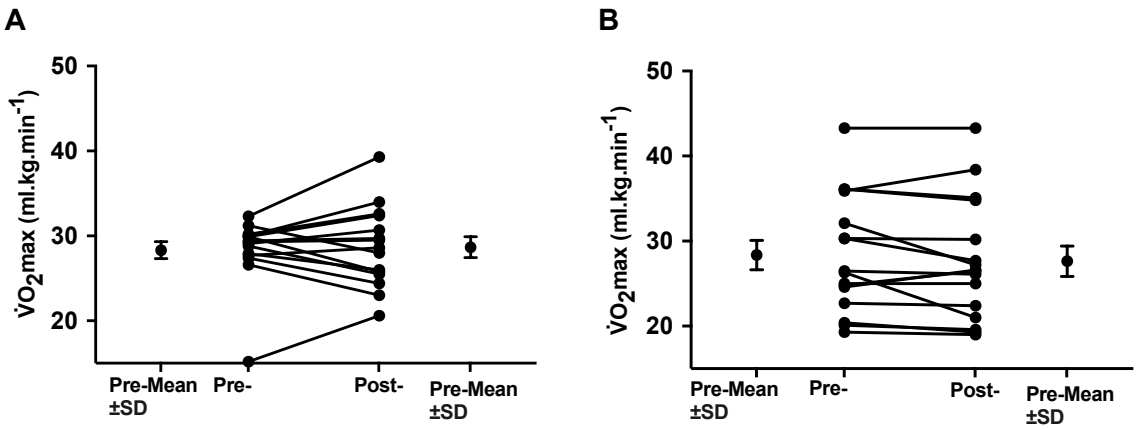


Figure 31. Changes in $\dot{V}O_2$max pre- and post-exercise interventions in A. Partly supervised group n=15 and B. Remotely monitored group n=15.

Data shown as means ± SD and individual changes

$\dot{V}O_2$max; maximum oxygen uptake. ml; millilitre. kg; kilogram. min; minute

Table 38. Differences between fitness and physical activity in partly supervised group and remotely monitored groups and changes pre- and post-exercise intervention

	Partly supervised group (n=15)			Remotely monitored group (n=15)			
	Pre	***Post***	***Main effect of time (one-way ANOVA)***	***Pre***	***Post***	***Main effect of time (one-way ANOVA)***	***Interaction effect (two-way ANOVA)***
$\dot{V}O_2$max (ml.kg.min^{-1})	28.2 ± 3.9	28.7 ± 4.8	F(1,14)=0.147, p=0.707	28.9 ± 6.8	28.2 ± 7.1	F(1,14)=1.690, p=0.215	F(1,28)=1.036, p=0.318
6-minute walk (m)	475 ± 43	518 ± 35*	F(1,14)=20.106, p=0.001	493 ± 72	509 ± 76	F(1,14)=2.888, p=0.111	F(1,28)=4.074, p=0.053
Sit to stand	16 ± 4	19 ± 5*	F(1,14)=7.166, p=0.018	16 ± 4	18 ± 5*	F(1,14)=6.364, p=0.024	F(1,28)=0.322, p=0.575
Get up and go (seconds)	5.0 ± 0.8	4.8 ± 0.8	F(1,14)=1.294, p=0.274	5.0 ± 1	5.0 ± 1	F(1,14)=4.565, p=0.051	F(1,28)=0.410, p=0.527

* significantly different from baseline, p<0.05. Data shown as means ± SD

ml; millilitre. kg; kilogram. min; minute. m; metres. ANOVA; analysis of variance.

No significant differences were seen between groups at baseline in terms of depression, anxiety and stress. A significant decrease of 4 was seen in depression post-intervention (Z= -2.770, p=0.006, large effect size 0.7) (Table 39).

Table 39. Differences between psychological factors in partly supervised group and remotely monitored groups and changes pre- and post-exercise intervention

	Partly supervised group (n=15)			Remotely monitored group (n=15)		
	Pre	***Post***	***Chi squared statistic***	***Pre***	***Post***	***Chi squared statistic***
Depression	9 ± 11	5 ± 9*	Z=−2.770, p=0.006	9 ± 11	8 ± 10	Z=1.333, p=0.248
Anxiety	3 ± 5	2 ± 3	Z=−1.080, p=0.280	3 ± 5	2 ± 3	Z=0.143, p=0.705
Stress	8 ± 9	6 ± 7	Z=−1.035, p=0.301	13 ± 9	12 ± 9	Z=0.692, p=0.405

* significant difference to baseline, p<0.05. Data shown as means ± SD

13/15 participants returned completed pre- and post-intervention diet diaries in the prescribed, partly supervised group and 13/15 in the remotely monitored group. Reasons for not returning diet diaries included losing the paper diary and forgetting to complete the diary within the 7-day timescale. No significant differences were observed in macro and micronutrient intake between groups and within groups (Table 40.).

Table 40. Differences between nutritional intake in partly supervised group and remotely monitored groups and changes pre- and post-exercise intervention

	Prescribed, Partly Supervised group (n=13)			Remote, Advisory group (n=13)			
	Pre	***Post***	***Main effect of time (one-way ANOVA)***	***Pre***	***Post***	***Main effect of time (one-way ANOVA)***	***Interaction effect (two-way ANOVA)***
Energy Intake (kcal per day)	1714 ± 379	1636 ± 521	F(1,24)=0.421, p=0.529	1715 ± 428	1891 ± 417	F(1,24)=3.724, p=0.076	F(1,24)=2.898, p=0.101
Carbohydrate intake (g.kg.day^{-1})	2.7 ± 0.8	2.6 ± 0.9	F(1,24)=0.001, p=0.821	3.1 ± 1.0	3.1 ± 0.9	F(1,24)=0.007, p=0.935	F(1,24)=0.010, p=0.921
Protein intake (g.kg.day^{-1})	1.2 ± 0.4	1.0 ± 0.2	F(1,24)=0.200, p=0.659	1.1 ± 0.5	1.1 ± 0.3	F(1,24)=0.032, p=0.860	F(1,24)=0.002, p=0.962
Fat intake (g.kg.day^{-1})	1.1 ± 0.4	1.0 ± 0.5	F(1,24)=0.217, p=0.646	1.0 ± 0.4	1.2 ± 0.3	F(1,24)=1.534, p=0.227	F(1,24)=2.164, p=0.154
Sugar (g.kg.day^{-1})	1.2 ± 0.4	1.1 ± 0.3	F(1,24)=0.512, p=0.481	1.2 ± 0.6	1.2 ± 0.3	F(1,24)=0.346, p=0.562	F(1,42)=1.529, p=0.229
Saturated fat (g.kg.day^{-1})	0.4 ± 0.2	0.4 ± 0.2	F(1,24)=0.875, p=0.359	0.4 ± 0.2	0.4 ± 0.2	F(1,24)=0.129, p=0.723	F(1,24)=2.414, p=0.533
Vitamin A (mg.day^{-1})	788 ± 1153	668 ± 481	F(1,24)=0.121, p=0.731	945 ± 502	986 ± 687	F(1,24)=0.031, p=0.863	F(1,24)=0.257, p=0.617
Vitamin C (mg.day^{-1})	110 ± 132	84 ± 70.2	F(1,24)=0.391, p=0.171	112 ± 44	82 ± 73	F(1,24)=1.675, p=0.208	F(1,24)=0.019, p=0.893
Vitamin D (mg.day^{-1})	12.9 ± 35.9	3.3 ± 3.1	F(1,24)=0.391, p=0.537	6.1 ± 8.4	7.6 ± 14.8	F(1,24)=0.096, p=0.759	F(1,24)=1.092, p=0.306
Vitamin E (mg.day^{-1})	7.3 ± 4.3	7.7 ± 3.8	F(1,24)=0.063, p=0.804	10.8 ± 6.4	9.9 ± 5.5	F(1,24)=0.128, p=0.723	F(1,24)=0.225, p=0.640
Calcium (mg.day^{-1})	637 ± 264	627 ± 226	F(1,24)=0.009, p=0.923	668 ± 336	643 ± 219	F(1,24)=0.049, p=0.827	F(1,42)=0.019, p=0.892
Sodium (mg.day^{-1})	1708 ± 530	1596 ± 650	F(1,24)=0.231, p=0.635	1757 ± 642	1956 ± 591	F(1,24)=0.672, p=0.420	F(1,24)=0.986, p=0.331
Iron (mg.day^{-1})	9.5 ± 3.0	9.5 ± 2.7	F(1,24)=0.000, p=0.994	11.0 ± 3.5	9.3 ± 3.6	F(1,24)=1.342, p=0.258	F(1,42)=0.937, p=0.343
Zinc (mg.day^{-1})	8.0 ± 4.6	6.8 ± 2.2	F(1,24)=0.685, p=0.416	8.5 ± 4.3	8.6 ± 5.6	F(1,24)=0.003, p=0.959	F(1,24)=0.710, p=0.408

Data shown as means ± SD. *kcal; kilocalorie. g; grams. ANOVA; analysis of variance.*

No significant differences were observed from pre- to post-intervention in either group in terms of haematological cell counts. At baseline, total leukocyte count was significantly higher in the partly supervised group compared to the remotely monitored group (5.6 ± 1.5 x 10^9/L versus 4.5 ± 1.0 x 10^9/L) ($F(1,28)=5.371$, $p=0.028$, effect size 0.2 small). Furthermore, there a significantly higher neutrophil (3.7 ± 1.3 x 10^9/L versus 2.7 ± 0.7 x 10^9/L), ($F(1,27)=6.276$, $p=0.019$, effect size 0.2 small) and monocyte count ($F(1,27)=4.719$, $p=0.039$, effect size 0.2 small) was observed in the partly supervised group (Table 41.). Post-intervention, monocyte count remained significantly higher in the partly supervised group versus the remotely monitored group ($F(1,27)=9.820$, $p=0.004$).

Table 41. Differences between haematological counts in partly supervised group and remotely monitored groups and changes pre- and post-exercise intervention

	Prescribed, Partly Supervised group (n=14)			**Remote, Advisory group (n=15)**			
	Pre	***Post***	***Main effect of time (one-way ANOVA)***	***Pre***	***Post***	***Main effect of time (one-way ANOVA)***	***Interaction effect (two-way ANOVA)***
Total leukocytes (x 10^9/L)	5.6 ± 1.5	5.2 ± 1.1	F(1,13)=0.869, p=0.368	4.5 ± 1.0**	4.5 ± 1.3	F(1,14)=0.007, p=0.937	F(1,27)=0.687, p=0.415
Red blood cells (x 10^9/L)	4.4 ± 0.3	4.3 ± 0.3	F(1,13)=2.151, p=0.166	4.3 ± 0.4	4.4 ± 0.3	F(1,14)=1.579, p=0.229	F(1,27)=3.615, p=0.068
Haemaglobin (x 10^9/L)	13.4 ± 0.8	13.5 ± 0.7	F(1,13)=0.184, p=0.675	13.0 ± 0.8	13.4 ± 1.2	F(1,14)=1.863, p=0.194	F(1,27)=0.704, p=0.409
Platelets (x 10^9/L)	234 ± 64	231 ± 47	F(1,13)=0.045, p=0.836	203 ± 75	202 ± 65	F(1,14)=0.001, p=0.976	F(1,27)=0.013, p=0.909
Lymphocytes (x 10^9/L)	1.4 ± 0.3	1.4 ± 0.3	F(1,13)=0.000, p=1.000	1.4 ± 0.4	1.3 ± 0.3	F(1,14)=3.680, p=0.076	F(1,27)=1.105, p=0.290
MXD (x 10^9/L)#	0.5 ± 0.2	0.6 ± 0.1	F(1,13)=3.205, p=0.097	0.3 ± 0.1**	0.4 ± 0.2**	F(1,14)=0.515, p=0.485	F(1,27)=0.616, p=0.439
Neutrophils (x 10^9/L)	3.7 ± 1.3	3.2 ± 0.9	F(1,13)=2.144, p=0.167	2.7 ± 0.7**	2.8 ± 1.1	F(1,14)=0.203, p=0.659	F(1,27)=2.049, p=0.164

** significantly different from prescribed, partly supervised group at the same time point, p<0.05. Data shown as means ± SD. #*Monocytes, eosinophils and basophils, however eosinophils and basophils only make up a small fraction (0.1-1.6 X 10^9/L according to manufacturers guidelines,* Sysmex, KX-21N, Kobe, Japan*).*

L; litre. ANOVA; analysis of variance

Significant differences were seen between groups in fasted glucose pre- and post-intervention. Baseline glucose levels were 0.68 mmol/L significantly higher in the partly supervised group ($F(1,27)=11.273$, $p=0.002$, effect size 0.3 small). Post-intervention glucose levels remained significantly higher in the partly supervised group ($F(1,27) = 11.368$, $p=0.002$, effect size small 0.3). No significant differences were seen in either group post-intervention compared to pre-intervention. No significant differences were observed within groups for cholesterol, NEFA, ESR, TAG, CRP and fasted glucose. Importantly, within the partly supervised group, one participant saw and increase in CRP post intervention to over 10mg/L compared to <1mg/L pre-intervention which has drawn the mean upwards (Table 42).

As levels of biomarkers measured are associated with increasing adiposity we examined if there were any significant correlations between biomarkers and DEXA measure body fat in all participants at all time points (pre- and post-intervention). Significant correlations were observed between body fat percentage ($r=0.395$, $p=0.002$) and CRP whereby an increased in body fat percentage was associated with higher CRP levels (data not shown). No other biomarkers significantly correlated with body fat percentage.

Table 42. Differences between biochemical markers in partly supervised group and remotely monitored groups and changes pre- and post-exercise intervention

	Partly supervised group (n=14)			Remotely monitored group (n=15)			
	Pre	***Post***	***Main effect of time (one-way ANOVA)***	***Pre***	***Post***	***Main effect of time (one-way ANOVA)***	***Interaction effect (two-way ANOVA)***
Cholesterol (mmol/L)	5.93 ± 0.86	5.88 ± 0.97	F(1,13)=0.105, p=0.715	6.19 ± 0.89	5.89 ± 1.19	F(1,14)=3.903, p=0.068	F(1,27)=1.544, p=0.225
TAG (mmol/L)	1.07 ± 0.44	1.12 ± 0.38	F(1,13)=0.639, p=0.438	1.23 ± 0.43	1.16 ± 0.36	F(1,14)=1.368, p=0.262	F(1,27)=1.933, p=0.176
Glycerol (mmol/L)	0.05 ± 0.02	0.05 ± 0.01	F(1,13)=1.215, p=0.290	0.06 ± 0.02	0.05 ± 0.02	F(1,14)=0.762, p=0.398	F(1,27)=0.007, p=0.934
Glycerol blanked TAG (mmol/L)	1.01 ± 0.43	1.07 ± 0.38	F(1,13)=0.830, p=0.379	1.17 ± 0.42	1.11 ± 0.36	F(1,14)=1.181, p=0.295	F(1,27)=1.991, p=0.170
CRP (mg/L)	1.53 ± 1.69	1.76 ± 2.69	F(1,13)=0.090, p=0.769	0.74 ± 0.56	1.38 ± 2.53	F(1,14)=1.041, p=0.325	F(1,27)=0.176, p=0.678
Glucose (mmol/L)	6.16 ± 0.58	6.29 ± 0.64	F(1,13)=0.410, p=0.533	5.48 ± 0.51**	5.49** ± 0.63	F(1,14)=0.017, p=0.897	F(1,27)=0.305, p=0.585
NEFA (mmol/L)	0.43 ± 0.13	0.37 ± 0.11	F(1,13)=1.649, p=0.222	0.48 ± 0.13	0.42 ± 0.10	F(1,14)=3.199, p=0.095	F(1,27)=0.049, p=0.827
ESR (mm.h^{-1})	7 ± 4	6 ± 5	F(1,13)=0.805, p=0.386	6 ± 5	6 ± 4	F(1,14)=0.468, p=0.505	F(1,27)=0.095, p=0.761

** significantly different from prescribed, partly supervised group at the same time point, p<0.05. Data shown as means ± SD

mmol; millimole. L; litre., mm; millimetres. mg; milligrams. h; hours. CRP; c-reactive protein. NEFA; non-esterified fatty acids. ESR; erythrocyte sedimentation rate. ANOVA; analysis of variance

6.3.4 Changes in characteristics mid- and post-interventions

13 out of 15 participants in the partly supervised group, and 14 out of 15 participants in the remotely monitored group attended the mid-intervention laboratory visit after 4 weeks of exercise. Reasons for 3 participants not attending were due to illness. No significant differences were seen between groups at any time point for any variable measured at the 3 time points (blood pressure, $\dot{V}O_2max$, body mass, W:H, BMI, sit to stand performance, 6-minute walk performance, and get up and go performance and levels of depression, anxiety and stress) ($p>0.05$)(Table 43.).

DEXA scans were not conducted at the mid-intervention visit, therefore the only body compositional data available at the all three time points were height, body mass, BMI and W:H. No significant differences over time were observed in either group ($p>0.05$) (Table 43).

All participants in the partly supervised group who attended the mid-intervention visit completed the exercise tests. 3 of the participants in the remotely monitored group that attended the mid-intervention visit did not complete exercise tests due to injury, therefore a total of 13 out of 15 participants in this group have exercise data pre-, mid- and post-intervention. No changes in $\dot{V}O_2max$ were observed in either group at any time point ($p>0.05$). A significant impact of time on 6-minute walk distance was observed in the partly supervised group ($F(1,17) = 15.062$, $p=0.001$). The distance walked was significantly lower at baseline (472 ± 44 m) compared to mid- (499 ± 40m) and post-intervention (517 ± 36m). Sit to stand scores were significantly changed by time in both the partly supervised group ($F(1,17)=4.275$, $p=0.044$) and the remotely monitored group ($F(2,24) = 6.756$, $p=0.005$). Get up and go time significantly decreased at mid-intervention in the remotely monitored group ($F2,24) = 6.756$, $p=0.005$) with no change post-intervention (Table 43).

No significant differences were observed in anxiety and stress in either group. A significant difference in depression scores were observed pre- to post-intervention in the partly supervised group only ($X(2) = 7.312$, $p=0.02$).

11/15 participants in the partly supervised group returned diet diaries at 3 time points and 14/15 of participants in the remotely monitored group. No significant differences were observed in energy intake, macronutrient or micronutrient intake within groups throughout the intervention (no main effect of time or interaction effect, $p>0.05$) (Table 43). Significant

differences were observed between groups at baseline in terms of carbohydrate intake which higher per kg body weight in the remote advisory group ($F(1,19)=30.958$ $p<0.001$). At mid-intervention intake of carbohydrate, protein fat and saturated fat intake was higher in the remote group ($F(1,19)=11.098$, $p=0.004$, $F(1,19)=10.708$, $p=0.004$, $F(1,19)=8.195$, $p=0.010$ and $F(1,19)=5.757$, $p=0.027$ respectively). Post-intervention, intake of all macronutrients (carbohydrates, sugars, protein, fat and saturated fat) was significantly higher in the remote advisory group ($F(1,19)=18.913$, $p<0.001$, $F(1,19)=9.086$, $p=0.007$, $F(1,19)=25.494$, $p<0.001$, $F(1,19)=13.861$, $p=0.001$ and $F(1,19)=8.688$, $p=0.008$ respectively) (Table 43).

Table 43. Differences between lifestyle characteristics in partly supervised group and remotely monitored groups and changes pre-, mid- and post-exercise intervention

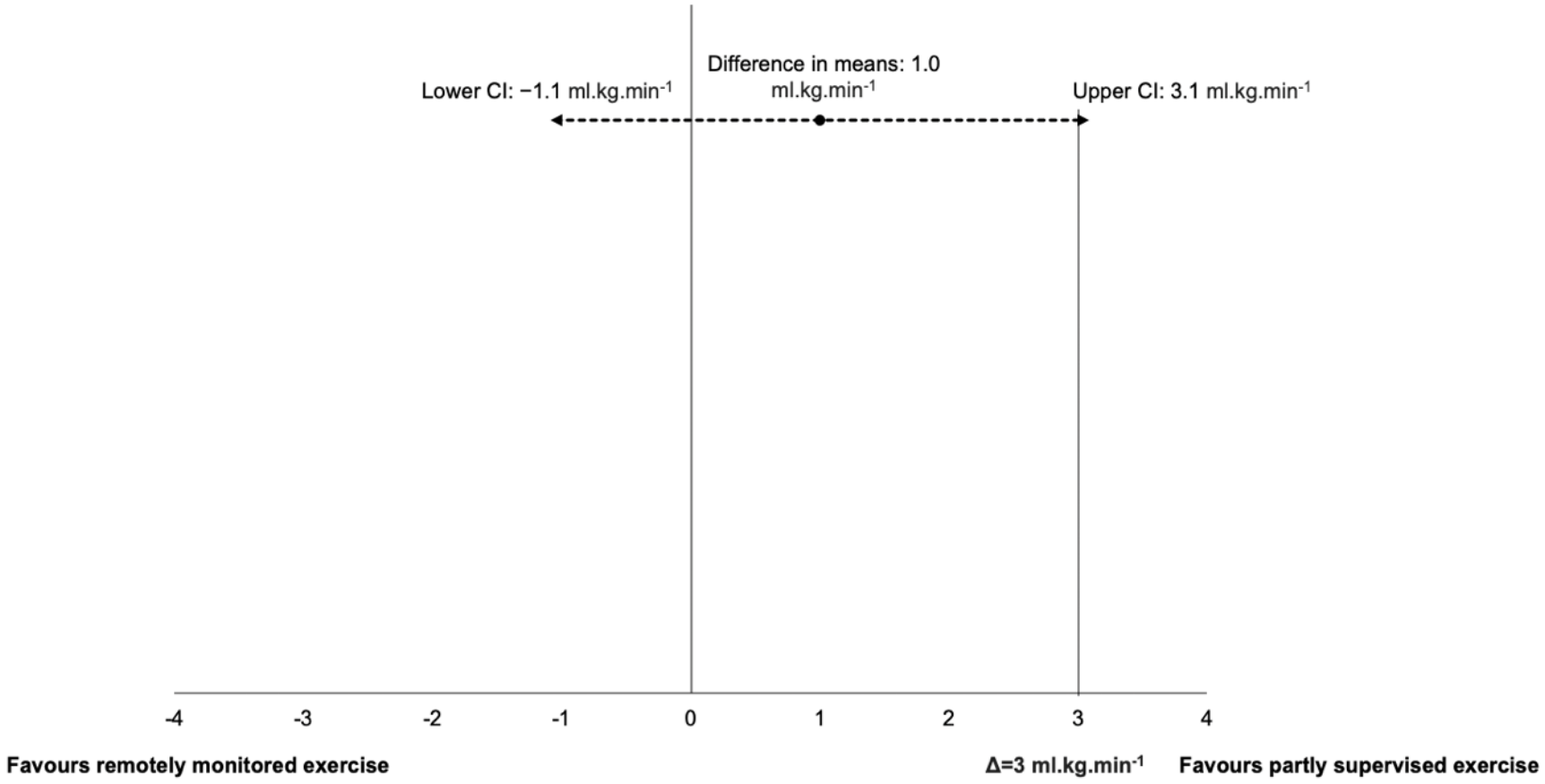
	Prescribed, Partly Supervised group			**Remote, Advisory group**		
	Pre	***Mid***	***Post***	***Pre***	***Mid***	***Post***
Characteristics						
Age (years)	57 ± 7	57 ± 7	57 ± 7	56 ± 6	56 ± 7	56 ± 6
Systolic blood pressure (mmHg)	135 ± 22	133 ± 23	127 ± 9*	125 ± 21	121 ± 24	121 ± 20
Diastolic blood pressure (mmHg)	80 ± 8	81 ± 6	80 ± 9	80 ± 11	78 ± 10	79 ± 12
Body mass (kg)	66.7 ± 8.1	65.9 ± 9.2	67.1 ± 8.7	67.5 ± 11.4	67.2 ± 11.3	67.7 ± 11.0
Body composition						
Height (m)	1.61 ± 0.06	1.61 ± 0.06	1.61 ± 0.06	1.64 ± 0.05	1.64 ± 0.06	1.64 ± 0.05
BMI (kg.m$_2$)	25.8 ± 3.1	25.4 ± 3.5	26.0 ± 3.4	24.9 ± 3.8	24.9 ± 3.9	25.0 ± 3.8
W:H	0.81 ± 0.06	0.83 ± 0.14	0.79 ± 0.05	0.80 ± 0.16	0.81 ± 0.05	0.79 ± 0.05
Fitness						
V̇O$_2$max (ml.kg.min$_{-1}$)	28.3 ± 4.2	28.9 ± 3.9	28.8 ± 5.1	30.2 ± 6.8	29.0 ± 6.8	29.3 ± 7.3
6-minute walk (m)	472 ± 44	499 ± 40*	517 ± 36*	497 ± 84	507 ± 72	518 ± 75
Sit to stand	16 ± 4	18 ± 4	19 ± 5*	16 ± 4	18 ± 5*	18 ± 6
Get up and go (seconds)	5.0 ± 0.8	4.8 ± 0.7	4.8 ± 0.6	5.2 ± 0.5	4.6 ± 0.7*	4.8 ± 0.9
Psychological factors						
Depression	9 ± 11	3 ± 6	5 ± 9*	9 ± 11	8 ± 10	6 ± 9
Anxiety	3 ± 5	2 ± 3	2 ± 3	4 ± 6	3 ± 4	2 ± 3
Stress	8 ± 9	6 ± 5	6 ± 6	13 ± 8	11 ± 10	10 ± 7
Nutritional Intake						
Energy Intake (kcal per day)	1734 ± 380	1803 ± 434	1680 ± 533	1726 ± 496	1742 ± 415	1930 ± 484
Carbohydrate intake (g.kg.day$_{-1}$)	2.4 ± 0.6*	2.8 ± 0.9*	2.3 ± 0.8*	4.2 ± 0.8	4.2 ± 0.9	4.2 ± 1.2
Protein intake (g.kg.day$_{-1}$)	1.0 ± 0.3	1.0 ± 0.3*	0.9 ± 0.2*	1.5 ± 0.8	1.4 ± 0.3	1.4 ± 0.3
Fat intake (g.kg.day$_{-1}$)	1.1 ± 0.4	1.0 ± 0.3*	0.9 ± 0.4*	1.4 ± 0.5	1.5 ± 0.5	1.5 ± 0.5
Sugar (g.kg.day$_{-1}$)	1.2 ± 0.4	1.4 ± 0.7	1.1 ± 0.2*	1.6 ± 0.8	1.8 ± 0.6	1.8 ± 0.8
Saturated fat (g.kg.day$_{-1}$)	0.4 ± 0.2	0.4± 0.2*	0.4 ± 0.2*	0.5 ± 0.2	0.6 ± 0.3	0.6 ± 0.2
Vitamin A (mg.day$_{-1}$)	885 ± 1236	762 ± 732	579 ± 469	895± 533	805 ± 562	986 ± 768
Vitamin C (mg.day$_{-1}$)	122 ± 141	100 ± 49	83 ± 77	107 ± 42	86 ± 39	66 ± 38
Vitamin D (mg.day$_{-1}$)	14.8 ± 38.9	3.4 ± 2.4	3.0 ± 2.9	4.4 ± 7.6	3.8 ± 3.0	3.5 ± 2.7
Vitamin E (mg.day$_{-1}$)	7.5 ± 4.7	7.5 ± 3.4	7.3 ± 3.7	9.1 ± 2.8	8.8 ± 4.5	8.2 ± 4.5
Calcium (mg.day$_{-1}$)	638 ± 290	947 ± 981	588 ± 157	664 ± 357	738 ± 179	624 ± 232
Sodium (mg.day$_{-1}$)	1740 ± 572	1766 ± 547	1511 ± 670	1809 ± 593	1901 ± 906	1901 ± 666
Iron (mg.day$_{-1}$)	9.4 ± 3.2	9.7 ± 2.5	9.0 ± 2.5	11.2 ± 3.7	10.4 ± 3.2	8.5 ± 2.8
Zinc (mg.day$_{-1}$)	8.0 ± 5.0	8.1 ± 4.9	6.5 ± 2.0	7.8 ± 3.9	6.8 ± 1.3	7.0± 4.1

* significant difference from remotely monitored group at same timepoint, p<0.05. Data shown as means ± SD

mmHg; millimoles of mercury. kg; kilogram. m; metres. W:H; waist to hip ratio. ml; millilitre. kg; kilogram. min; minute.

6.3.5 Non-inferiority analysis

Non-inferiority analysis was conducted on main outcome measure $\dot{V}O_2max$ and secondary outcome measure, body fat (both DEXA measured and BMI). Furthermore, as a significant effect of time was seen on systolic blood pressure and 6-minute walk time, non-inferiority analysis was also conducted on these variables. As previous analysis on significant changes over time and between groups suggested no significant differences in data non-inferiority analysis was not conducted on any other outcomes. For a summary of change scores and CI please see Appendix 4. As the upper CI (3.1ml.kg.min^{-1}) lies above the non-inferiority margin (3.0 ml.kg.min^{-1}), the remotely monitored intervention is deemed not non-inferior to the prescribed, partly supervised intervention (Figure 32.). The 95% CI surrounding the variance in difference between groups were calculated as −1.1 to 3.1 ml.kg.min^{-1}. As demonstrated in section 6.2.7, Δ, based previous literature (Shinkai *et al.*, 1994b) was set at 3 ml.kg.min^{-1}.



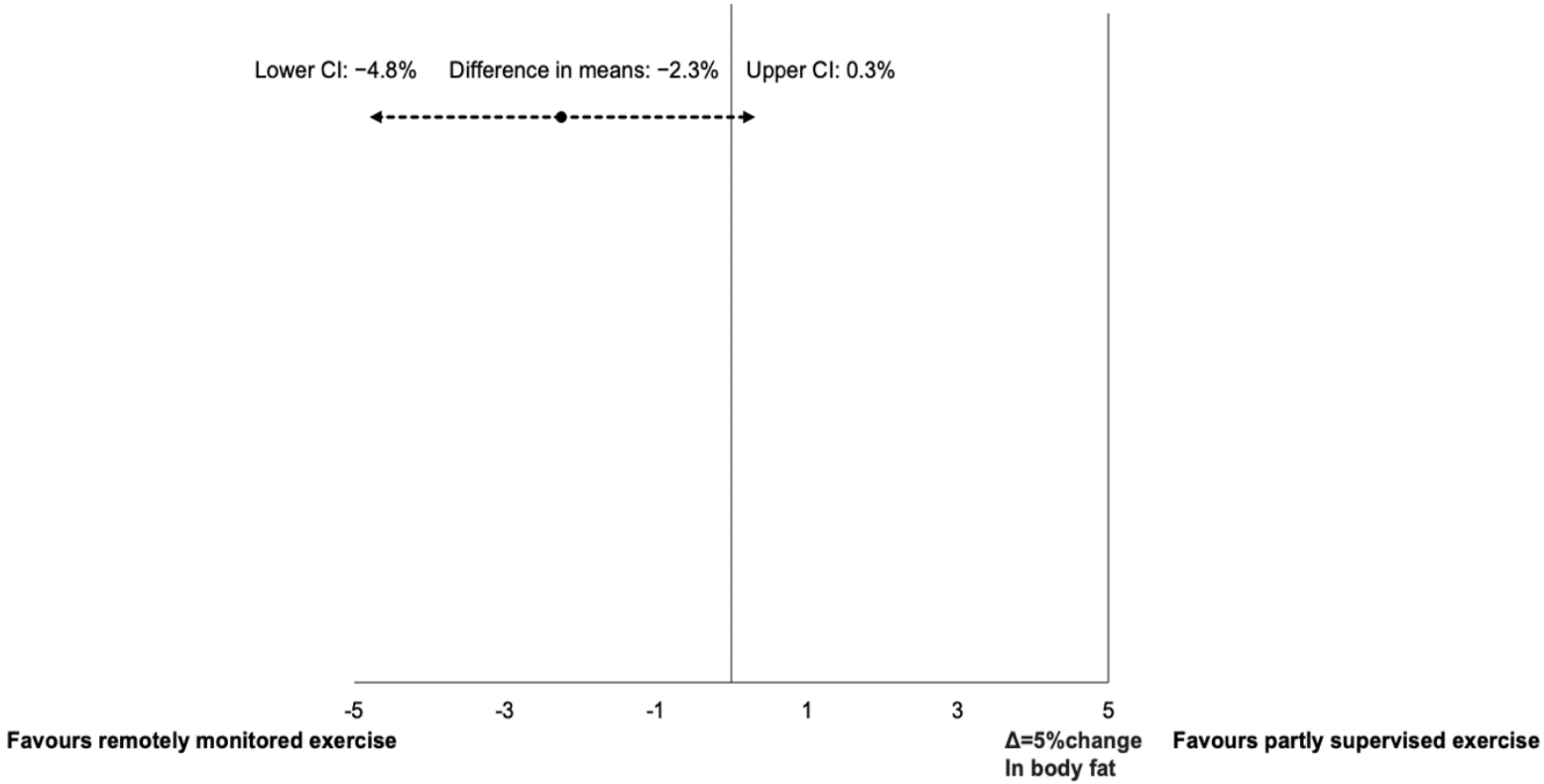
3903

3904 Figure 32. Non-inferiority analysis of the remotely monitored group to partly supervised group regarding $\dot{V}O_2max$

3905 **Δ**; margin. CI; confidence interval

3906

In terms of body fat percentage, Δ was set at −5% as this has previously been determined as the lowest clinically significant change in body composition (Donnelly *et al.*, 2009). The variance of difference between the difference in the groups was −2.3% therefore the 95% CI were set at −4.8 to 0.3%. As the entire CIs lies below (higher body fat is worse) Δ (5%), the remote, advisory group is non-inferior (Figure 33.).



3912

3913

3914 Figure 33. Non-inferiority analysis of the remotely monitored group to partly supervised group regarding DEXA measured body fat percentage

3915 **Δ**; margin. CI; confidence interval

3916

Reductions in systolic blood pressure of ≥2 mmHg can significantly reduce the incidence of CVD in both hypertensive and normotensive individuals, subsequently small reductions of this magnitude are considered clinically meaningful (Turnbull, 2003; Wong *et al.*, 2015), therefore for both systolic blood pressure Δ was set at 2mmHg. For systolic blood pressure, the variance of difference between the difference in the groups was −5 mmHg therefore the 95% confidence intervals were set at −12 to 3 mm Hg. As the CI cross the non-inferiority margin (2mmHg), the remote intervention is deemed not non-inferior to the partly supervised group (Figure 34.).

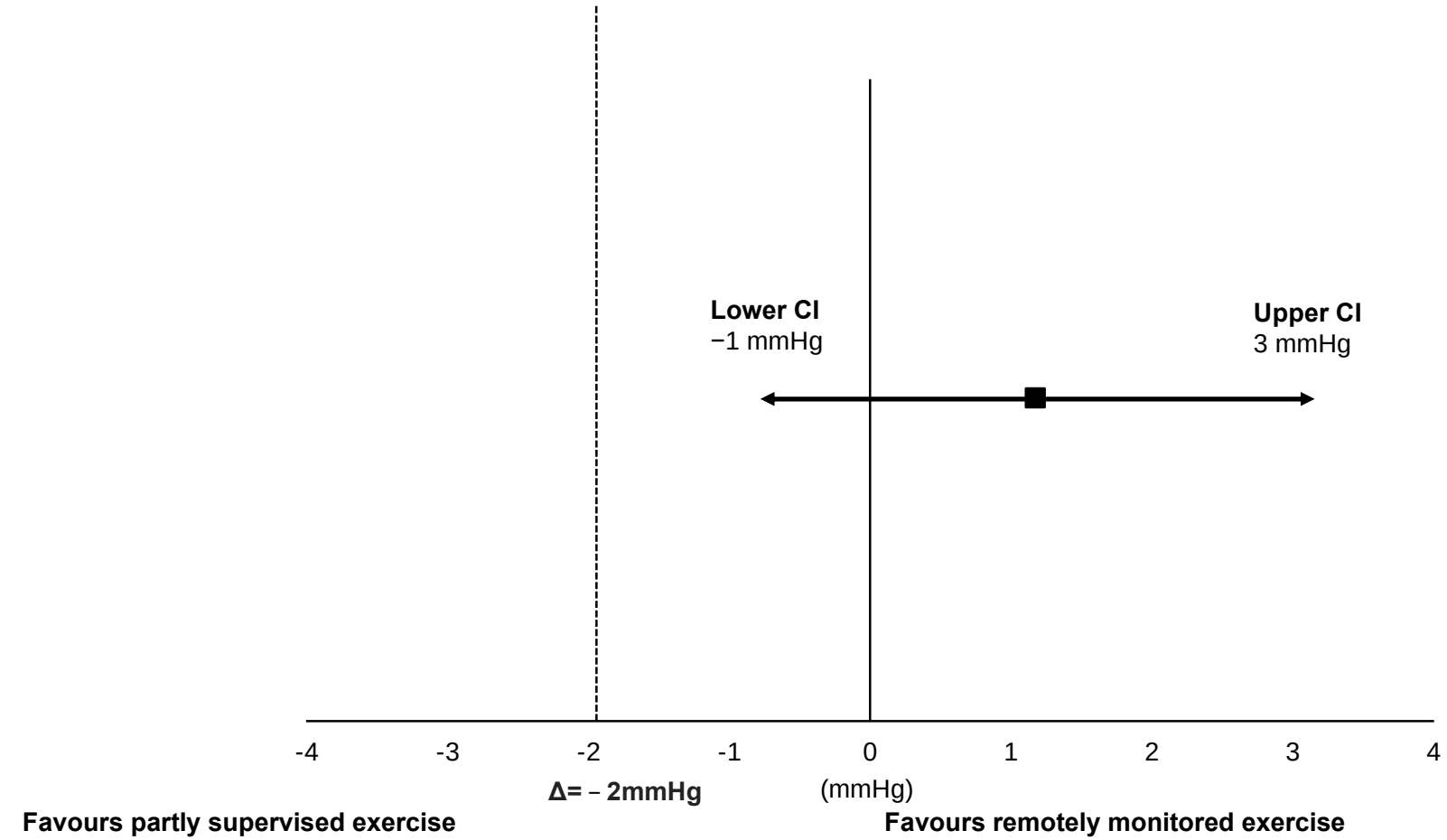
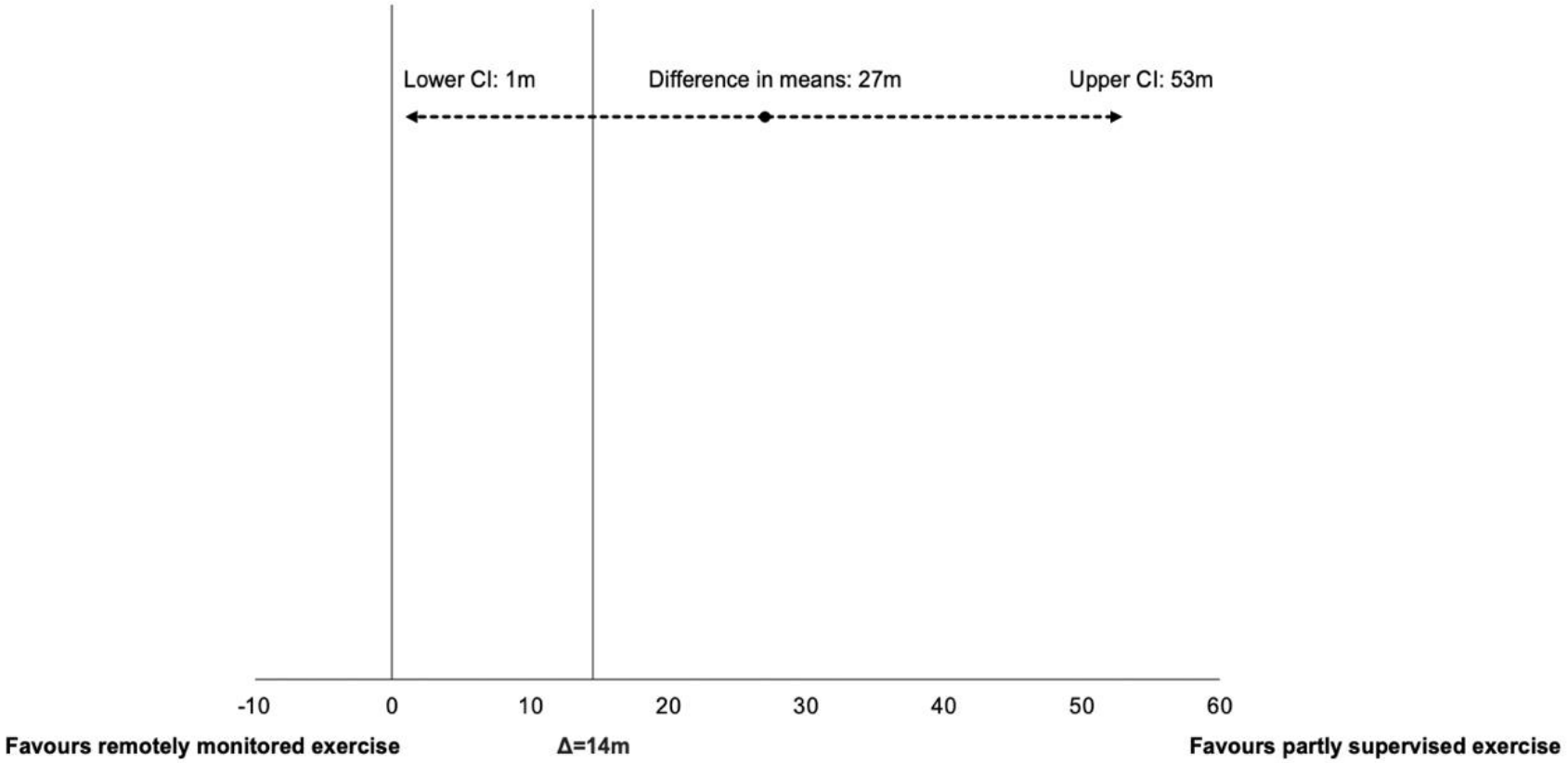


Figure 34. Non-inferiority analysis of the remotely monitored group to partly supervised group regarding systolic blood pressure

***Δ**; margin. CI; confidence interval. mmHg; millimoles of mercury.*

Improvements in 6-minute walk time of 14m are considered clinically meaningful (Bohannon and Crouch, 2017), therefore for both systolic and diastolic blood pressure Δ was set at 14m. For 6-minute walk distance, the variance of difference between the difference in the groups was 27m therefore the 95% confidence intervals were set at 1-53m. As the CIs cross Δ, the remote, advisory group is not non-inferior (Figure 35.).



3935

3936 Figure 35. Non-inferiority analysis of the remote, advisory group to prescribed, partly supervised group regarding 6-minute walk distance

3937 ***Δ****; margin. CI; confidence interval*

3938

6.3.6 Data from maximal exercise tests

To confirm the accuracy of the $\dot{V}O_2$max tests conducted throughout the research, criteria for meeting $\dot{V}O_2$max were analysed and are shown in Table 44. In the partly supervised group, no significant differences were seen in any variables at any time point (pre-, mid- and post-intervention). Respiratory exchange ratio (RER) was above 1 at maximal exercise pre- and post-intervention but not mid-intervention (0.99 ± 0.29) however 14% less women achieved an RER above 1.1 during maximum exercise post-intervention when compared to pre- and mid-intervention. Maximum HR was significantly higher pre-intervention versus mid- and post- intervention ($F(2,16) = 9.659$, $p=0.002$) by 18 and 4 bpm respectively. In the remotely monitored group, RER on average was over 1 at all time points, however more women had an RER over 1.1 pre-intervention compared to other time points ($p>0.05$). RPE was significantly lower mid-intervention (by 1) compared to partly supervised group ($F(1,24) = 4.680$, $p=0.041$). On average, HRmax was significantly lower than pre- and post-intervention HRmax by 11 and 6 bpm respectively ($F2,20) = 9.952$, $p=0.01$. No significant differences were seen within or between groups in terms of percentage of women within 10 bpm of predicted HRmax.

Table 44. Differences between data from cardiorespiratory exercise testing in partly supervised group and remotely monitored groups

	Partly supervised group			Remotely monitored group		
	Pre (n=15)	***Mid (n=11)***	***Post (n=15)***	***Pre (n=15)***	***Mid (n=11)***	***Post (n=16)***
RER	1.06 ± 0.07	0.99 ± 0.29	1.03 ± 0.08	1.05 ± 0.13	1.03 ± 0.08	1.06 ± 0.08
RER > 1 (%)	73% (11/15)	82% (9/11)	60% (9/15)	87% (13/15)	64% (7/11)	69% (11/16)
RER > 1.1 (%)	27% (4/15)	27% (3/11)	13% (2/15)	33% (5/15)	27% (3/11)	25% (4/16)
RPE	18 ± 2	17 ± 5	18 ± 1	18 ± 2	18 ± 2**	18 ± 1
HRmax (bpm)	164 ± 12	146 ± 46#	161 ± 13#	165 ± 15*	154 ± 14	160 ± 12*
HRmax within 10 bpm calculated max (207 – (0.7 x age)) (%)	66% (10/15)	45% (5/11)	47% (7/15)	80% (12/15)	36% (4/11)	69% (11/16)
HRmax within 10 bpm calculated max (220– age) (%)	80% (12/15)	56% (6/11)	73% (11/15)	80% (12/15)	55% (6/11)	81% (13/16)

* significantly different from mid time point within group, p<0.05. ** significantly different from partly supervised group at same time point. # significantly different from baseline p<0.05.

RER; respiratory exchange ratio. RPE; rating of perceived exertion. HRmax; maximum heart rate. bpm; beats per minutes

6.4 DISCUSSION

This study aimed to evaluate whether 8-weeks of a technology-enabled, remotely monitored physical activity intervention with the use of a fitness tracking wristwatch, produces changes to cardiorespiratory fitness that are not meaningfully inferior to changes in health in response to a partly supervised exercise intervention in female breast cancer survivors. The main outcome of this study was that 8-weeks of remotely monitored exercise produced not non-inferior changes to $\dot{V}O_2$max, functional fitness and systolic blood pressure within the remotely monitored group (whereby changes were better in the partly supervised group) but did produce non-inferior changes to body composition. Furthermore, neither intervention led to any significant changes in $\dot{V}O_2$max or body composition. No changes were seen in scores for anxiety and stress but scores for depression were significantly reduced in the partly supervised group. No changes in cell counts or biochemical markers of health were observed in either group. Adherence and enjoyment were both significantly higher in the prescribed, partly supervised group.

Previously, in 45 breast cancer survivors, 1–36 months following treatment for early stage breast cancer (chemotherapy, and radiotherapy), after undertaking a supervised aerobic (50-80% HR reserve) and resistance exercise intervention for 12 weeks, $\dot{V}O_2$max significantly improved from 26.97 ± 4.94 to 32.32 ± 6.22 ml.kg.min^{-1} versus a decline in $\dot{V}O_2$max over the same period of time in the control group (Casla *et al.*, 2015). The increase in $\dot{V}O_2$max is larger than the increase observed in the current study (0.5 ml.kg.min^{-1}) within the partly supervised group, however, $\dot{V}O_2$max was predicated in previous research from a sub-maximal rather than maximal exercise protocol and the exercise intervention was 4-weeks longer. Interesting the decline in $\dot{V}O_2$max previously observed was 1.5 ml.kg.min^{-1}. Whilst $\dot{V}O_2$max did not improve in the current study within the remotely supervised the intervention may have prevented the natural decline in $\dot{V}O_2$max that may occur during 8-weeks. However, this cannot be established without the use of a control group. In another study in female breast cancer survivors, 12 weeks of supervised aerobic and resistance exercise, three times per week, improved aerobic fitness by 6-8% (Milne *et al.*, 2008). However, aerobic fitness in this study was assessed using the Aerobic Power Index, a submaximal exercise test on a cycle ergometer rather than a maximal exercise test on a treadmill used in the current research which may account for differences in results.

Furthermore, it may be that in the current study participants were not meeting actual $\dot{V}O_2$max due to the nature of the exercise test protocol. The maximal exercise test in the current study was conducted on a treadmill, a piece of equipment were not accustomed to

or had perhaps never used before, (participants were asked before commencement of exercise on the treadmill whether they had previously used the equipment). It is also unlikely that the cohort had experienced maximal exercise recently, thus, may have found it difficult to determine when they were at their maximum or 1-minute prior to maximum. To deduce this, we investigated end point data from the maximal exercise test. On average, a respiratory exchange ratio (RER) > 1.0 was observed during the final stage of exercise for both groups at all time points where $\dot{V}O_2$max was measured. Whilst this has previously been used as a criterion for reaching $\dot{V}O_2$max (Paterson *et al.*, 1999; Aspenes *et al.*, 2011; Davis *et al.*, 2002), other evidence suggests this value should be higher at >1.1 (Nelson *et al.*, 2010; Brown *et al.*, 2002) or even >1.15 (Howley *et al.*, 1995; Issekutz Jr *et al.*, 1962). New recommendations have been made that the criterion for RER is sex and age dependent, for females, >1.0 for those >65 years, >1.05 for those 49 years ><69 years and >1.1 for those <49 years (Edvardsen *et al.*, 2014). When using these criteria, only 61% and 66% of all maximal exercise tests in the remotely monitored and partly supervised groups respectively met this criterion.

Another criterion for reaching $\dot{V}O_2$max is HRmax, which should be within 10 beats of age predicted HRmax (Aspenes *et al.*, 2011; Davis *et al.*, 2002; Paterson *et al.*, 1999). In the current study 70% of all $\dot{V}O_2$max tests conducted in the partly supervised exercise group had end stage heart rate data that was within 10 beats of age predicted HRmax using the classic formula of 220-age. This was 72% in the remotely monitored group. It has however been questioned whether this formula is accurate, so this analysis was also completed using the formula 207-(0.7 x age) (Gellish *et al.*, 2007b). Results demonstrated that only 53% and 62% of all maximal exercise tests in the partly supervised group and the remotely monitored groups respectively met this end point criteria. Whilst blood lactate concentration or determining a plateau in oxygen uptake (other criteria used from reaching $\dot{V}O_2$max (Aspenes *et al.*, 2011; Davis *et al.*, 2002; Paterson *et al.*, 1999)) were not recorded in the current study, the evidence suggests that not all women had reached true maximum during exercise testing which may be affecting changes in $\dot{V}O_2$max. Furthermore, the lack of improvement in the remotely monitored group compared to the slight improvement in $\dot{V}O_2$max in the partly supervised group may be down to increased experience of gas sampling procedures in the former group who exercised on the same equipment twice weekly throughout the intervention.

Another factor that can affect improvements in $\dot{V}O_2$max is baseline $\dot{V}O_2$max (Sisson *et al.*, 2009). This may have played a role in the lack of change in the current study as baseline

$\dot{V}O_2$max was 28.3 ± 4.2 and 30.2 ± 6.8 ml.kg.min^{-1} for the partly supervised group and the remotely monitored group respectively, which is higher than previously reported. In a previous study of 30 breast cancer survivors of similar age and BMI (age; 51 ± 6, BMI; 29.2 ± 5.3) average $\dot{V}O_2$max was 25.4± 5.3 mL·kg^{-1}·min^{-1}, which is 11% and 17% lower than the baseline measures in the partly supervised and remotely monitored group respectively in the current study, despite average time since treatment being less (12 ± 6 months versus 50 ± 27 months). Furthermore, previous research has shown $\dot{V}O_2$max of breast cancer survivors is similar to the 20th percentile threshold value for age and gender group matched normative values, whereas in the current study both groups are similar to 50th percentiles.

Another explanation for the lack of change in $\dot{V}O_2$max may be the intensity of the exercise intervention as increased benefits to $\dot{V}O_2$max are seen with exercising at higher intensities when comparing exercise at 50% $\dot{V}O_2$ reserve, 75% $\dot{V}O_2$ reserve and 95% $\dot{V}O_2$ reserve (Gormley *et al.*, 2008). In the current study, breast cancer survivors exercised at 50-70% $\dot{V}O_2$max or HRmax, but in fact, only the final 2 weeks of both interventions were spent at the higher end of this range. In breast cancer survivors, the tolerance and effect of exercising at higher intensities is relatively unknown and a recent review on exercise guidelines for cancer survivors recommends moderate-intensity aerobic training at least three times per week, for at least 30 minutes, for at least 8 to 12 weeks (Campbell *et al.*, 2019b). Furthermore, a limitation of the current study is that it compares exercise intensity determined by % $\dot{V}O_2$max and %HRmax when some evidence suggests that the intensity of exercise performed at a given percentage may differ and instead recommends using $\dot{V}O_2$ and HR reserve (Mann *et al.*, 2013; Swain and Leutholtz, 1997).

Free living physical activity, outside of exercise sessions may also be contributing to changes in $\dot{V}O_2$max. In the partly supervised group, whilst vigorous exercise and total physical activity increased per week, moderate exercise minutes decreased as reported by IPAQ, perhaps suggesting participants stopped doing activities they were previously engaged in. In the remotely monitored group, whilst exercise minutes increased, self-reported physical activity decreased, suggesting that participants were being less active outside their exercise sessions. It must be remembered that the IPAQ is a self-reported tool and therefore very subjective (Cleland *et al.*, 2018). This is demonstrated by the average number of exercise minutes per week at week 8 being 74 minutes when in fact we know participants undertook a minimum of 2 x 50-minute supervised exercise sessions and were asked to do 1 x 50-minute exercise session alone (equal to a total of 150 minutes) and further demonstrated by Bland-Altman analysis conducted previously within this thesis.

Furthermore, no significant differences were seen in energy expenditure and step count at week 1 versus week 8 of the intervention in the remotely monitored group. However, the Polar A370 wristwatch has yet to be validated for predicting energy expenditure or measuring step count, so this data should also be treated with caution.

Other factors that were not assessed in the current study include the genetic determinants of trainability. Individual variability in response to training was observed in the current study with both groups containing participants that declined, maintained or improved $\dot{V}O_2$max, a trend which has been previously observed whereby some people respond and other do not respond to training (Mann *et al.*, 2014b; Bouchard *et al.*, 2015; Mori *et al.*, 2009). A landmark study, the HERITAGE study investigated the effect of 20 weeks of moderate exercise in 473 adults from 99 families and it was concluded that the difference in change of $\dot{V}O_2$max was 2.5 times greater between versus within families (Bouchard *et al.*, 1999). This study identified heritable factors such as changes in stroke volume, blood volume, capillary density and muscle mitochondrial content can estimate 47% of the response to exercise training (Bouchard *et al.*, 1999; Clausen, 1977; Holloszy and Coyle, 1984; Levine, 2008). Furthermore, a recent review of 35 studies concluded that there are at least 97 genes associated with exercise training response (Williams *et al.*, 2017). Thus, it is important to remember that $\dot{V}O_2$max is not only influenced by environmental factors that were measured in the current study, but also genetic factors which were not measured.

The change to $\dot{V}O_2$max was not deemed non-inferior in the remote, advisory group compared to the partly supervised group. Whilst on the surface this result may lead to recommendations that a partly supervised, prescribed exercise intervention may be preferred to a remotely delivered intervention it must be remembered no significant differences were observed between baseline and post-intervention $\dot{V}O_2$max in either group. Furthermore, the closeness of the upper CI to the margin was small (0.1 ml.kg.min^{-1}). Given the difficulties and inaccuracies that are common place when assigning such margins (Fleming, 2008), this result should be treated with caution.

Some studies have assessed body composition in response to exercise training. Casla et al. (2015) demonstrated in their exercise intervention study in breast cancer survivors that, compared to controls, significant improvements were observed in body fat percentage and lean mass. No differences were observed in BMI or W:H, similarly to the current study. Whilst improvements in body fat percentage were observed previously, this was measured using bioelectrical impedance rather than the more accurate DEXA scan as used in the

current study (Völgyi *et al.*, 2008). In the current study neither group observed changes to body fat percentage and furthermore, the remotely monitored group was deemed non-inferior to the partly supervised group for this variable.

A potential explanation for a lack of change in BMI and W:H in the current study is that values were close to healthy normal values in both groups to begin with 25.8 ± 2.9 and 24.8 ± 3.9 kg.m$_2$ in the partly supervised and the remotely monitored group respectively and W:H 0.80 ± 0.06 and 0.81 ± 0.22 respectively. Furthermore, any potential body mass composition changes may have been offset by compensatory eating (Finlayson *et al.*, 2009) through an increase in energy expenditure (as observed in the remotely monitored group), especially as many participants were involved in the exercise intervention over the festive season (Helander *et al.*, 2016; Yanovski *et al.*, 2000). Whilst the partly supervised group did not report an increase in energy intake when comparing pre- to post-intervention data, an increase was seen pre- to mid-intervention and not all diet diaries were returned. I has been well documented that individuals often change dietary habits and under report energy intake when asked to record their diet, therefore the data gathered from the self-reported diet diaries may not accurately represent diet (Macdiarmid and Blundell, 1998; Bingham, 1991; Poslusna *et al.*, 2009). Most studies that report significant reduction in BMI, W:H and body fat used dietary interventions or a combination of physical activity, exercise and diet rather than exercise alone (Playdon *et al.*, 2013).

No significant differences were observed in total energy intake, macronutrient intake or micronutrient intake between or within groups in the current study. This is perhaps unsurprising given the nature of the exercise intervention which did not include any dietary intervention or advice. Previous studies that have manipulated diet and/or energy intake by reducing energy intake from fat and increasing fruit and vegetable intake such as the WHEL or WINS study have demonstrated a significant change in dietary intake (Pierce *et al.*, 2007; Hoy *et al.*, 2009). It has previously been reported that increasing energy expenditure may be related to an increased compensatory energy intake due to homeostatic (hunger) or hedonic (reward) processes (Melanson *et al.*, 2013). However, numerous studies, similarly to the results of the current study, report no changes in energy intake or macronutrient intake following exercise interventions (Melanson *et al.*, 2013). The largest contributor to discrepancies in results is likely related to the weakness of the diet diary as a tool for reporting habitual diet (Melanson *et al.*, 2013). Under reporting of total intake, protein, fat and sugar have been reported in dietary validation studies (Heitmann and Lissner, 1995) and to complexify the issue, under reporting is not consistent across individuals, with obese

individuals likely to further under report values. Future research should attempt to use the doubly labelled water technique to reduce this error.

When looking at micronutrient intake, vitamin D intake was below recommended thresholds (15mg.day^{-1}) in both groups at both timepoints. This is particularly important as breast cancer survivors taking aromatase inhibitors for previous hormone positive cancer, are at increased risk of bone loss and fracture risk (Shapiro, 2005). This should be taken with results that show calcium intake was below recommended values (100mg.day^{-1}) for both groups at both time points, given the relationship between calcium in conjunction with vitamin D and bone health (Newberry *et al.*, 2014). Furthermore, some links have been made between reduced vitamin D status and breast cancer recurrence and it may be that vitamin D is important for all breast cancer survivors (Goodwin *et al.*, 2009b), especially as previously ~35-75% of breast cancer survivors have been shown to have deficient vitamin D status (Friedman *et al.*, 2012; Hines *et al.*, 2010). Whilst most vitamin D is absorbed through sunlight, vitamin D intake through the diet should be encouraged in breast cancer survivors, particularly in winter months when sunlight is limited. and many oncologists now prescribe supplements to breast cancer survivors to improve disease specific and all-round health.

No changes were seen in biochemical markers (CRP, NEFA, glucose, TAG, glycerol, ESR or cholesterol) post-intervention in the current study. In a previous study in breast cancer patients, fasted glucose was on average 6.1 ± 1.4mmol/L. This is similar to values seen in the current study, all of which are indicative of pre-diabetic values (DiabetesUK, 2020). Exercise training has previously shown to reduce fasting glucose through various metabolic mechanisms (Norton *et al.*, 2012). However, in the current study no reduction in fasting blood glucose was observed despite the trend being previously reported following physical activity interventions in sedentary adult participants (Ross *et al.*, 2004). Moderate and vigorous exercise over 8-months, a much longer training intervention than the current study, also elicited no change in fasted blood glucose, despite improvements in beta cell function and insulin sensitivity (Slentz *et al.*, 2009). This demonstrates that rather than reducing blood glucose, maintenance of blood glucose in a population that would see an increase in this value over time may also be protective (Slentz *et al.*, 2009). Insulin sensitivity was not assessed in the current study, so we are unable to confirm whether this is the case.

Total cholesterol was unchanged and on average above healthy levels (5.2 mmol/L) (NIH). This is unsurprising as whilst exercise has been shown to reduce or offset increases in LDL

(low density lipoprotein) cholesterol it has been shown to increase HDL (high density lipoprotein) cholesterol (Mann *et al.*, 2014a). A study of a similar duration (12 weeks) which implemented 150 minutes per week of exercise at 65%$\dot{V}O_2max$ demonstrated a similar small, non-significant decline in total cholesterol by 0.3 mmol/L. HDL and LDL cholesterol was not measured in the current study, so we are unable to confirm this, but it may explain the reason behind the lack of change in total cholesterol. An improvement in the cholesterol profile of breast cancer survivors may lead to a reduction in future breast cancer risk and cardiovascular disease (Garcia-Estevez and Moreno-Bueno, 2019; Nelson, 2013).

Elevated levels of TAG in plasma are positively and independently associated with cardiovascular disease (Luz *et al.*, 2008). Exercise has previously been shown to offset increases in TAG (Mann *et al.*, 2014a). An 8 week exercise intervention in breast cancer survivors consisting of twice weekly supervised and one unsupervised aerobic exercise sessions increasing in duration and intensity fortnightly, similarly to the protocol used in the partly supervised group reported no differences in TAG pre- (1.4 ± 0.7 mmol/L) and post-intervention (1.4 ± 0.9 mmol/L) (Guinan *et al.*, 2013). Similar values were observed within the current study, which on average across groups and time points, ranged from 1.01 and 1.17 mmol/L. Another study in 494 breast cancer survivors reported TAG values ranging from 1.00-1.62 mmol/L, with higher levels in those with higher BMI (Elme *et al.*, 2013). Similarly, we saw no change post-intervention in TAG but did not see a relationship between body fat % and TAG level. Previous research has shown whilst exercise elicits beneficial changes in lipid profiles by increasing HDL, only higher intensity exercise can reduce TAG by initiating clearance of plasma LDL cholesterol and triglycerides (Mann *et al.*, 2014a). Furthermore, it may be that fat loss is necessary for changes to occur (McTiernan *et al.*, 1998), which was not seen in the current study, explaining the lack of change.

CRP is an important biomarker of inflammation and has been implicated in reduced disease-free survival and higher risk of mortality among breast cancer patients (Villasenor *et al.*, 2014; Pierce *et al.*, 2009; Allin *et al.*, 2011). In previous research in breast cancer survivors, CRP levels varied from 1.07-1.70mg/L 3-15 months following treatment (Sabiston *et al.*, 2018), comparable to levels measured pre- and post-intervention in both groups in the current study. Exercise may reduce CRP levels in breast cancer survivors (Fairey *et al.*, 2005b; Friedenreich *et al.*, 2016; Ballard-Barbash *et al.*, 2012) as acute bouts of exercise cause a short term increase in CRP, thus exercise training may induce an anti-inflammatory counter response to these increases (Kasapis and Thompson, 2005). Furthermore, exercise can decrease resting CRP by reducing adiposity, improving insulin sensitivity and

lowering non-HDL cholesterol (Ford, 2002). A decrease in resting CRP was not observed in the current study, however CRP levels in previous studies that have reported decreases were elevated at baseline (on average >5mg/L) (Fairey *et al.*, 2005b) which may explain discrepancies. Interestingly we also found that CRP levels correlated with DEXA measured body fat percentage, supporting previous literature (Lin *et al.*, 2010). This supports mechanistic links between adiposity and suggests that weight loss, or more specifically fat loss, may be a driving factor in reducing inflammation.

A marker of chronic inflammation, ESR, was measured in the current study (Bray *et al.*, 2016b). In early stage breast cancer patients (50 ± 11 years old), 68% had an ESR < 25mm/h (Petekkaya *et al.*, 2014). In the current study 100% of the cohort had an ESR under this 'normal' threshold, perhaps due to not having a current diagnosis of the disease. Other studies in breast cancer patients have demonstrated elevated ESR levels in patients (48 ± 7 mm/h) versus age matched controls (7 ± 1 mm/h) (Ofunre *et al.*, 2017). The values from healthy aged matched controls are comparable to levels measured in the current study, supporting that breast cancer survivors no longer have raised levels of inflammation. The normal ESR levels seen in the current study and previously reported in patients may be explained by the early stage diagnoses in both cases as metastasis has been shown to be related to higher ESR levels (Henry-Amar *et al.*, 1991). Little evidence exists between the effects of exercise training on ESR. A study in adolescent and child inflammatory bowel disease patients with an exercise intervention of 30 minutes, 5 days a week, for a total of 8 weeks, reduced ESR from 12 ± 8 to 10 ± 8 mm/h (Legeret *et al.*, 2019). Participants in the current study did not have a diagnosis of inflammatory unlike previous participants with inflammatory bowel disease. Another study investigated 10 weeks of high intensity walking in 12 physically inactive adults aged 64 ± 7 years with rheumatoid arthritis. This exercise elicited significant reductions in ESR by 58% from 11 ± 12 to 7 ± 9 mm/h post- intervention (Bartlett *et al.*, 2018). Again, the lower values reported in the current study are probably explained by an absence of inflammatory disease in breast cancer survivors (Bray *et al.*, 2016a) or due to the longer exercise intervention (12- compared to 8-weeks) and higher intensity of exercise (80-90% $\dot{V}O_2$ reserve). The anti-inflammatory effects of regular exercise may be mediated via both a reduction in visceral fat mass (with a subsequent decreased release of adipokines) (Gleeson *et al.*, 2011). Furthermore, each acute bout of exercise causes a release of cortisol, adrenaline and IL-6, downregulates pro-inflammatory macrophages and migration towards adipose tissue, downregulates pro-inflammatory cytokine production, and increases the number of T-reg cells, all of which further contribute to the anti-inflammatory effects (Gleeson *et al.*, 2011).

In the current study significant reductions in systolic blood pressure were observed following the partly supervised exercise intervention only perhaps due to greater improvements in fitness (as measured by $\dot{V}O_2$max, 6-minute walk test and sit to stand) or reductions in depression (Dimeo *et al.*, 2012; Rubio-Guerra *et al.*, 2013) when compared to those in the remotely monitored study. In line with this, changes in systolic blood pressure were not non-inferior in the remotely monitored group, suggesting the partly supervised group only is able to lead to meaningful reductions in systolic blood pressure. Systolic blood pressure in breast cancer survivors has previously been reported with values such as 125 ± 12 and 117 ± 11 mmHg (Guinan *et al.*, 2013) which are similar to values seen in the current study in the remotely monitored group which were 125 ± 20 mmHg. Reductions in systolic blood pressure as small as 2 mmHg are considered clinically meaningful (Collaboration, 2002), thus whilst both groups demonstrated a clinically meaningful decline, the decline in the remotely monitored group was not non-inferior to the larger decrease observed with the partly supervised group. Other exercise interventions have shown similar declines. An 8-week exercise intervention in breast cancer survivors consisting of three aerobic exercise sessions per week increasing in duration and intensity fortnightly, demonstrated an average reduction in systolic blood pressure by 7 mmHg (Guinan *et al.*, 2013). Another study in 966 cancer survivors implementing a 10-week exercise intervention showed a significant reduction in systolic blood pressure by 5 mmHg (Santa Mina *et al.*, 2017). Due to the increased risk of cardiovascular events with higher blood pressure (Mehlum *et al.*, 2018), and the increased risk of cardiovascular disease in women who have undergone breast cancer treatment (Hooning *et al.*, 2007), it may be particularly important to reduce blood pressure using exercise interventions. The results of this study suggest that a partly supervised, prescribed exercise intervention is preferred over a remotely monitored intervention to do this. The mechanisms underlying a reduction in systolic blood pressure by exercise training are unclear as blood pressure is affected by a multitude of factors (such as diet, exercise, body fat, race, socioeconomic status, psychological factors, age and genetics). Animal studies demonstrate aerobic exercise may prevent increases in blood pressure through beneficial alterations in insulin sensitivity and autonomic nervous system function (Moraes-Silva *et al.*, 2013).

In the current study measures of functional fitness improved in both interventions. 6-minute walk time improved in both groups by 43m and 21m in partly supervised and remotely monitored groups respectively, however the improvements were not deemed non-inferior, whereby the partly supervised group observed better improvements. Previous research has

demonstrated that improvements over 14m may have clinical relevance in terms of cardiorespiratory disease (Bohannon and Crouch, 2017). The 6-minute walk time reflects the functional capacity of individuals and is a prognostic marker for adverse effects of heart disease (Du *et al.*, 2009; Ingle *et al.*, 2014b; Ingle *et al.*, 2014a). Whilst the patients in the current study did not currently have heart disease, breast cancer survivors are at increased risk of cardiovascular disease (Buttros *et al.*, 2019) thus an improvement in 6-minute walk time may in turn reduce the risk of this, potentially suggesting a partly supervised exercise intervention as a preferred choice. In healthy women 49-80 years old, the average distance covered in the 6-minute walk test was 555 ± 81m (Casanova *et al.*, 2011). This is lower than the averages in both groups pre- and post-intervention, perhaps explained by debilitating treatments received by women in the current study. However, this is in line with data from other breast cancer survivors. A study of breast cancer survivors undertaking 10-weeks of exercise training reported a significant increase in distance covered by 55m, from 440 ± 23m to 495 ± 23m (Santa Mina *et al.*, 2017). Another study consisting of 12 weeks of aerobic, resistance and flexibility training in breast cancer survivors reported an increase in 6-minute walk distance from 417 ± 81m to 477 ± 97m (Foley and Hasson, 2016). Both groups in the current study saw a significant increase in sit to stand scores by 2. Normative values for women over 60 years old in Hong Kong and USA were 12 and 15 respectively (Macfarlane *et al.*, 2006; Rikli and Jones, 1999). The values seen pre-intervention in the current study, despite the average age of participants being much lower, were similar to this, but post-intervention were higher at 18 and 19 for the partly supervised and remotely monitored group respectively.

Depression is often elevated in women following cancer (Niedzwiedz *et al.*, 2019). Depression decreased in the partly supervised group only. Exercise interventions have continuously been shown to reduce symptoms of depression (Patsou *et al.*, 2017). A review article reported significant reductions in exercising groups in terms of depression with a small to moderate effect compared to control groups (Patsou *et al.*, 2017), in line with the results of this study. This supports the concept that exercise offers a way in which to manage mood cost-effectively during survivorship. A randomised controlled study in females aged 35-70 years who had previously been treated for stage I-III breast cancer, exercise (Naumann *et al.*, 2012), and exercise in combination with diet (Saxton *et al.*, 2014) was shown to decrease depression as assessed by the Beck Depression Inventory. Moderate aerobic exercise has been shown to be most effective with durations of up to 135 minutes per week for up to 12-weeks (Patsou *et al.*, 2017), whilst the current study demonstrates that even 8-weeks of partly supervised exercise can significantly alter

feelings of depression. On average, both groups in the current study had normal scores for depression at baseline. Larger effects may be seen in those experiencing higher levels of depression. In the current study one participant in the remotely moderated group reduced depression by 28 (severe) to 20 (moderate) and one in the partly supervised group reduced from 32 (severe) to 12 (mild), further supporting this.

Adherence in the current study was comparable to previously reported adherence rates. In an 18-week supervised exercise intervention in breast cancer patients undergoing treatment, adherence was 83%, only 4% lower than seen in the partly supervised group in the current study (Witlox *et al.*, 2019). In post-menopausal women free from current or prior breast cancer diagnosis undertaking supervised exercise, women completed a higher amount of supervised sessions (95%) versus those in a unsupervised exercise arm (79%) (Courneya *et al.*, 2012). This demonstrates a similar trend to the current study, where adherence was significantly lower in the remotely monitored group. Many predictors to adherence of exercise interventions have been identified which were not assessed in the current study such as education, beliefs about planned behaviours, outcome expectations, motivations for exercise, lifetime exercise history and physical fatigue (Ormel *et al.*, 2018; Kampshoff *et al.*, 2016). Furthermore, in the remotely monitored group, participants had to identify and use exercise settings, another predictor of adherence, rather than exercise facilities being provided in the partly supervised group for 2/3 sessions per week.

The main limitation of this study is that physical activity, including free living physical activity was not objectively measured, thus we cannot confirm whether there was a significant change in both exercise minutes and intensity and free-living physical activity pre- and post-exercise intervention. The lack of change in either of these, due to compensatory changes to lifestyle, may explain why there were a lack of differences seen in $\dot{V}O_2$max. Future studies should include the use of a validated physical activity monitor to measure this. There are various limitations of the $\dot{V}O_2$max exercise protocol, such as participants not being acquainted with maximal exercise and being worried about exercising to true maximum. Future studies should introduce a familiarisation session and also consider doing maximal exercise training on a cycle ergometer and use a breath by breath gas analysis system to measure $\dot{V}O_2$max. Future research comparing $\dot{V}O_2$ and HR prescribed exercise interventions should use $\dot{V}O_2$ and HR reserves to set exercise intensity rather than $\dot{V}O_2$ and HRmax values. Finally, when interpreting results, the limitations associated with non-inferiority designed studied (such as limitations with margins defined), should be kept within the forefront of the mind.

Future research should establish the optimal and most easy to adopt exercise intervention for improving $\dot{V}O_2$max in breast cancer survivors including interval, resistance and high intensity exercise training and track women over time to measure ACM and disease relapse. Furthermore, interventions should use a combination of exercise and diet intervention to elicit beneficial changes in fitness, body composition and health, to decipher whether weight loss or exercise itself is most important in terms of benefits to health in this population. This way clinicians can specifically advise breast cancer survivors on the most effective exercise or exercise and diet they can undertake to improve health and lower risk of ACM and disease relapse.

8-weeks of both exercise interventions did not elicit improvements in $\dot{V}O_2$max or body composition. The remotely monitored intervention was deemed non-inferior to the partly supervised intervention when referring to body composition but was not deemed non-inferior in terms of changes to $\dot{V}O_2$max, 6 minute walk distance and systolic blood pressure.

CHAPTER 7: General Discussion

7.1 Summary of key findings in healthy women

In Chapter 3 it was demonstrated that 86% of healthy women volunteers from Bath, UK (sample may not be representative of entire UK population) have specific T-lymphocytes that can recognise and respond (as assessed by IFN-γ production) to tumour-associated antigens, despite being free from a current cancer diagnosis. At first look this may seem surprising, as previously T-lymphocyte response to tumour-associated antigens have not been well documented amongst healthy individuals. However, when looking at cancer defence mechanisms and treatment strategies in more detail, it becomes apparent that the ability to generate such responses may be beneficial to the host. Evidence exists to support the notion that T-lymphocytes provide an important defence against cancer, detecting cells expressing tumour-associated antigens, a process that has been taken advantage of in treatment interventions such as using autologous T-lymphocytes or engineered cell therapies (Zamora *et al.*, 2018; Ilyas and Yang, 2015). This is further supported by literature reporting improved prognosis in breast cancer patients who possess higher immune responses to tumour-associated antigens (Tiriveedhi *et al.*, 2014; Muraro *et al.*, 2015a; Blixt *et al.*, 2011; Muraro *et al.*, 2015c; Tiriveedhi *et al.*, 2013). Literature has demonstrated MamA vaccination in stage IV metastatic breast cancer patients increased the frequency of tumour-infiltrating CD4+ cells, decreased the frequency of T-reg cells and increased T-lymphocyte IFN-γ release in response to MamA post-vaccination, with preliminary evidence suggesting that this response improves progression-free survival (Tiriveedhi *et al.*, 2013; Tiriveedhi *et al.*, 2014). Sustained levels of CD8+ T-lymphocyte specific response to tumour-associated antigens MamA and SUR throughout neoadjuvant chemotherapy have previously been related to pCr in breast cancer patients (Muraro *et al.*, 2015b) and finally, higher levels of MUC1 autoantibodies were significantly associated with reduced rate and delay in metastases in early stage breast cancer patients (Blixt *et al.*, 2011). Furthermore the hallmarks of cancer state that in order to survive, cancer cells must avoid immune destruction (Hanahan and Weinberg, 2011), suggesting that immune detection of cancer cells through recognition and response to tumour-associated antigens on cancer cell surfaces will in fact destroy the cancer cell, thus in order for cancer to progress, the cancer cell must avoid this immune detection.

As healthy women in the current thesis were free from a cancer diagnosis, this suggests that having functional immune responses towards tumour-associated antigens may demonstrate that beneficial immune detection has previously occurred within the host.

However, whether this is happening and if so, to what magnitude, is unknown. The possession of tumour-associated antigen specific T-lymphocytes able to secrete IFN-γ after overnight stimulation suggests that the response is a 'memory' response. This is supported by the high number of IFN-γ secreting T-lymphocytes responding to viral antigens to which we know hosts are seropositive towards. As proven by ELISA, we can confirm whether individuals have previously encountered a virus, and therefore are likely to possess memory specific T-lymphocytes able to respond to the virus specific antigens. In the current thesis, it was observed that if individuals were seropositive for a virus, SFUs in response to virus specific antigens was increased, confirming the response within the ELISpot is likely to represent memory T-lymphocyte responses. This suggests that if there is a T-lymphocyte response to a tumour-associated antigen then the tumour-associated antigen has previously been encountered and left behind specific memory, so that on future exposure to the specific antigen, a rapid immune response occurs (Pennock *et al.*, 2013b). However, another subset of T-lymphocytes can also respond to tumour-associated antigen; naïve T-lymphocytes. It may be that a naïve T-lymphocyte, which has not yet been activated, has a specific TCR for the tumour-associated antigen which, when encountered, becomes activated and thus creates an immune response (Pennock *et al.*, 2013b). Due to the limit time of culture (overnight) and the results of T-lymphocyte response to previously exposed viruses, it is likely that the memory T-lymphocytes are responsible for the overall response to the tumour-associated antigens in the current assay. As we cannot be sure that all IFN-γ secreting T-lymphocytes are memory T-lymphocytes, future studies should use cell separation and phenotyping techniques using magnetic beads, flow cytometry or cultured ELISpot assays which re-stimulate cells after a period of culture, to confirm this. Knowing whether naïve or memory T-lymphocytes are responsible for the IFN-γ release in response to tumour-associated antigens will allow researchers to determine the importance of possessing both naïve and memory T-lymphocyte specific responses to tumour-associated antigens. For example, in healthy women, it could be investigated whether lifestyle factors and CMV serostatus are associated with differences in possessing naïve T-lymphocytes specific to tumour-associated antigens or the magnitude of a memory response to tumour-associated antigens. In patients this would allow us to identify which response (naïve or memory) is more robustly associated with pathological response.

Whilst possession of memory responses to tumour-associated antigens may be beneficial in terms of cancer cell detection and elimination, ongoing re-exposure to tumour-associated antigens may in time lead to dysfunctional T-lymphocytes with a decreased ability to proliferate and function due to increased expression of inhibitory markers (Xia *et al.*, 2019).

In turn, this may increase susceptibility to successful cancer cell growth due to an inability to eliminate cancer cells (Xia *et al.*, 2019). Due to the limitations of observational studies as implemented in Chapter 3 of this thesis (data is only collected from participants at one time point, offering only a snapshot in time), it is beyond the scope of this thesis to determine whether possessing a larger number of IFN-γ specific T-lymphocytes affects future risk of breast cancer. Future studies should adopt a longitudinal design to track participants over time to determine whether the number of specific tumour-associated antigen T-lymphocytes changes (due to potential re-exposure occurs) and whether this is related to breast cancer risk by recording future diagnoses.

10 different tumour-associated antigens were used as antigenic stimulants within Chapter 3, each eliciting a varying number of women who possessed specific T-lymphocytes that recognised each antigen. This suggests that the magnitude of immune response to antigens may depend on whether antigens are self (e.g. MUC1) or non-self (e.g. CEA) and whether antigens are associated with one or more different cancer types. Self-antigens may be less well recognised due to deletion of self-reactive T-lymphocytes within the thymus (Dzhagalov *et al.*, 2013) and antigens associated with more than one type of cancer are more likely to have previously been encountered within the body due to increased chance of exposure.

In Chapter 3, the tumour-associated antigen that had the highest number of women possessing specific T-lymphocytes towards it was MamA. MamA is a breast cancer specific antigen (Fleming and Watson, 2000b; Kundu *et al.*, 1996) with proven immunogenicity (Tiriveedhi *et al.*, 2013; Jaramillo *et al.*, 2002; Kim *et al.*, 2016; Fleming and Watson, 2000b). As breast cancer is accountable for 44% of all cancers in women in their 40s, and the average age of participants in the current study was 42 years old, it may be that this population, despite being free from a cancer diagnosis, may have possessed, detected and eliminated breast cancer cells displaying the MamA antigen previously, and thus possess robust MamA specific memory T-lymphocytes which are responsible for IFN-γ release on simulation in the ELISpot. The immune response to the tumour-associated antigen may also represent strong immune defence against cancer, explaining why individuals remain free from cancer diagnoses. The least number of women possessed specific T-lymphocytes able to respond upon stimulation with the tumour-associated antigen SUR. Central and peripheral tolerance mechanisms are in place to eliminate or control T-lymphocyte response to self-antigens, thus, as survivin is an overexpressed self-antigen, it would be expected that T-lymphocyte response to this antigen would be low or absent (Bright *et al.*, 2014). As the second most weighted factor in the prioritisation of cancer antigens to be used

in cancer treatments such as vaccines is immunogenicity (Cheever *et al.*, 2009), this work helps to identify which antigens elicit the greatest and smallest immune responses.

The number of tumour-associated antigen specific T-lymphocytes was much lower than anti-viral specific T-lymphocytes for those who were seropositive (had had prior exposure and infection to) to CMV, EBV and VZV. This is not surprising given that some tumour-associated antigens are overexpressed self-antigens (whilst viral antigens are not) and that exposure to viral antigens (through reactivation throughout life) may be much higher than exposure to tumour-associated antigens. This is supported by evidence demonstrating that individuals are often infected with viruses at a young age (high prevalence before age 20 for EBV, CMV and VZV (Balfour *et al.*, 2013; Lachmann *et al.*, 2018; Gershon *et al.*, 2015)) compared to cancer incidence occurring at older ages (risk of breast cancer dramatically increases over the age of 40 years (Kamińska *et al.*, 2015)). Measuring immune responses to previously exposed viruses offers a marker of function of cellular immunity.

Evidence such as an increased risk of cancer in immunodeficient mice and humans, positive associations between tumour infiltrating lymphocytes and prognosis in cancer patients and immune avoidance mechanisms adopted by successful cancer cells (Corthay, 2014) demonstrates the importance of immune function in cancer defence. Furthermore, the immune system can be manipulated by aspects of lifestyle whereby regular moderate activity or exercise can enhance the immune function in terms of improved vaccine response, delay immunosenescence and reduce the risk of infection (Davison *et al.*, 2016). Therefore, it is important to assess immunosurveillance in respect to lifestyle.

However, in Chapter 3 it was demonstrated that a limited number of lifestyle characteristics were related to the possession of tumour-associated antigen specific T-lymphocytes. This may be explained by a variety of factors. Most likely is the fact that lifestyle factors (exercise, physical activity, body composition and diet) are attributable risk factors for only a small percentage of cancer diagnoses (Parkin, 2011; Brown *et al.*, 2018). Other factors such as breast density, genetic determinants, and oestrogen exposure (age of menstruation, menopause and first pregnancy) (Engmann *et al.*, 2017; Dixon, 2012) may play a more important role in breast cancer risk. Furthermore, non-lifestyle factors may play a more predominant role in modulating immune function such as intrinsic factors (genetics, and comorbidities), perinatal factors (gestational age, birth weight, feeding method, and maternal factors), and extrinsic factors (microbiota, infections, and antibiotics) (Zimmermann and Curtis, 2019).

In Chapter 4 it was hypothesised that CMV positive serostatus, due to association with accelerated immunosenescence, would be associated with lower T-lymphocyte specific response to tumour-associated antigens when compared to CMV seronegative counterparts. What was actually demonstrated, for the first time, was in fact the opposite, that CMV seropositive individuals had consistently higher responses to tumour-associated antigens when compared with seronegative individuals.

Whilst this is surprising, we must remember that the volunteers in this study were free from cancer diagnosis, perhaps due to luck, but also perhaps due to an increased ability of the specific T-lymphocytes. If we presume that the response elicited in an ELISpot is a memory T-lymphocyte response (as discussed previously), it may be that CMV seropositive individuals may more frequently encounter tumour-associated antigens, due to a variety of factors including lifestyle, compared to negative counterparts who may not possess specific memory T-lymphocytes (due to no prior exposure). Furthermore, seronegative individuals may possess specific naïve T-lymphocytes should exposure occur, an idea that warrants further investigation through the use of cultured ELISpot methods which cultures cells so that naïve T-lymphocytes can proliferate and generate specific memory responses to antigens on re-stimulation.

A further aim of Chapter 4 was to determine any lifestyle differences and the impact of them on immune function in CMV seropositive and seronegative women. Due to the cumulative effect of CMV serostatus and lifestyle on immune function we hypothesised that individuals with healthier lifestyles in CMV seropositive individuals would have improved T-lymphocyte function in response to tumour-associated and viral antigens. What was observed was that in CMV seropositive women, individuals with some characteristics of unhealthier lifestyles (e.g. decreased fitness and physical activity level and increased body fat) demonstrated a higher number of T-lymphocyte responding cells to viral and tumour-associated antigens or a higher proportion of women able to respond to tumour-associated antigens. There is an increased risk of cancer in individuals with unhealthy lifestyles (Katzke *et al.*, 2015) (smoking, alcohol consumption, obesity, diet, and physical inactivity) and therefore an increased chance of a normal cell mutating into a cancer cell. Thus, individuals with unhealthy lifestyles, due to this increased likelihood of encountering a cancerous cell, may have had increased prior exposure to tumour-associated antigens compared to their healthier counterparts. Women in this study were free from cancer diagnosis, potentially down to the ability of their immune system to detect and destroy these cells, in the process

producing an increasing amount of memory cells specific for tumour-associated antigens. Conversely, in those who lead healthy lifestyles it may be that there has been little or no exposure to tumour-associated antigens, therefore few or no memory T-lymphocytes reside within the body and thus smaller memory T-lymphocyte responses exist.

Collectively Chapters 3 and 4 demonstrate that healthy women possess tumour-associated antigen specific T-lymphocytes. CMV seropositive individuals have higher numbers of tumour-associated antigen specific T-lymphocytes compared to their seronegative counterparts and in CMV seropositive individuals only, some characteristics of less healthy lifestyles are linked with higher numbers of tumour-associated antigen specific T-lymphocytes.

7.2 Summary of key findings in breast cancer patients

In Chapter 5, research examined T-lymphocyte IFN-γ release in response to tumour-associated antigens in breast cancer patients and relationships with lifestyle factors through neoadjuvant chemotherapy with reference to clinical response. Multiple challenges were experienced throughout data collection surrounding recruitment. Initially, a sample size of 40 breast cancer patients was expected, whereby on average, recruitment of 12 patients per year was deemed feasible. Delays in the start date of recruitment due to equipment familiarisation and collaboration with the NHS recruitment site hindered recruitment. On top of this, the number of patients diagnosed per year and prescribed neoadjuvant chemotherapy treatment was much lower than expected, so, despite recruitment percentage of total women diagnosed being higher than expected, total recruitment, within the time frame of this PhD was not sufficient to conduct original analysis. This was the first time a breast cancer specific project was collaborated between the research group and NHS site, thus teething problems were encountered. For example, there were multiple consultants within the oncology team, not all of whom were fully prepared and briefed on the study, thus some potential patients may have fallen through the net by either not receiving information about the study or being given only very little information on the study.

Future research projects should be explained to all consultants who may come across potential patient participants with a plan on what to advise to potential participants and who to inform. Asking consultants to look ahead at patients attending consultations and to communicate this with members of the research team will allow a member of the research team to be present at consultations to discuss the study in more detail and answer any questions patients may have about participation. Creating such rapport may be beneficial

to recruitment. Furthermore, this may help to overcome another problem experienced which was the short time period between consultation and chemotherapy commencement, as no time would be lost trying to contact the patient by phone or email and the initial laboratory visit could be scheduled at the time of diagnosis. Another strategy that may help is to gain access to eligible patients' contact information before their consultation to allow more time before the beginning of their chemotherapy regime to schedule in a laboratory visit. Encouragingly, consultants are offering an element of choice in terms of cancer treatment (surgery, chemotherapy, radiotherapy) and the timing of treatment to patients, highlighting the advantages and disadvantages of each. Anecdotally, it was noted that many women with breast cancer diagnosis did not want to wait for surgery to have neoadjuvant chemotherapy so chose to avoid this treatment plan, whilst new evidence has begun to question the efficacy of neoadjuvant chemotherapy (Vaidya *et al.*, 2018), which is likely to have decreased the number of patients being eligible for this study. Finally, future research should consider using multiple sites from which patients can enrol on the study as only one site was used in the current study.

Other challenges faced in this study surrounded sample collection. To conduct total ELISpot analysis ~40 ml of blood was to be taken by venepuncture at each time point to allow for at least 11 x 10_6 PBMCs to be collected for use. All qualified phlebotomists on campus took blood samples, however they were not used to the difficulties you can face when taking blood for people who are currently or have previously undergone chemotherapy, such as being limited to take blood from one side of the body only due to involvement of lymph nodes and the damage of veins. Thus, on various occasions, venepuncture was unsuccessful and subsequent analysis could not be undertaken. To ensure that this does not happen in future, blood samples should be taken at an NHS site by a phlebotomist who has an abundance of experience of the challenges to be faced.

Finally, patient retention was a challenge within the current study. Despite ongoing communications via phone, email and attendance at appointments, some patients were lost at follow up. Whilst they did not withdraw consent, they did not respond to any communications or attend the final laboratory visit. In any study you are likely to lose patients at follow up and in the current study the loss of ~33% is slightly high, but this may be due to the low sample size. Strategies need to be put in place to reduce loss to follow up such as increased attendance at appointments and reminders from the NHS site about attendance to trial visits.

Whilst the original aims of Chapter 5 were not met, the individual changes in lifestyle factors, blood cell counts and T-lymphocyte responses to tumour- associated and viral antigens with reference to individual clinical outcomes were reported. Unsurprisingly predicted $\dot{V}O_2$ max declined in all patients in line with previous research, likely due to the toxic effects of chemotherapeutic agents (Jones *et al.*, 2007a; Jones *et al.*, 2007b), or reductions in physical activity level over treatment as demonstrated within Chapter 5. Women following breast cancer are at a greater risk of death from cardiovascular disease than women who have not received treatment (Patnaik *et al.*, 2011). As $\dot{V}O_2$ max, a predictor of cardiovascular risk (Fernström *et al.*, 2017; Khan *et al.*, 2014), declines through treatment, it may be important to establish a safe and efficient exercise protocol for women undergoing chemotherapy to undertake to maintain or at least, limit the decrease seen. Some exercise intervention studies have been undertaken that suggest that exercise is a safe, feasible, and an efficacious intervention in breast cancer patients who are undergoing different types of treatment (Fairman *et al.*, 2016) and thus may be included in future treatment regimes.

BMD also declined with neoadjuvant chemotherapy, consistent with trends previously reported in breast cancer patients who have undergone adjuvant and neoadjuvant chemotherapy (Tang Axelsen *et al.*, 2018). Such decreases in BMD can have negative consequences on health such as increased risk of fracture and osteoporosis (Hernlund *et al.*, 2013). However, in Chapter 3 we demonstrated individuals who had tumour-associated antigen specific T-lymphocytes for one or more tumour-associated antigens had, on average, a lower BMD. Whilst lower BMD may be associated with improved immune responses, care must be taken to avoid clinically low levels and results much be interpreted with care as the low sample size allows extreme values to alter the mean. Weight bearing exercise can improve or reduce declines in BMD (Benedetti *et al.*, 2018), further supporting the use of exercise intervention during treatment.

Regarding cell counts, no immediate observations were made between cell counts and prognosis, however much research has supported the use of markers such as NLR (Wei *et al.*, 2016a) , Hb (Bottini *et al.*, 2003) and PLR (Zhu *et al.*, 2017) to predict prognosis of treatment and are therefore important in assessing treatment response prediction for cancer patients and are quick and easy measures to potentially assess tumour growth, progression, invasion, and metastasis. A big limitation of the current study is the small sample size but also the lack of variety in response to treatment as all patients had either a complete or partial response to treatment, therefore it is difficult to identify any potential characteristics or markers that may predict treatment response. This limitation also holds

true when interpreting data regarding T-lymphocyte response to tumour-associated antigens which demonstrated a trend of increasing from diagnosis, mid-chemotherapy and post-chemotherapy. As all patient involved had either a partial or complete response to treatment, this supports findings that individuals who possess tumour-associated antigen specific T-lymphocytes have improved treatment prognosis (Cobbold *et al.*, 2013b; Muraro *et al.*, 2015a; Muraro *et al.*, 2011; Inokuma *et al.*, 2007b; Roscilli *et al.*, 2014b; Epel *et al.*, 2008; Stadler *et al.*, 2016; Kao *et al.*, 2001; Criscitiello, 2012; Galvis-Jimenez *et al.*, 2013). However, this is difficult to confirm when there are no comparisons to poor prognosis patients involved in the current study.

A limitation of the current study is that cells were taken from the periphery at rest. As tumour-infiltrating lymphocytes are a good prognostic marker to cancer treatment (Hellwig, 2019), assessing the functionality of these cells which are in contact with the tumour itself, encountering tumour-associated antigens displayed on cancerous cell surfaces, which may be obtained through biopsy, may be important. Having information regarding the immune response from the periphery and within the tumour microenvironment can also confirm how the two interact and affect one another. This would also confirm whether the ability of cells to migrate to the tumour leads to a difference in cell function of those in the periphery and those infiltrating the tumour. T-lymphocytes within the lymph should also be investigated as when chemotherapy causes tumour cell death, T-lymphocytes are most likely to be required to undertake most functions at the lymph nodes, where there is an increased amount of antigen presentation from the dead cells, or within the tumour itself, recognising and subsequently eliminating cells with specific antigens.

This Chapter highlights that breast cancer patients (as well as healthy women observed in Chapters 3 and 4) can elicit tumour-associated antigen specific T-lymphocyte responses before, during and after neoadjuvant chemotherapy. Chapter 5 sets the scene for future research to examine lifestyle and immune function changes and the implications this may have on treatment outcomes in breast cancer patients receiving neoadjuvant chemotherapy, alongside addressing feasibility issues.

7.3 Summary of key findings in breast cancer survivors

Breast cancer treatment often results in a decline in aspects of lifestyle, such as fitness (as demonstrated in Chapter 5), and evidence demonstrates that breast cancer survivors are at an increased risk of cardiovascular disease (Patnaik *et al.*, 2011). Therefore, Chapter 6

investigated the effects of two exercise interventions in this population and how they affected various aspects of health.

The aim of Chapter 6 was to evaluate whether 8-weeks of a technology-enabled, remotely monitored physical activity intervention with the use of a physical activity tracking wrist-watch, produces changes to health that are not meaningfully inferior to changes in response to a partly supervised exercise programme in female breast cancer survivors. It was hypothesised that the technology-enabled, remotely monitored physical activity intervention would result in non-inferior improvements in health compared to 8-weeks of a partly supervised, exercise. It is concluded that neither exercise intervention caused significant changes to $\dot{V}O_2max$ or body composition. Whilst changes observed in the remotely monitored group were non-inferior to changes in the partly supervised group in terms of body fat, changes observed in the remotely monitored group were not non-inferior to changes to the partly supervised group in terms of $\dot{V}O_2max$, 6-minute walk time and systolic blood pressure.

A non-inferiority design was chosen in the current randomised controlled trial. This trial design is not often used in sport and exercise science research, however, is increasingly used in clinical research, particularly in drug trials. The use of this type of trial has a variety of advantages. Whilst the absence of a control group may be considered a limitation, this study design ensures all participants receive something that can potentially improve health. Secondly it allows us to directly compare two interventions that want to achieve the same thing. Discovering whether one intervention is non-inferior allows us to identify the easiest, most enjoyable intervention that can also elicit meaningful changes in markers of health, which in turn may be the types of intervention most easily adopted in real life.

However, non-inferiority trials also introduce difficulty. Defining Δ, the largest value that can be deemed not clinically significant introduces subjectivity. In exercise science, many measures do not have pre-determined clinical margin thus, it becomes the responsibility of the researcher to determine these margins based on experience and justification from literature, a process that can introduce discrepancies. Whilst this is a relatively robust method for selecting the margin it relies on previous literature accurately quantifying the effectiveness of the active comparator; in this case the effect of partly supervised exercise on $\dot{V}O_2max$ (Fleming, 2008). Whilst literature documents that exercise can increase $\dot{V}O_2max$, the extent to which differs depending on the exercise intervention (duration and intensity) and the participant group (trained, untrained, males, females etc). Conversely, in

some settings it may be shown that the active comparator does not always benefit - again adding problems. To overcome this, future research should use 3 arm inferiority trials which include a placebo group to compare to the active comparator. In a clinical setting e.g. pharmaceutical drugs, there may be evidence for the active comparator being administered to the same study population under the same regimen, however in the exercise science setting, it is unlikely that the exact exercise intervention has been previously carried out robustly in the same population of interest, thus some discrepancies may occur through between trial differences in participant characteristic, schedule, adherence and end points (Fleming, 2008). No real guidelines exist for defining what is the crucial non-inferiority margin. Criteria for defining the non-inferiority margin should be clearly developed for researchers adopting the non-inferiority design. At present, it is possible that an intervention may be deemed non-inferior when it is really 10-20% less effective than the active comparator, a concept that seems unethical (Rief and Hofmann, 2019), thus results should be interpreted with caution and reference to the raw data.

The methodology chosen regarding non-inferiority analysis and interpretation can lead to confusion. For example, depending on methodology selected the same data can be confirmed as superior or equivalent (Rief and Hofmann, 2019). Again, no clear guidance is set out for statistical analysis and interpretation of non-inferiority trials, especially in the sports science setting. In order to produce robust conclusions, recommendations for non-inferiority analysis in a sports science setting should be established to avoid erroneous non-inferiority results being circulated through literature.

Finally, the motivation of a non-inferiority trial may introduce poorly designed and poorly conducted trials which in turn increase the likelihood of a type I error and a conclusion to be drawn that an intervention is non-inferior when really it is not (Newberry *et al.*, 2014; Schumi and Wittes, 2011).

A main finding of this research was that neither exercise intervention elicited changes in $\dot{V}O_2$max. This may be due to a variety of reasons including the design and implementation of the $\dot{V}O_2$max exercise tests, familiarity with maximal exercise (as exercise tests were run to volitional exhaustion) and a reduction of impact of the exercise interventions. The exercise interventions may not have elicited improvements in $\dot{V}O_2$max due to the short duration of the interventions (8-weeks) and low intensity of some exercise sessions (if measured $\dot{V}O_2$max and HRmax did not represent 'actual' max due to problems with maximal testing, then prescribed exercise at % max is actually lower than required). In the remotely

monitored group specifically, it may be people just increased the intensity or duration of normal lifestyle activities, such as walking the dog, to contribute to exercise training, rather than undertaking completely new structured exercise bouts, thus the increase in exercise was not as large as actually prescribed. This same principle can be applied to the unsupervised session in the partly, supervised group. The lack of changes in body composition are likely due to the short duration of the exercise interventions or the majority of women taking part already having a normal body composition. The lack of change in body composition may also contribute to the lack of changes in other markers of health, particularly biochemical markers where changes are often driven by adiposity (Veghari *et al.*, 2015; Ahmad *et al.*, 2018; Visser *et al.*, 1999; Akter *et al.*, 2017; George *et al.*, 2017).

Finally, changes in systolic blood pressure are not non-inferior in the remotely monitored exercise intervention. As systolic blood pressure is a risk factor for cardiovascular disease (Bundy *et al.*, 2017) and cardiovascular disease is at increased risk in breast cancer survivors (Garcia-Estevez and Moreno-Bueno, 2019; Nelson, 2013), a reduction in blood pressure may be significant to health in this population, thus suggesting the partly supervised intervention may be preferred when targeting this variable of health. Therefore, future research should use increased sample sizes, calculated using systolic blood pressure as the primary objective to confirm this and superiority studies should be run to confirm whether the partly supervised exercise is indeed superior to the remotely monitored intervention.

7.4 Conclusions

The immune response is important in cancer defence, during treatment for cancer and again in cancer defence following treatment. Lifestyle factors are modifiable factors of health that can improve immune function. Chapter 5 in this thesis demonstrated that such lifestyle factors worsen during neoadjuvant chemotherapy for breast cancer, thus women following treatment should implement exercise interventions to improve their health. In the Chapter 6 we demonstrated that a remotely monitored exercise intervention produced non-inferior changes in body composition and $\dot{V}O_2max$ compared to partly supervised exercise, however neither intervention improved many aspects of health significantly.

7.5 Future research

Future research in healthy participants should include longitudinal study designs to measure T-lymphocyte immune response to tumour-associated antigens and breast cancer risk by tracking women over time, measuring cancer diagnoses to better understand whether possession or magnitude of T-lymphocyte response to tumour-associated antigens and changes over time are related to disease risk.

Future research using ELISpot assays should include the use of cell separation or phenotyping techniques to separate specific T-lymphocyte responses to tumour-associated antigens. This will determine which cells, or the proportion of cells, are responsible for the immune response so that these can be investigated to either target in immunotherapy treatment strategies or improve cell functionality to ensure immune defences are efficient to eliminate potential tumours. Furthermore, T-lymphocytes should be collected from various sites, including the tissue and lymph through biopsy, to elicit whether differences are apparent between sampling site and which is most relevant to breast cancer risk and/or prognosis to use as a future marker of response to treatment. Studies should also incorporate sampling post-exercise, when immune cell mobilisation and migration is taking place to determine the importance of individual exercise bouts of varying intensity, type and duration on T-lymphocyte response to tumour-associated antigens. This will offer insight into whether it is the culmination of multiple exercise bouts or exercise training and the physiological adaptations that it brings is most important when assessing immune response to tumour-associated antigens. This will then allow exercise interventions to be developed which optimise immune responses to tumour-associated antigens in participants, which may lead to improve cancer protection or treatment efficacy. To assess the impact of lifestyle on T-lymphocyte response to tumour-associated antigens, more extreme populations (e.g. obese) should be targeted to identify differences.

In breast cancer survivors, future research should use non-inferiority or equivalence trials including placebo arms to confirm the most enjoyable and well-adhered to exercise intervention for improving $\dot{V}O_2max$ and other markers of health including interval, resistance and high intensity exercise training, with and without the use of dietary interventions. This will allow breast cancer survivors to incorporate beneficial, but easily executed exercise following breast cancer treatment. Furthermore, after exercise interventions, women should be tracked over time to measure disease relapse, disease-free survival and ACM to confirm that the beneficial effects of exercise reduce disease relapse and ACM.

7.5 Conclusions

In conclusion lifestyle factors of healthy individuals had little relationship with possession of T-lymphocytes that strongly recognise breast cancer tumour-associated antigens, suggesting other variables may contribute to this more so. Such variables should be investigated to identify which aspects of lifestyle or genetics can be targeted to improve immune cell response to tumour-associated antigens, to potentially reducing cancer risk. The majority of healthy women possessed T-lymphocytes able to respond to tumour-associated antigens. This, alongside data demonstrating CMV seropositive individuals have a higher number and proportion of tumour-associated antigen specific T-lymphocytes than seronegative individuals and data demonstrating improved prognosis in breast cancer patients with higher T-lymphocyte responses to tumour-associated antigens, suggests this immune memory response may be beneficial in terms of cancer protection due to the absence of diagnosis in this population. To confirm this future research should track women over time and record future diagnoses. The tenuous links between improved lifestyle characteristics and reduced memory T-lymphocyte response to tumour-associated antigens in CMV seropositive individuals may suggest a reduction in previous antigen exposure and perhaps cancer cell development in healthier individuals, supporting the notion of adopting a healthy 'anti-cancer' lifestyle, however much more data needs to be collected to confirm this. Furthermore, we conclude that tumour-associated antigens have varying immunogenicity, and, in this healthy population MamA was the most immunogenic. This is potentially useful when designing immunotherapeutic strategies for breast cancer to ensure the most immunogenic antigens are targeted to improve treatment outcomes. Using the limited sample size, patients demonstrating positive clinical outcomes exhibited increasingly stronger recognition of breast cancer tumour-associated antigens by T-lymphocytes through treatment. As the immune function plays a large role in cancer prognosis it may be that interventions are developed to increased T-lymphocyte response to tumour-associated antigens in the hope to improve treatment prognosis. Furthermore, immune cell response to tumour-associated antigens may be used as a treatment predictor during the early or mid-stages of chemotherapy to guide treatment options. The low sample size makes it difficult to draw conclusions on whether lifestyle factors are related to treatment outcomes, however, cardiorespiratory fitness declines after chemotherapy may have potential negative consequences on health, especially cardiovascular health given the already increased risk in this population. This reduction in fitness, which is a marker of cardiovascular health, can potentially be targeted through the intervention of exercise programmes during treatment. Furthermore, lessons can be learned from the difficulties encountered in recruiting and

running a study in this patient group, advising approaches taken in future studies to increase research success. In breast cancer survivors, neither an 8-week remotely monitored, or partly supervised exercise intervention causes significant changes in $\dot{V}O_2$max or body composition (remotely monitored is non-inferior) thus interventions need to be adapted to improve these variables. A remotely monitored exercise intervention was not deemed non-inferior than partly supervised exercise in regard to systolic blood pressure, $\dot{V}O_2$max and 6-minute walk time. Given the relationship with these outcomes, health and ACM it is recommended to improve these values a prescribed, partly supervised exercise intervention is undertaken by breast cancer survivors following treatment to potentially reduce disease recurrence and ACM.

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APPENDIX 1

In the current thesis (Chapters 3 and 4) T-lymphocyte responses to tumour-associated antigens were deemed positive if the mean number of spots from the stimulated wells was greater than the minimum number of spots in viral wells where participants were seropositive towards the virus. This method is immunologically robust but has rarely been used before, therefore we have provided data within this appendix using 2 more methods of defining positivity; over 2 x the number of spots averaged in the negative control wells and over 4 x the number of spots averaged in the negative control wells.

Healthy women have immune responses to tumour-associated antigens

Using serostatus to viruses to define positivity, immune responses to tumour-associated antigens were present in 43/50 healthy women with only 14% of women lacking an immune response to any of the 10 tumour-associated antigens (Table 1.) When using a threshold to define positivity as over 2 x negative control well, immune responses to tumour-associated antigens were present in 48/50 healthy women with only 4% of women lacking an immune response to any of the 10 tumour associated antigens (Table 1.) When using a threshold to define positivity as over 4x negative control well, immune responses to tumour associated antigens were present in 43/50 healthy women with only 14% of women lacking an immune response to any of the 10 tumour associated antigens (Table 1.)

Table 1. Number and percentage of women defined as positive to tumour-associated antigens as per 3 different methods

Approach to define positivity	**Anti-viral serostatus**	**2 x negative control**	**4 x negative control**
Number positive	43	48	43
Number negative	7	2	7
Percentage positive	86	96	86
Percentage negative	14	4	14

N=50

Immuno-dominance of tumour associated antigens

Using anti-viral serostatus to define positivity, MamA and CEA were the most immune-dominant antigens with 52% of women eliciting an immune response towards them followed by ERB ECD (50% women showing an immune response), CycB1 (48%), ERB ICD and TARP (46%), MUC1 (44%), SUR and Cl6 (38%). MMP11 was the least immune-dominant antigens with only 28% of women eliciting an immune response towards this antigen (Figure 1). When using a threshold to define positivity as over 2 x negative control well, MamA was the most immune-dominant antigen with 70% of women eliciting an immune response towards them. MMP11 was the least immune-dominant antigens with only 44% of women eliciting an immune response towards this antigen (Figure 1.) When using a threshold to define positivity as over 4 x negative control well, MamA was the most immune-dominant antigen with 58% of women eliciting an immune response towards them. MMP11 was the least immune-dominant antigen with only 28% of women eliciting an immune response towards this antigen (Figure 1.)

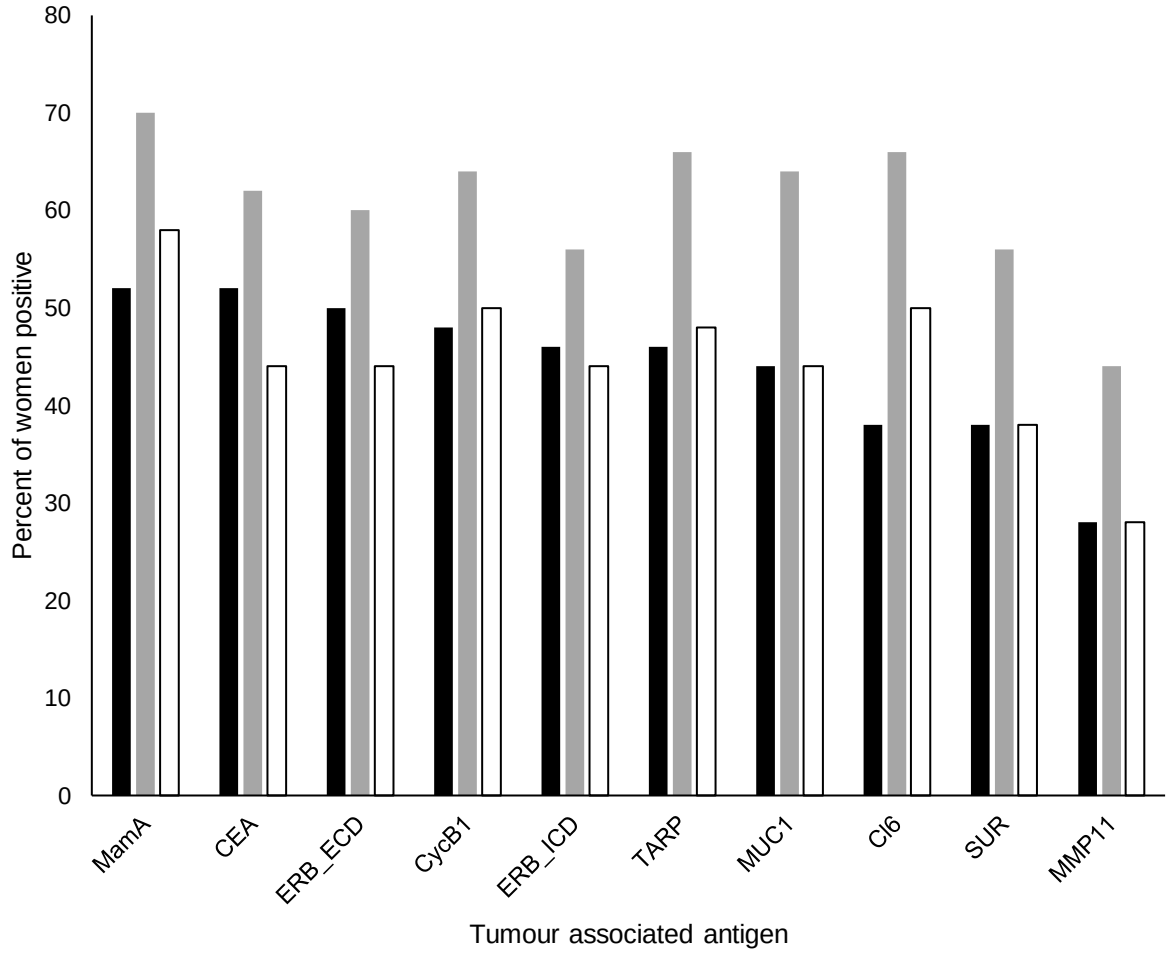

Figure 1. Percent of women positive to each individual tumour associated antigen as per 3 methods of defining positivity. ■ Anti-viral method for defining T-lymphocyte positivity ▒ >2 x negative control method for defining T-lymphocyte positivity □ >4 x negative control method for defining T-lymphocyte positivity.

N=50, MamA, mammaglobin-A. CEA, carcinoembryonic antigen. Cl6, claudin-6. CycB1, cyclin-B1. ERB_ECD, receptor tyrosine-protein kinase erbB-2 extracellular domain. ERB_ICD, receptor tyrosine-protein kinase erbB-2 intracellular domain. MMP1, stromelysin-3. TARP, TCRgamma alternate reading frame protein. MUC1, mucin-1. SUR, survivin

<u>Differences between responders and non-responders</u>

Using anti-viral serostatus to define a positive response, no significant differences were found between responders and non-responders surrounding body composition, fitness, physical activity levels and dietary intake other than bone mineral density being significant

higher ($F(1,48)=4.049$, $p=0.05$ and $F(1,48)=4.132$, $p=0.048$ respectively) in non-responders by around 10% and T-score by 351% (Table 2.). Regarding haematological measures platelet counts were significantly lower ($F(1,48) = 4.693$, $p=0.035$) in non-responders (mean of 181 vs 120 x 10_9/L respectively for responders and non-responders). Significant differences were found between groups when assessing specific IgG antibody response to VZV which was 56% lower in the non-responders vs responders (Table 3.). No significant difference were found between responders to tumour-associated antigens and non-responders to tumour-associated antigens in terms of anti-viral positivity and magnitude of response to EBV, CMV, VZV or FLU as assessed by ELISpot assay (Table 4.).

When using the 2 x negative control approach to define positivity, significant differences were found between responders and non-responders surrounding energy expenditure ($F(1,46)=5.539$, $p=0.023$) (Table 2.). No significant differences were found between positive and negative responders for other aspects of lifestyle or psychological measures. Regarding haematological measures no significant differences were seen (Table 3.). No significant difference were found between responders to tumour-associated antigens and non-responders to tumour-associated antigens in terms of anti-viral positivity and magnitude of response to CMV, VZV or FLU as assessed by ELISpot assay, but a significant difference was seen in those who were positive and negative in terms of ELISpot response to EBNA1 EBV ($F(1,46) = 29.064$, $p<0.01$ (Table 4.).

When using the 4x negative control approach significant differences were found in body composition in terms of visceral fat, with total visceral fat mass (2% higher in non-responders) ($F(1,48) = 57.828$, $p<0.01$), visceral outer wall middle fat mass ($F(1,48) = 7.864$, $p=0.007$), visceral fat cavity inner mass and percentage fat ($F(1,48) = 263.321$, $p<0.01$ and $F(1,48) = 286.57$, $p<0.01$ respectively). Other significant difference were found in android fat percentage ($F(1,48) = 89.708$, $p<0.01$), android to gynoid ratio ($F(1,48) = 195.971$, $p<0.01$) and T and Z scores being significantly different($F(1,48) = 24.992$, $p<0.01$ and $F(1,48) = 26.161$, $p<0.01$ respectively). In terms of physical activity, significant differences were found in IPAQ reported vigorous activity (183 minutes per week in responders vs 930 minutes per week in non-responders) ($F(1,48) = 18.226$, $p<0.01$), IPAQ reported moderate activity ($F,1,48) = 6.889$, $p=0.012$) and total MET minutes per week (839 in responders vs 2990 in non-responders) ($F(1,48) = 26.439$, $p<0.01$) (Table 2.). Significant differences were also seen in age ($F(1,48) = 4.830$, $p=0.033$ with age being 22% higher in those negative to tumour-associated antigens. Regarding haematological measures CMV IgG was significantly different between responders and non-responders (around 69%

higher in non-responders) ($F(1,21) = 5.956$, $p=0.024$) (Table 3.). No significant difference were found between responders to tumour associated antigens and non-responders to tumour associated antigens in terms of anti-viral positivity and magnitude of response to CMV, VZV or FLU as assessed by ELISpot assay but a significant difference was seen in those who were positive and negative in terms of ELISpot response to EBNA1 EBV ($F(1,46) =4.647$, p=036 (Table 4.).

9413 Table 2. Lifestyle characteristics of women who show positivity to at least one tumour-associated antigens and those negative to all tumour-
9414 associated antigens defined by 3 different methods

Characteristics	**Anti-viral serostatus**		**2 x negative control**		**4 x negative control**	
	Positive (n=43)	**Negative (n=7)**	**Positive (n=48)**	**Negative (n=2)**	**Positive (n=43)**	**Negative (n=7)**
Age (years)	43 ± 12	40 ± 12	42 ± 12	52 ± 15	43 ± 12	52 ± 17*
Height (m)	1.6 7± 0.08	1.64 ± 0.07	1.66± 0.07	1.70 ± 0.12	1.67± 0.07	1.60 ± 0.15
Weight (kg)	70.2 ± 16.5	63.5 ± 9.9	68.7 ± 15.4	72.4 ± 19.3	70.0 ± 16.0	61.9 ± 13.8
Predicted VO_2 max ($ml.kg.min^{-1}$)	36.3 ± 8.8	41.7 ± 33.9	37.8 ± 9.1	32.6 ± 6.1	37.2 ± 9.0	33.7 ± 1.0
Blood pressure (mmHg)	116/74	117/75	116/75	120/73	116/75	125/69
Body composition characteristics						
BMI ($kg.m_2$)	25.0 ± 5.1	23.7 ± 3.4	24.8 ± 5.1	24.8 ± 3.9	24.9 ± 5.0	24.1 ± 0.8
W:H	0.78 ± 0.04	0.79 ± 0.11	0.78 ± 0.06	0.78 ± 0.04	0.78 ± 0.06	0.77 ± 0.02
Body fat %	32 ± 8	31 ± 5	31.8 ± 7.4	31.6 ± 7.2	31.6 ± 7.4	36.2 ± 0.4
Fat mass (kg)	21.6 ± 8.3	22.8 ± 7.2	21.5 ± 7.9	23.1 ± 9.9	21.7 ± 8.2	22.2 ± 5.3
Fat mass index ($kg.m_2$)	7.81 ± 2.97	7.89 ± 2.03	7.81 ± 2.89	7.94 ± 2.68	7.79 ± 2.89	8.63 ± 0.41
BMD (g/cm_2)	1.15 ± 0.13	1.26 ± 0.12*	1.17 ± 0.12	1.15 ± 0.19	1.17 ± 0.13	1.14 ± 0.21
Lean mass (kg)	46.0 ± 7.6	58.3 ± 32.3	47.5 ± 14.5	48.5 ± 11.2	48.0 ± 14.1	39.0 ± 8.6
T-score	0.52 ± 1.54	1.79 ± 1.40*	0.74 ± 1.46	0.41 ± 2.27	0.71 ± 1.56	0.35 ± 2.62*
Z-score	0.56 ± 1.22	1.53 ± 1.19	0.70 ± 1.19	0.71 ± 1.69	0.70 ± 1.26	0.60 ± 1.56*
Breast fat (%)	29 ± 11	29 ± 11	28.7 ± 10.3	30.0 ± 12.0	28.5 ± 10.5	37.5 ± 1.9
Android mass (kg)	5.02 ± 1.77	4.13 ± 11.27	4.90 ± 1.71	4.89 ± 1.85	4.92 ± 1.74	4.19 ± 0.74
Android fat (%)	30 ± 10	30 ± 10	30.4 ± 10.2	30.0 ± 10.4	30.2 ± 10.2	41.6 ± 21.6*

	Anti-viral serostatus		2 x negative control		4 x negative control	
	Positive (n=43)	**Negative (n=7)**	**Positive (n=48)**	**Negative (n=2)**	**Positive (n=43)**	**Negative (n=7)**
Gynoid mass (kg)	8.3 ± 4.9	8.2 ± 5.1	8.75 ± 4.72	5.39 ± 5.34	8.36 ± 4.84	6.39 ± 8.0
Gynoid fat (%)	37 ± 7	35 ± 3	36.9 ± 6.8	37.4 ± 6.9	36.8 ± 6.8	41.5 ± 0.1
Visceral fat body mass (kg)	2.1 ± 0.9	2.0 ± 0.1	2.73 ± 8.41	1.54 ± 0.82	2.0 ± 0.85	1.61 ± 1.41
Visceral fat body fat (%)	32 ± 11	32 ± 10	32.3 ± 10.4	32.2 ± 10.7	32.0 ± 10.4	37.4 ± 6.0
Visceral fat outer wall middle mass (kg)	2.5 ± 0.6	2.3 ± 0.1	2.46 ± 0.58	2.51 ± 0.58	2.48 ± 0.59	2.13 ± 0.18*
Visceral fat outer wall middle fat (%)	30 ± 10	30 ± 9	29.8 ± 9.4	29.9 ± 10.4	29.6 ± 9.6	37.8 ± 5.2
Visceral fat cavity inner mass (kg)	2.0 ± 0	1.9 ± 0.5	1.99 ± 0.4	1.97 ± 0.4	2.0 ± 4.45	1.86 ± 6.47*
Visceral fat cavity inner fat (%)	27 ± 9	27 ± 8	27.1 ± 9.1	26.5 ± 10.5	26.8 ± 9.3	32.5 ± 4.0*
Visceral fat mass (g)	362 ± 232	347 ± 225	359.0 ± 218.4	366.8 ± 305.8	359.5 ± 233.5	372.6 ± 38.3*
Fat mass ratio Trunk (%):Limb (%)	0.760 ± 0.151	0.831 ± 0.275	0.773 ± 0.174	0.747 ± 0.161	0.76 ± 0.17	0.92 ± 0.06
Android:Gynoid ratio	0.798 ± 0.177	0.857 ± 0.233	0.810 ± 0.185	0.784 ± 0.186	0.80 ± 0.19	0.88 ± 0.17*
Physical Activity Levels						
<1.8 METS (minutes)	703 ± 114	733 ± 43	707 ± 102	704 ± 139	701 ± 105	838 ± 65
1.8><3 METS (minutes)	106 ± 58	72 ± 15	99 ± 52	115 ± 76	103 ± 55	52 ± 3
3><6 METS (minutes)	126 ± 68	119 ± 33	126 ± 64	118 ± 68	128 ± 63	54 ± 38
>6 METS (minutes)	26 ±23	36 ±16	28 ±22	23 ± 27	28 ±23	17 ± 24
>10.2 METS (minutes)	0 ± 1	1 ± 2	1 ± 2	0 ± 0	0 ± 1	0 ± 0
Energy expenditure (kcal/day)	2498 ± 421	2405 ±230	2474 ± 374	2551 ± 554*	2512 ± 385	1864 ± 89
PAL	1.74 ± 0.27	1.77 ± 0.15	1.74 ± 0.25	1.73 ± 0.30	1.75 ± 0.25	1.49 ± 0.19
Steps per day	10121 ± 3287	11688 ± 4672	10684 ± 3574	8922 ± 2790	10505 ± 3512	8630 ± 3776

	Anti-viral serostatus	2 x negative control	4 x negative control			
	Positive (n=43)	**Negative (n=7)**	**Positive (n=48)**	**Negative (n=2)**	**Positive (n=43)**	**Negative (n=7)**
Sedentary time (% of waking)	74 ± 11	78 ± 4	75 ± 10	75 ± 14	74 ± 10	88 ± 6
IPAQ Light MET (min per week)	620 ± 788	431 ± 334	511 ± 506	1171 ± 1498	628 ± 752	596 ± 856
IPAQ Moderate MET (min per week)	398 ± 611	274 ± 238	305 ± 333	889 ± 1251	395 ± 588	499 ± 724*
IPAQ Vigorous MET (min per week)	307 ± 531	141 ± 105	183 ± 187	930 ± 1096	290 ± 507	364 ± 629*
Total MET min per week	1325 ± 1599	847 ± 434	839 ± 760	2990 ± 1905	1313 ± 1526	1447 ± 1877*
Nutrition characteristics						
Energy Intake	1933 ± 482	1689 ± 530	1896 ± 450	1911 ± 739	1905 ± 497	1741 ± 378
Carbohydrate (% of total energy intake)	56 ± 10	56 ± 12	56 ± 10	55 ± 13	56 ± 10	63 ± 115
Protein (% of total energy intake)	22 ± 6	22 ± 5	22 ± 6	21 ± 6	22 ± 6	16 ± 1
Fat (% of total energy intake)	22 ± 6	22 ± 7	22 ± 6	24 ± 9	22 ± 6	20 ± 14
Sugars (g)	86 ± 53	73 ± 29	83 ± 45	90 ± 80	84 ± 51	80 ± 21
Saturated fat (g)	29 ± 11	33 ± 28	29 ± 15	32 ± 12	30 ± 14	29 ± 21

*N=50, * P<0.05 significantly different from positive responders*

W:H, waist to hip ratio. BMD, bone mineral density. PAL, physical activity level.

9419 Table 3. Haematological and anti-viral characteristics of women who show positivity to at least one tumour-associated antigens and those negative
9420 to all tumour-associated antigens

	Anti-viral serostatus		**2 x negative control**		**4 x negative control**	
Characteristic	**Positive (n=43)**	**Negative (n=7)**	**Positive (n=48)**	**Negative (n=2)**	**Positive (n=43)**	**Negative (n=7)**
Total Leukocytes (x 10^9/L)	5.3 ± 1.6	4.9 ± 0.6	5.2 ± 1.5	6.5 ± 2.3	5.2 ± 1.5	5.6 ± 1.7
Platelets (x 10^9/L)	182 ± 72	120 ± 50*	174 ± 74	156 ± 9	170 ± 777	195 ± 25
Lymphocyte (x 10^9/L)	1.6 ± 0.5	1.5 ± 0.4	1.6 ± 0.5	1.5 ± 0.6	1.6 ± 0.5	1.6 ± 0.5
Monocytes# (x 10^9/L)	0.4 ± 0.2	0.3 ± 0.1	0.4 ± 0.2	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.3
Neutrophil (x 10^9/L)	3.3 ± 1.3	3.1 ± 0.7	3.2 ± 1.2	4.4 ± 2.8	3.3 ± 1.2	3.5 ± 1.2
EBV positive (number, %)	41/43, 95%	7/7, 100%	46/48, 96%	2/2, 100%	41/43, 95%	7/7, 100%
EBV specific IgG (IU/ml)	436 ± 230	436 ± 256	578 ± 405	276 ± 51	530 ± 401	406 ± 388
CMV positive (number, %)	19/43, 44%	1/7, 14%	23/48, 44%	0/2, 0%	21/43, 44%	2/7, 57%
CMV specific IgG (IU/ml)	14 ± 7	13 ± 5	13.09 ± 7.70	n/a	12.0 ± 7.05	24.6 ± 4.6*
VZV positive (number, %)	41/43, 95%	7/7, 100%	46/48, 96%	2/2, 100%	41/43, 95%	7/7, 100%
VZV specific IgG (IU/ml)	1558 ± 951	687 ± 542*	1467 ± 985	429 ± 234	1431± 973	1962 ± 1005
RANTES (ng/ml)	11.28 ± 6.04	11.77 ± 7.56	11.35 ± 6.31	11.22 ± 1.80	11.46 ± 6.51	10.64 ± 3.87
Resistin (ng/ml)	0.99 ± 0.51	0.90 ± 0.25	0.98 ± 0.49	0.80 ± 0.13	0.99 ± 0.50	0.86 ± 0.37
Leptin (ng/ml)	15.29 ± 14.58	9.76 ± 6.03	13.71 ± 12.72	33.70 ± 31.05	14.08 ± 13.0	17.18 ± 19.05
Osteopontin (ng/ml)	25.19 ± 8.88	21.81 ± 7.49	24.37 ± 8.78	16.39 ± 2.47	24.42 ± 9.18	21.77 ± 5.35

9421 *N=50, * P<0.05, #Monocytes, eosinophils and basophils, however eosinophils and basophils only make up a small fraction.*
9422 WBC, white blood cell. MXD, mixed cells (including monocytes, basophils and eosinophils). EBV, Epstein Barr Virus. EBNA1, Epstein Barr
9423 nuclear antigen 1. BZLF1, BamHIZ leftward reading frame 1. MP1, matrix protein1. NP, nucleoprotein. CMV, Cytomegalovirus. pp65,
9424 phosphoprotein 65. IE1, immediate-early protein 1. VZV, Varicella Zoster Virus. IE63, immediate-early protein 63. gE, glycoprotein E.

Table 4. Average specific T-lymphocyte responses against Flu, EBV, CMV and VZV viral antigens per 3 different methods of defining positivity to tumour-associated antigens

Viral associated antigens	**Anti-viral serostatus**		**2 x negative control**		**4 x negative control**	
	Positive (n=43)	**Negative (n=7)**	**Positive (n=48)**	**Negative (n=2)**	**Positive (n=43)**	**Negative (n=7)**
FLU MP1	6 ± 8	5 ± 3	6 ± 7	8 ± 1	6 ± 8	7 ± 5
FLU NP	21 ± 61	6 ± 7	16 ± 54	85 ± 111	17 ± 57	29 ± 59
EBV EBNA1	16 ± 28	20 ± 14	13 ± 15	95 ± 104*	13 ± 15	36 ± 59*
EBV BZLF1	25 ± 63	86 ± 144	33 ± 89	75 ± 99	31 ± 82	54 ± 63
CMV pp65	148 ± 134	181	174 ± 154	n/a	158 ± 147	337 ± 189
CMV IE1	126 ± 135	58	118 ± 132	n/a	105 ± 129	256 ± 85
VZV IE63	4 ± 5	4 ± 4	4 ± 5	4 ± 3	4 ± 5	3 ± 2
VZV gE	4 ± 5	6 ± 7	4 ± 5	5 ± 3	5 ± 6	4 ± 2

*Data shown as means ± standard deviation number of spot forming units per 250,000 peripheral blood mononuclear cells. EBV, Epstein Barr Virus. EBNA1, Epstein Barr nuclear antigen 1. BZLF1, BamHIZ leftward reading frame 1. MP1, matrix protein1. NP, nucleoprotein. CMV, Cytomegalovirus. pp65, phosphoprotein 65. ie1, immediate-early protein 1. VZV, Varicella Zoster Virus. ie63, immediate-early protein 63. gE, glycoprotein E. Only those who are defined as positive to individual viruses by ELISA were included in the analysis. *Significant difference from positive ($p<0.05$)*

Table 5. Average specific T-lymphocyte responses against tumour-associated antigens per 3 different methods of defining positivity to tumour-associated antigens

	Anti-viral serostatus	**2 x negative control**	**4 x negative control**
Tumour-associated antigen			
MamA	4 ± 4	4 ± 4	4 ± 4
CEA	5 ± 10	4 ± 9	5 ± 11
Cl6	3 ± 3	3 ± 3	3 ± 3
CycB1	4 ± 8	4 ± 7	4 ± 7
ERB_ECD	5 ± 6	4 ± 6	4 ± 6
ERC_ICD	5 ± 8	4 ± 7	4 ± 8
MMP11	2 ± 3	2 ± 3	1 ± 1
TARP	4 ± 6	3 ± 5	3 ± 5
MUC1	5 ± 8	4 ± 7	4 ± 7
SUR	5 ± 9	4 ± 8	4 ± 10

Data shown as means ± standard deviation number of spot forming units per 250,000 peripheral blood mononuclear cells. MamA, mammaglobin-A. CEA, carcinoembryonic antigen. Cl6, claudin-6. CycB1, cyclin-B1. ERB_ECD, receptor tyrosine-protein kinase erbB-2 extracellular domain. ERB_ICD, receptor tyrosine-protein kinase erbB-2 intracellular domain. MMP1, stromelysin-3. TARP, TCRgamma alternate reading frame protein. MUC1, mucin-1. SUR, survivin. Only those who are defined as positive to individual antigens were included in the analysis.

APPENDIX 2

Tanita scales measured body fat percentage values measured through bioelectrical impedance were obtained for 48/50 participants. No significant differences were found between body fat percentage values measured by DEXA and by Tanita scales $t(47)=-0.369$, $p=0.714$. Furthermore, a significant correlation was observed between DEXA and Tanita measured body fat percentage ($p=0.001$), $r=470$, $r_2=0.221$. The mean differences and limits of agreements between bioelectrical impedance and DEXA from the Bland-Altman plots for body fat percentage were 0.4 ± 5.9%, lower limit of agreement −11.1% upper limit of agreement 11.9%. (Figure 1.).

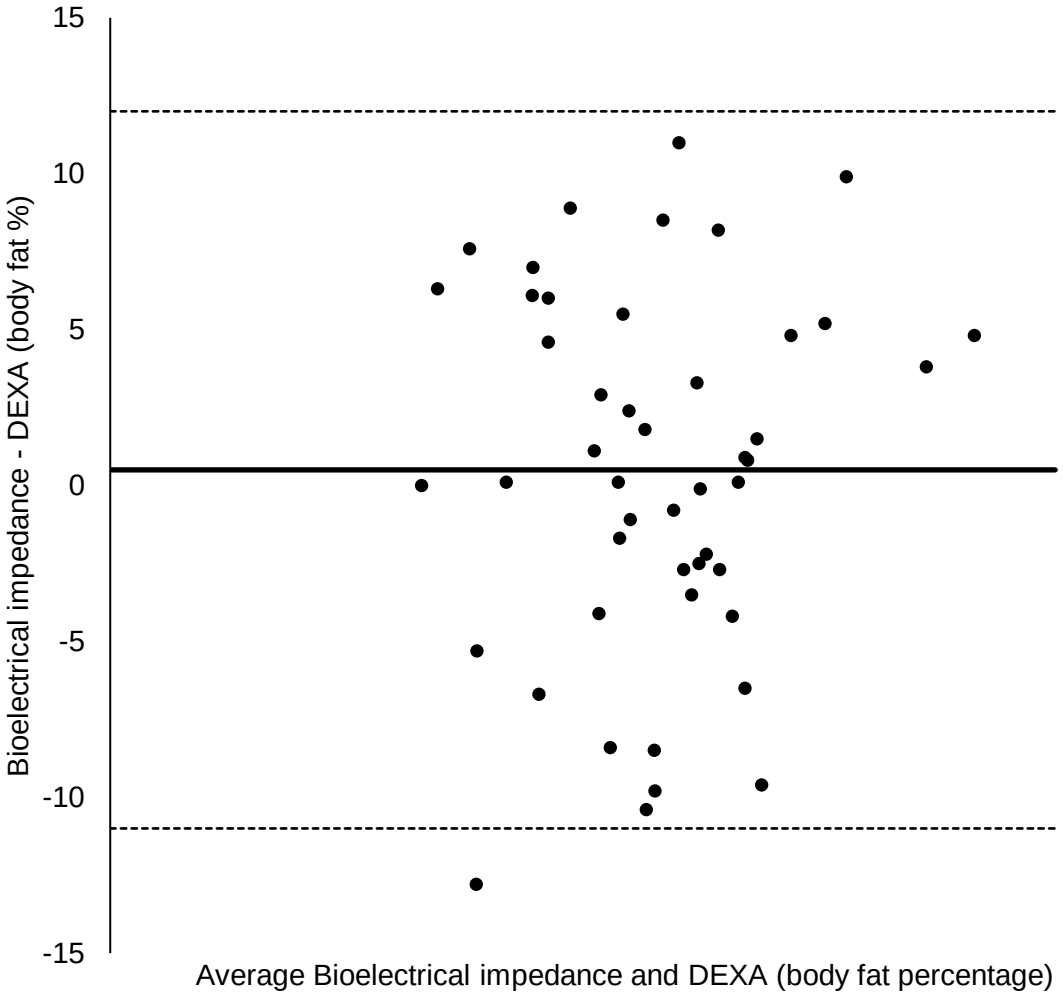

Figure 1. Bland-Altman plot between two measures of body fat percentage; bioelectrical impedance and DEXA. Data shown for individuals at all time points.

DEXA; dual energy x-ray absorptiometry. N=50

APPENDIX 3

Table 1. demonstrates that there are no differences in significance when parametric and non-parametric tests are used when analysing cell count data.

Table 1. Friedman's ANOVA and repeated measures ANOVA statistics from cell count data

	Friedman's ANOVA	**Repeated measures ANOVA**
White blood cells	$\chi^2(2) = 1.130$, p = 0.568	*F*(2.000, 10.000) = 1.850, P = 0.207
Reb blood cells	$\chi^2(2) = 3.391$, p = 0.183	*F*(1.465, 7.327) = 4.432, P = 0.062
Haemaglobin	$\chi^2(2) = 6.000$, p = 0.050	*F*(2.000, 10.000) = 6.690, P = 0.014
	Pre (Mdn = 129g/L) versus post (Mdn = 115g/L), T = 20.000, p = 0.046	Pre versus post, *F*(10.208), p = 0.024
	Pre (Mdn = 129g/L) versus mid (Mdn = 118g/L), T = 0.000, p = 0.043	Pre versus mid *F*(12.514), p = 0.017
Platelets	$\chi^2(2) = 1.652$, p = 0.438	*F*(2.000, 10.000) = 0.137, P = 0.874
Lymphocytes	$\chi^2(2) = 0.087$, p = 0.957	*F*(1.246, 6.230) = 0.434, P = 0.576
Neutrophils	$\chi^2(2) = 0.400$, p = 0.819	*F*(1.450, 7.251) = 1.779, P = 0.218
NLR	$\chi^2(2) = 2.348$, p = 0.309	*F*(1.450, 7.251) = 0.015, P = 0.960
PLR	$\chi^2(2) = 1.130$, p=0.568	*F*(1.449, 7.243) = 1.215, P = 0.331

Mdn; median. NLR; neutrophil lymphocyte ration. PLR; platelet lymphocyte ratio.

APPENDIX 4

The tables herein demonstrate no significant differences between the remotely monitored and partly supervised groups, supporting non-inferiority of the remotely monitored group.

Table 1. Differences in change of physical activity pre- and post-intervention in both intervention groups.

	Prescribed, partly supervised group (n=15)			**Remote, advisory group (n=15)**			
	Change pre-post (mean ± SD)	**Lower bound 95% CI**	**Upper bound 95% CI**	**Change pre-post (mean ± SD)**	**Lower bound 95% CI**	**Upper bound 95% CI**	**ANOVA statistic**
Minutes of moderate exercise per week	−17 ± 107	−76	42	5 ± 48	−20	29	F(1,28)=0.529, p=0.473
Minutes of vigorous exercise per week	27 ± 49	0	54	3 ± 44	−26	71	F(1,28)=0.030, p=0864
Light activity (MET.min^{-1} per week)	−535 ± 1506	−1359	309	−961 ± 2082	−2038	106	F(1,28)=0.485, p=0.492
Moderate activity (MET.min^{-1} per week)	740 ± 3700	−1309	2789	−720 ± 2151	−1737	487	F(1,28)=1.578, p=0.219
Vigorous activity (MET.min^{-1} per week)	388 ± 1173	−262	1038	−211 ± 1659	−864	1075	F(1,28)=0.270, p=0.607
Total activity (MET.min^{-1} per week)	602 ± 4297	−1776	2983	−1892 ± 3472	−3369	395	F(1,28)=2.180, p=0.151

CI; confidence interval. SD; standard deviation. METs; metabolic equivalents. min; minutes

9479 Table 2. Differences in change of physiological characteristics pre- and post-intervention in both intervention groups.

	Prescribed, partly supervised group (n=15)			**Remote, advisory group (n=15)**			**ANOVA statistic**
	Change pre-post (mean ± SD)	**Lower bound 95% CI**	**Upper bound 95% CI**	**Change pre-post (mean ± SD)**	**Lower bound 95% CI**	**Upper bound 95% CI**	
Age	0 ± 0	0	0	0 ± 1	0	0	F(1,28)=0.509, p=0.484
Systolic blood pressure (mmHg)	−9 ± 10	−14	−4	−5 ± 11	−11	2	F(1,28)=1.341, p=0.257
Diastolic blood pressure (mmHg)	−2 ± 8	−6	3	−1 ± 6	−5	2	F(1,28)=0.042, p=0.840

9480 *CI; confidence interval. SD; standard deviation. mmHg; millimoles of mercury.*

9481

9482 Table 3. Differences in change of body composition pre- and post-intervention in both intervention groups.

	Prescribed, partly supervised group (n=15)			Remote, advisory group (n=15)			ANOVA statistic
	Change pre-post (mean ± SD)	**Lower bound 95% CI**	**Upper bound 95% CI**	**Change pre-post (mean ± SD)**	**Lower bound 95% CI**	**Upper bound 95% CI**	
BMI (kg.m$_2$)	0.1 ± 0.9	−0.4	0.6	0.0 ± 0.6	−0.3	0.4	F(1,28)=0.100, p=0.754
W:H	−0.01 ± 0.04	−0.02	0.01	0.04 ± 0.15	−0.02	0.01	F(1,28)=1.324, p=0.260
DEXA measured body fat (%)	0.0 ± 1.0	−0.2	0.0	0.6 ± 5.1	−1.8	1.1	F(1,28)=0.639, p=0.431
Fat mass (kg)	0.3 ± 1.2	−0.4	1.0	−0.1± 2.2	−1.3	1.1	F(1,28)=0.438, p=0.513
Lean mass (kg)	0.2 ± 1.4	−0.4	0.8	0.3 ± 1.0			F(1,28)=0.037, p=0.850
BMD (g.cm$_2$)	−0.016 ± 0.044	0.000	0.000	−0.009 ± 0.035	−0.028	0.011	F(1,28)=0.945, p=0339
T score	−0.2 ± 0.5	−0.4	0.3	−0.1 ± 0.5	−0.3	0.2	F(1,28)=0.000, p=1.000
Z score	−0.1 ± 0.5	−0.2	0.3	−0.1 ± 0.4	−0.4	0.3	F(1,28)=2.800, p=0.105

9483 *CI; confidence interval. SD; standard deviation. BMI; body mass index. kg; kilogram. m; metres. W:H; waist to hip ratio. DEXA; dual energy x-ray*

9484 *absorptiometry. BMD; bone mineral density. g; grams. cm; centimetres.*

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9486

9487 Table 4. Differences in change of fitness pre- and post-intervention in both intervention groups.

	Prescribed, partly supervised group (n=15)			Remote, advisory group (n=15)			ANOVA statistic
	Change pre-post (mean ± SD)	**Lower bound 95% CI**	**Upper bound 95% CI**	**Change pre-post (mean ± SD)**	**Lower bound 95% CI**	**Upper bound 95% CI**	
$\dot{V}O_2$max (ml.kg.min^{-1})	0.3 ± 3.5	−1.6	2.3	−0.7 ± 2.2	−2.0	0.5	F(1,28)=1.036, p=0.318
Six-minute walk (m)	43 ± 37	22	63	16 ± 36	−4	36	F(1,28)=4.073, p=0.053
Sit to stand	3 ± 4	1	5	2 ± 3	0	4	F(1,28)=0.322, p=0.575
Get up and go (seconds)	−0.2 ± 0.8	−0.7	0.2	−0.4 ± 0.8	−-0.9	0	F(,128)=0.392, p=0.536

9488 *CI; confidence interval. SD; standard deviation. $\dot{V}O_2$max; maximum oxygen uptake. ml; millilitres. kg; kilogram. min; minutes. m; metres.*

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Table 5. Differences in change of nutritional intake pre- and post-intervention in both intervention groups.

	Prescribed, partly supervised group (n=13)			Remote, advisory group (n=14)			ANOVA statistic
	Change pre-post (mean ± SD)	**Lower bound 95% CI**	**Upper bound 95% CI**	**Change pre-post (mean ± SD)**	**Lower bound 95% CI**	**Upper bound 95% CI**	
Energy Intake (kcal per day)	−78 ± 431	−228	183	192 ± 324	−21	373	F(1,25)=2.898, p=0.101
Carbohydrate intake (% per day)	0 ± 4	−2	3	−1 ± 7	−5	2	F(1,25)=0.661, p=0.424
Protein intake (% per day)	−8 ± 21	−2	3	12 ± 42	−4	3	F(1,25)=0.295, p=0.592
Fat intake (% per day)	1 ± 4	−2	1	0 ± 6	0	4	F(1,25)=6.679, p=0.016
Sugar (g per day)	−1 ± 3	−21	5	2 ± 3	−15	34	F(1,25)=1.757, p=0.197
Saturated fat (g per day)	−4 ± 11	−10	4	3 ± 8	−2	8	F(,125)=2.793, p=0.107

CI; confidence interval. SD; standard deviation. g; grams. kcal; kilocalorie

9495 Table 6. Differences in cell counts pre- and post-intervention in both intervention groups.

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	Prescribed, Partly Supervised group (n=15)			Remote, advisory group (n=15)			ANOVA statistic
	Change pre-post (mean ± SD)	**Lower bound 95% CI**	**Upper bound 95% CI**	**Change pre-post (mean ± SD)**	**Lower bound 95% CI**	**Upper bound 95% CI**	
Total leukocytes (x 10^9/L)	−0.3 ± 1.4	−0.8	0.6	0.0 ± 1.0	−0.5	0.6	F(1,27)=0.687, p=0.415
Red blood cells (x 10^9/L)	−0.1 ± 0.3	−0.2	0.1	0.1 ± 0.3	−0.1	0.3	F(1,27)=3.461, p=0.074
Haemaglobin (x 10^9/L)	0.1 ± 0.7	−0.3	0.6	0.4 ± 1.1	−0.2	1.0	F(1,25)=0.704, p=0.409
Platelets (x 10^9/L)	−2.8 ± 49	−26	29	−0.5 ± 58	−33	32	F(1,25)=0.013, p=0.909
Lymphocytes (x 10^9/L)	0.0 ± 0.3	−0.1	0.2	−0.1 ± 0.2	−0.2	0.0	F(1,25)=1.165, p=0.290
MXD (x 10^9/L)#	0.1 ± 0.2	0	0.2	0.0 ± 0.2	−0.1	0.1	F(1,25)=0.616, p=0.439
Neutrophils (x 10^9/L)	−0.4 ± 1.1	−0.8	0.3	0.1 ± 0.9	−0.4	0.6	F(1,25)=2.049, p=0.164

9497

9498 *CI; confidence interval. SD; standard deviation. L; litre*

9499 #*Monocytes, eosinophils and basophils, however eosinophils and basophils only make up a small fraction (0.1-1.6 X 10^9/L according to*

9500 *manufacturers guidelines,* Sysmex, KX-21N, Kobe, Japan*).*

9501

Table 7. Differences in cell counts pre- and post-intervention in both intervention groups.

	Prescribed, Partly Supervised group (n=15)			Remote, advisory group (n=16)			ANOVA statistic
	Change pre-post (mean ± SD)	**Lower bound 95% CI**	**Upper bound 95% CI**	**Change pre-post (mean ± SD)**	**Lower bound 95% CI**	**Upper bound 95% CI**	
Cholesterol (mmol/L)	−0.04 ± 0.52	−0.34	0.26	−0.30 ± 0.59	−0.64	0.03	F(1,27)=1.574, p=0.220
TAG (mmol/L)	−0.05 ± 0.23	−0.09	0.19	−0.07 ± 0.24	−0.20	0.06	F(1,27)=1.876, p=0.182
Glycerol (mmol/L)	−0.01 ± 0.02	−0.02	0.01	−0.05 ± 0.02	−0.02	0.01	F(1,27)=0.032, p=0.860
Glycerol blanked TAG (mmol/L)	−0.07 ± 24	−0.08	0.18	−0.07 ± 24	−0.10	0.08	F(1,27)=1.797, p=0.191
CRP (mg/L)	0.23 ± 2.86	−1.41	1.88	0.64 ± 2.44	−0.71	1.99	F(1,27)=0.172, p=0.682
Glucose (mmol/L)	0.13 ± 0.76	−0.31	0.58	0.01 ± 0.33	−0.17	0.20	F(1,27)=0.330, p=0.570
NEFA (mmol/L)	−0.06 ± 0.23	−0.15	0.05	−0.07 ± 0.14	−0.15	0.01	F(1,27)=0.081, p=0.778
ESR ($mm.h^{-1}$)	−1 ± 4	−3	1	−1 ± 3	−2	1	F(1.27)=0.095, p=0.761

CI; confidence interval. SD; standard deviation. mmol; millimole. L; litre. TAG; triglycerides. CRP; C-reactive protein. Mg; milligram. NEFA; non-esterified fatty acids. ESR; erythrocyte sedimentation rate. mm; millimetres. h; hour.