

Frailty, sarcopenia and immunesenescence: shared mechanisms and clinical insights

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Abstract

Frailty, the increased vulnerability of an individual to stressors, and sarcopenia, the loss of muscle mass with age, share many of the same clinical outcomes, associations and suggested pathophysiology. The pathophysiology of both conditions is incompletely characterised but it is postulated the immune system is central to development and propagation. Previous work has demonstrated dysregulation in the function of neutrophils with an individual's increasing age and the PI3kinase signalling pathway is implicated in this process. This study aimed to describe neutrophil function and inflammation in a group of frail older adults and correlate parameters of frailty with immunesenescence.

40 healthy young, 40 healthy older, and 37 frail older adults were recruited to three groups; all individuals were extensively characterised clinically. A further 73 healthy young adults were recruited for ultrasound assessment of muscle.

Ultrasound was reviewed as a diagnostic technique in the identification of sarcopenia using a simple scanning protocol to produce the bilateral anterior thigh thickness (BATT). The BATT was measured in a reference population, 113 in total, and proposed criteria for the identification of low muscle mass in older adults was based on this reference population. Ultrasound echogenicity correlated with muscle strength in a frail older adult population.

Muscle biopsy using a percutaneous needle biopsy technique was found to be not feasible in this study's recruited frail older adult population. This was secondary to both contraindications to muscle biopsy, such as anticoagulation and muscle depth.

Neutrophils exhibit a frailty related decline in migratory accuracy towards chemoattractants; this was both independent of age and associated with physical (grip strength) and cognitive parameters (Stroop Interference) of frailty. Incubation of neutrophils from frail older adults with PI3kinase inhibitors class 1A δ and class 1B γ restored migratory accuracy and this presents a novel therapeutic target for management of frailty.

For Mum and Dad, who nurtured my inner scientist,

And, for Mark, who did the washing up for five months.

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Abbreviations

ACEi	Angiotensin converting enzyme inhibitor
ACE-III	Addenbrooke's cognitive examination III
ADL	Activities of daily living
ALM	Appendicular lean muscle mass
AP	Autologous plasma
BAD	BCL-2 antagonist of cell death
BATT	Bilateral anterior thigh thickness
BCA	Bicinchoninic acid
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BSA	Bovine serum albumin
CFS	Clinical frailty scale
CGA	Comprehensive geriatric assessment
CI	Confidence intervals
COPD	Chronic obstructive pulmonary disease
CRF	Case report form
CRP	C-reactive protein
cs	Comparative scale
CT	Computed tomography
CXCL-8	Interleukin 8
CXCR-1	Interleukin 8 receptor one
CXCR-2	Interleukin 8 receptor two
CXCR-4	Interleukin 8 receptor four
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DHR	Dihydrorhodamine

DXA	Dual-energy x-ray absorptiometry
eFI	Electronic frailty index
EFS	Edmonton frail scale
ELISA	Enzyme linked immunosorbent assay
EWGSOP	European working group on sarcopenia in older people
F	Female
FDA	Food and drug administration
FGF	Fibroblast growth protein
FI	Frailty index
fMLP	N-formylmethionine-leucyl-phenylalanine
FO	Frail older adults
FoxO	Forked box proteins
FP	Frailty phenotype
G-CSF	Granulocyte colony stimulating factor
GH	Growth hormone
GM-CSF	Granulocyte macrophage colony stimulating factor
GPCR	Heterotrimeric G protein coupled receptor
GPS	Glutamine-Penicillin-Streptomycin
GTP	Guanosine triphosphate
HMGcoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HO	Healthy older adults
hs-CRP	High sensitivity C-reactive protein
HY	Healthy young adults
IADL	Activities of independent daily living
ICC	Intra-class correlation coefficient
IFN- γ	Interferon gamma
IGF-1	Insulin like growth factor one

I K-W	Independent Kruskal-Wallis
IL-1ra	Interleukin 1 receptor antagonist
IL-1 β	Interleukin 1 beta
IL-2	Interleukin two
IL-4	Interleukin four
IL-5	Interleukin five
IL-6	Interleukin six
IL-7	Interleukin seven
IL-8	Interleukin eight
IL-9	Interleukin nine
IL-10	Interleukin ten
IL-12	Interleukin twelve
IL-13	Interleukin thirteen
IL-15	Interleukin fifteen
IL-17	Interleukin seventeen
IP-10	Interferon gamma inducible protein
IQR	Interquartile range
IRS	Insulin receptor substrate
IWGS	International working group on sarcopenia
LFA-1	Lymphocyte function associated antigen one
M	Male
MCP-10	Monocyte chemoattractant protein ten
MFI	Median fluorescence intensity
MIP1 α	Macrophage inflammatory protein alpha
MIP1 β	Macrophage inflammatory protein beta
MMSE	Mini mental state examination
MNA-sf	Mini nutritional assessment – short form

MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
MUAC	Middle upper arm circumference
MuRF-1	Muscle RING finger protein
MW	Mann Whitney
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NF- κ B	Transcription factor nuclear factor kappa light chain enhancer of activated B cells
nNOS	Neuronal nitric oxide synthase
PAF	Platelet activation factor
PBS	Phosphate buffer solution
PBMCs	Peripheral blood mononuclear cells
PDGF	Platelet derived growth factor
PI3K/PI3kinase	Phosphoinositide 3-kinase
PIP3	Phosphoinositide 3,4,5-triphosphate
PP	Pooled plasma
PSGL-1	P selectin glycoprotein 1
PTEN	Phosphate and tensin homologue
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SHIP	SH2 domain containing inositol phosphatase 1
SPPB	Short physical performance battery
Thr	Threonine
TNF α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor

1. Introduction

1.1 Introduction

Research into healthy ageing is increasingly recognised as vitally important. Life expectancy is increasing and it is estimated that in the UK by 2030 men aged 65 will live on average to 88 and women to 91 (1). A rising life expectancy coupled with falling birth rates increases the proportion of older people in the population as a whole. By 2050, the World Health Organisation predicts that there will be two billion people aged over 60, and 395 million people living to be 80 (2).

The concept of healthy ageing is an emerging topic and is based on healthy life expectancy: the average number of years that a person can expect to live in full health by taking into account years lived in less than full health due to disease and/or injury (2). Healthy life expectancy is therefore lower than actual life expectancy. Healthy life expectancy at birth in England is currently 63.4 for men and 64.1 for women compared to a life expectancy of 79.5 for men and 83.2 and women (3). Men can therefore expect to live 16 years and women 19 years in poor health. Whilst both life expectancy and healthy life expectancy have increased the difference between the two has not diminished; time spent living in poor health has not improved with advances in medicine.

Frailty is a term that has been used to describe some of the components of unhealthy ageing.

1.2 Frailty

1.2.1 Definition

The concept of frailty has been recognised for many years but its emerging importance as a hallmark of ageing has led to a more rigorous medical definition of physical frailty.

‘A medical syndrome with multiple causes and contributors that is characterized by diminished strength, endurance, and reduced physiologic function that increases an individual’s vulnerability for developing increased dependency and/or death’ (4).

The definition recognises that frailty increases an individual’s risk for an adverse outcome such as falls, infections or death. It also alludes to frail individuals living closer to the threshold of an adverse outcome due to their reduced physiological reserve.

1.2.2 Models of frailty

The definition describes the syndrome but does not explain or interpret the condition and therefore two intrinsically different models of frailty have been proposed in an attempt to operationalise the syndrome.

The first model, the Frailty Phenotype model, was proposed by Fried and colleagues in 2001 (5). This proposes that a step-wise increase in self-reported disability is analogous with an increasing frailty state and correlates with adverse health outcomes such as death, hospitalisation and falls (5). Fried demonstrates that the presence of 3 components, out of a total of 5, has predictive power in identifying mortality risk. She extrapolates this to suggest that the presence of 3 components in a person identifies them as frail and the presence of 1 or 2 components as pre-frail (5). The model has been thoroughly validated by Fried and independent groups against poor outcomes but no

attempt has been made to assess the reliability of the phenotype (6). The variations in the measurement of the different components within each study leads to some ambiguity on the accuracy of the validation studies. The concurrent or predictive validity has been assessed in 17 different samples or cohorts (6).

Conversely Rockwood and colleagues suggested that frailty was not a present or absent phenomenon but best described as a dynamic, continuous process. He proposed the Frailty Index in 2001 (7). The most striking difference was that any variable could be included as long as it was associated with adverse health outcomes, increased in prevalence with age into the tenth decade and had a prevalence of at least 1% which did not saturate in older age (7). Using this model he proved there was a greater correlation of time to death with frailty index rather than age (7). The frailty index has also been widely tested for validity against poor outcomes but not for reliability (6). The concurrent or predictive validity has been assessed in 13 different samples or cohorts (6).

1.2.3 Complementary but different models

The Frailty Phenotype, as suggested by the name, is a model of physical parameters including only physical consequences. The Frailty Index can be considered to be a more global assessment of health and using this model frailty is often described as an accumulation of deficits rather than a present or absent phenomenon.

One of the difficulties in comparing the models and ultimately deciding upon the most elegant and clinically applicable model is that each model describes a slightly different population. The frailty index has better discriminatory ability for people with moderate and severe frailty (8). This is probably due to its broader approach to the diagnosis of

frailty and the inclusion of cognitive and psychosocial markers rather than solely relying on physical markers. There are also higher rates of frailty reported with the frailty index, likely due to the continuous nature of the model (9). This may make it a better objective marker of deficit accumulation and in the assessment of the efficacy of an intervention. The frailty phenotype may have utility in identifying older adults at risk of disability. It has been suggested that these assessment instruments should not be considered as alternatives, but rather as complementary. However, a recent systematic review of frailty interventions in community dwelling older adults found that none of the fourteen studies included used the Frailty Index to identify frailty (10). This suggests researchers are not utilising the models appropriately perhaps because of a perceived difficulty with utilising the Frailty Index in practice.

1.3 Sarcopenia

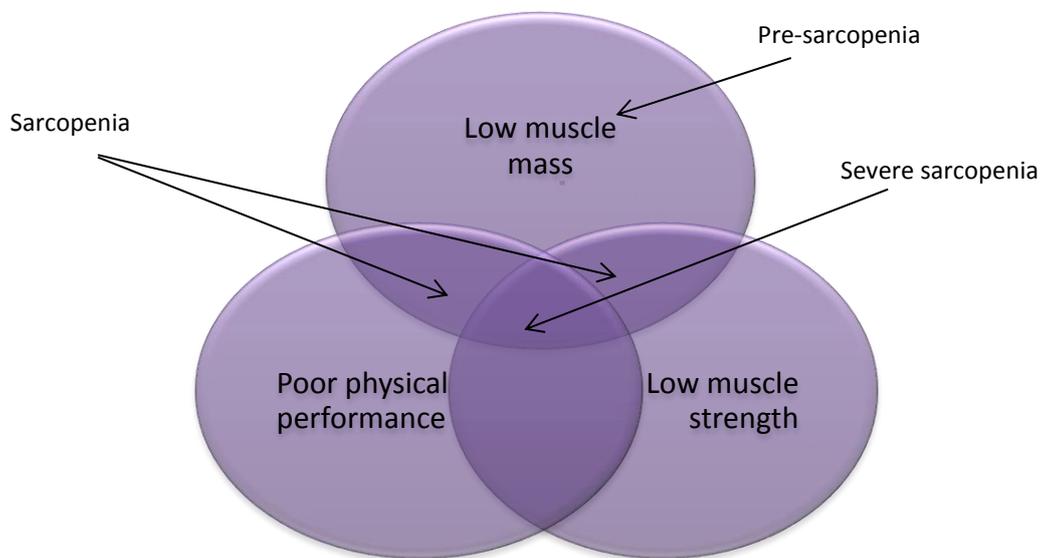
Sarcopenia, the syndrome of loss of muscle mass and strength, is considered by many as the physical manifestations of frailty or perhaps even a pre-cursor state if it is present without frailty. Definitions of sarcopenia have evolved over time reflecting greater understanding of the condition. The current broadly accepted definition includes the effects on function as well as including muscle mass and strength.

‘A syndrome characterised by progressive and generalised loss of skeletal muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life and death.’(11)

Similar to the frailty phenotype the European Working Group on Sarcopenia in Older People (EWGSOP) recommend categorising sarcopenia into pre-sarcopenia, sarcopenia,

and severe sarcopenia depending on the presence of certain criteria. They suggest that pre-sarcopenia is characterised by low muscle mass with no impact on muscle strength or physical performance (11). Whereas sarcopenia is low muscle mass with either low muscle strength or low physical performance and severe sarcopenia is the presence of all three criteria (11).

Figure 1.1 – The categorisation of sarcopenia



The categorisation of sarcopenia by severity: pre-sarcopenia, sarcopenia and severe sarcopenia. Pre-sarcopenia is characterised by low muscle mass with no impact on muscle strength or physical performance. Sarcopenia is low muscle mass with either low muscle strength or low physical performance and severe sarcopenia is the presence of all three criteria.

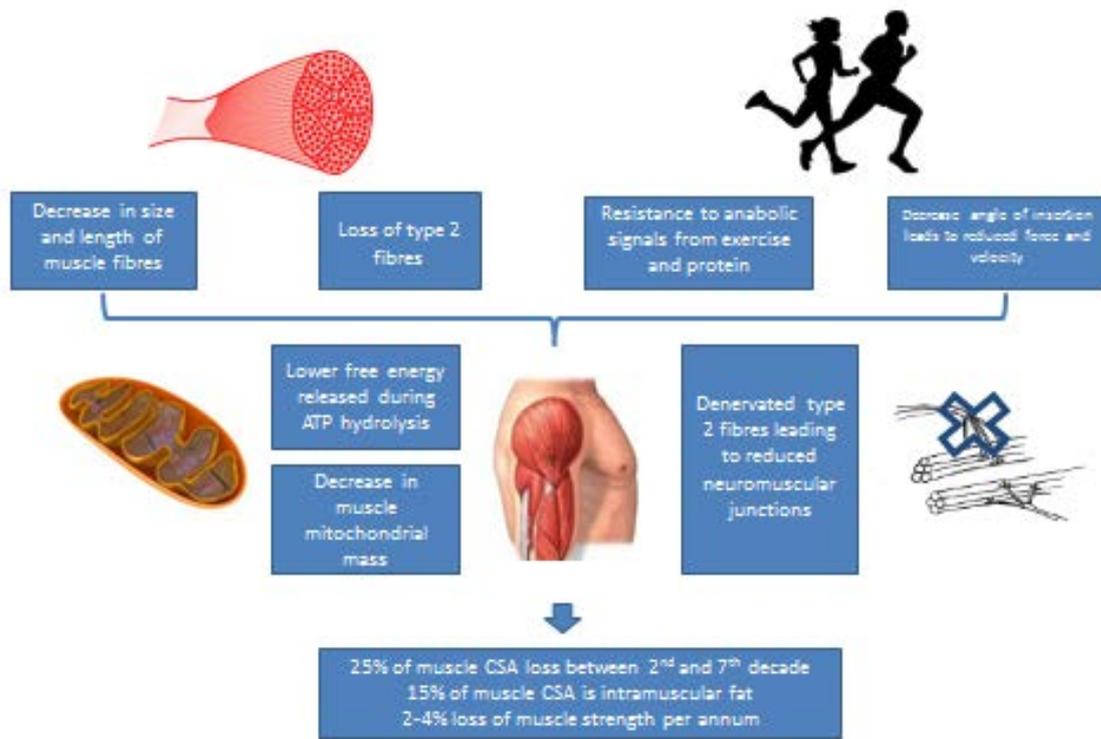
The inclusion of both muscle mass and strength within the definition of sarcopenia has partially occurred in response to extensive research which has shown that whilst loss of muscle mass is associated with loss of muscle strength, the relationship is not linear; the

decline in strength is more rapid than the concomitant loss of muscle mass. Goodpaster *et al* demonstrated in a community dwelling population aged between 70 and 79 that muscle mass declines at 0.5-2% per annum compared to 2-4% loss of muscle strength (12). Thus age-related changes in the quality of the muscle may be as important as the reduced mass and reduced muscle quality, notably loss of type II fibres, reduced mitochondrial mass and increased fat infiltration, may contribute to loss of muscle strength and power (13).

However, ambiguity in the literature regarding definitions of strength and power may account for the non-linear relationship between the loss of muscle mass and strength. Isometric strength is the force or torque produced by a muscle at a constant length contraction; there is no change in the muscle fibre length and no movement of the joint occurs. This correlates well with muscle mass. Alternatively, power is dynamic, force during movement, and is affected by not only muscle mass but other neuromuscular components such as denervated type 2 muscle fibres. These terms are often used interchangeably and therefore data should be interpreted with reference to the original methods.

Figure 2 demonstrates a pictorial depiction of the potential contributors to sarcopenia. Potentially a more inclusive definition of sarcopenia may incorporate the loss of muscle mass, reduced muscle quality and a loss of functional strength.

Figure 1.2 - Age related changes occurring within the muscle from a whole muscle level through to a cellular level and their impact on muscle mass, quality, strength and power



Age related changes occurring within the muscle from a whole muscle level through to a cellular level and their impact on muscle mass, quality, strength and power.

1.4 Identification of frailty and sarcopenia in clinical practice

Identification of frailty and sarcopenia in research is different to the diagnosis of the syndromes in clinical practice due to constraints both on time and resources. In research the gold standard for identifying frailty is either Frailty Phenotype or Frailty Index and, for sarcopenia it is measuring all three criteria. In clinical practice frailty has often been a diagnosis of the physician's judgement based on their interpretation of the condition and sarcopenia is rarely diagnosed. However, recently diagnostic and screening tools have evolved to improve the accuracy of diagnosis in both frailty and sarcopenia.

The accuracy of different screening tools in the diagnosis of frailty was recently evaluated in a systematic review (14). Whilst this was a systematic review of a relatively large number of participants, 3261, all of these participants are from a total of just three studies, therefore introducing bias. Additionally all of the screening tests were also measured solely against the frailty phenotype which reports lower rates of frailty than the frailty index. It is therefore not clear whether the reference population is an accurate representation of frailty. The review demonstrated that both the sensitivity and specificity of the PRISMA 7 were high but wide confidence intervals suggest this may not be reproducible. The gait speed, using a 0.8m/s cut off, also had a very high sensitivity but lower specificity as did a timed get up and go test. Gait speed and timed get up and go reflect a physical phenotype of frailty whereas; PRISMA 7 encompasses multiple domains, mobility, social support and health and is therefore more akin to the Frailty Index. The systematic review also reported on a general practitioner's clinical judgement of frailty in an individual, the Groningen Frailty Indicator, a 15 item questionnaire, polypharmacy and self-reported health. These tools all had lower sensitivity and

specificity values. All these tools had been assessed only once in a single population and therefore it is problematic to extrapolate these results to consider all populations (14).

Two diagnostic tests that assess both the presence and absence of frailty as well as severity are the Clinical Frailty Scale (CFS) and the Edmonton Frail Scale (EFS). The CFS can be completed following a comprehensive geriatric assessment and to an extent reflects clinical judgement (15). It has been validated as an adverse outcome predictor in hospitalised older adults (16, 17). The EFS was designed to be utilised by practitioners not trained in comprehensive geriatric assessment (18). It has been used to identify frailty in a number of studies (19, 20).

A single screening test has been proposed for sarcopenia, SARC-F. This is a short questionnaire comprising of 5 questions focused on the negative consequences of sarcopenia. It has been validated in a number of American cohorts in one single study. The study demonstrated an increased risk of mortality, hospitalisation and falls. The authors suggested that SARC-F could be utilised much like a FRAX score, identifying those who should have further assessment to make a diagnosis of sarcopenia (21). The EWGSOP suggest an algorithm for the diagnosis of sarcopenia which negates the need to measure muscle mass in all individuals (22). Whilst this reduces the resources required for the diagnosis of sarcopenia it does mean individuals with pre-sarcopenia, a condition that is likely to have greater potential for reversal, will not be diagnosed.

The recent introduction of the electronic frailty index (EFI) to general practice in the UK could change how frailty is identified in the community potentially removing the need for

screening tests. The EFI is also based on the Frailty Index and incorporates assessment of multiple domains (23).

1.5 Clinical relationship between frailty and sarcopenia

The relationship between frailty and sarcopenia is not yet fully characterised but these conditions share many of the same clinical outcomes, associations and suggested pathophysiology. Sarcopenia is considered a component of physical frailty but frailty is not considered a component of sarcopenia and there is considerable overlap between the defining criteria of the Frailty Phenotype and sarcopenia. Sarcopenia is often considered a pre-cursor syndrome or the physical component to frailty.

Sarcopenia is reported to be twice as common as frailty in the general population (24), However the prevalence of both conditions is dependent on population and definition. Using the EWGSOP definition and criteria of sarcopenia the prevalence ranges from 4.6%, community dwelling men in the UK aged 68-76 (25), to 68%, male Italian nursing home residents over 70 (26). A recent systematic review of frailty prevalence in a community population (27) reported the range to be 4.0%, independently mobile men over 65 (28), to 59.1%, community dwelling Dutch older than 70 (29). The calculated weighted average of frailty as defined using the Frailty Phenotype is 9.9% (27).

Research has shown that in frail community dwelling adults the most common positive Fried criteria was slow gait speed (43%) and weakness (54%), the functional defining criteria of sarcopenia (30). It has also been reported that the relative risk of developing weakness and low activity was higher than developing any other frailty defining criteria over 7.5 years of follow up in initially non-frail women (31). This suggests that

theoretically while it is possible to have frailty without sarcopenia clinically it is unlikely. However, two recent reports investigating the concordance of frailty and sarcopenia in community populations demonstrated that frailty was more common than sarcopenia and the concordance between frailty and sarcopenia was poor (32, 33). This is the only literature investigating the concordance of frailty and sarcopenia but is contrary to previous understanding and may reflect the population studied and the tools used rather than generalisable data. Nevertheless, if repeated studies demonstrate that concordance between the two conditions is incomplete it could suggest different pathophysiological pathways for sarcopenia and frailty which result in a common endpoint.

Importantly both frailty and sarcopenia are considered partly reversible conditions. Several epidemiological studies have shown that whilst transition between lesser and greater frailty is always the most frequent outcome, a number of participants will transition from greater to lesser frailty (34-37). Health status and baseline function is important, as those with poor mobility at baseline in a longitudinal study experienced faster acceleration of their frailty than those with good mobility (34). These studies did not investigate factors such as recent acute illness which could cause periods of temporary frailty but do demonstrate the fluctuating nature of the syndrome. The reversal of frailty has also been demonstrated in intervention studies. A multifactorial intervention in community dwelling frail adults resulted in a significant difference in frailty prevalence between the two groups who were identical at baseline. Whilst the intervention significantly reduced the prevalence of frailty and improved mobility this was not associated with change in mortality or hospitalisation, important clinical outcomes. Of note, the intervention was specific to the individual depending on their

positive frailty criteria at baseline assessment, for example those scoring for weight loss would be assessed and managed by a dietician, consistent with a personalised medicine approach. Importantly, this study reported an actual reduction in diagnosis of frailty in the intervention group not just a slowing of progression (38). It is also possible to improve components of frailty and sarcopenia. Several resistance exercise programmes have documented improvements in gait speed and strength in groups of frail older adults (39, 40), with programmes of 8-12 weeks able to reverse loss of muscle strength equivalent to that lost over 20 years (39). However, there is a degree of variability in the reported outcomes with resistance exercise programmes and this can be explained by the incorrect use of terminology. Fiatarone reported improvements in muscle strength with a short resistance exercise programme but the outcomes measured were actually power and likely to reflect neuromuscular improvements rather than improvement in isometric strength or muscle mass (39). Changes in muscle mass and isometric strength are likely to take much longer particularly in older adults and this is important to consider in both study design and clinical interventions.

Clinical outcomes in frailty and sarcopenia are similar. They are both associated with increased mortality, hospitalisation, falls, loss of independence and mobility. The Frailty Index had a better correlation with time to death than age thus proving its value as a tool in geriatric medicine in the original concept paper. The original Frailty Phenotype paper reported that at three year follow up of older adults identified as frail at baseline: 18% had died, 59% had been hospitalised and 28% had fallen. The odds and hazard ratios of falls, fractures and death in severely frail individuals has been reported as: 1.54 (1.34-1.76), 1.07 (0.14-1.22) and 1.50 (1.41-1.60) (41). This compares to the 27.3% of older

adults with sarcopenia who had fallen in a 2 year follow up period (42) and a reported odds ratio of 4.42 for falls in older adults with sarcopenia (43). The reported hazard ratio of death is 2.99 in older adults with sarcopenia (44). The differences in reporting styles and utilised definitions make it difficult to compare the two syndromes and draw conclusions about causality, concordance and chronicity.

1.6 The ageing immune system, frailty and sarcopenia

The pathophysiology of both sarcopenia and frailty is complex. Proposed models for both syndromes incorporate multiple causes, inter-relationships and elaborate pathways (11, 45). However, both syndromes are incompletely characterised and there is insufficient understanding of the underlying cellular mechanisms driving the development and maintenance of states of frailty and sarcopenia. Sarcopenia is better understood than frailty largely due to its effects being concentrated on a single system, the neuromuscular system, and for this reason it has been suggested as a physical model of frailty.

The role played by the immune system in frailty is believed to be fundamental in the development and maintenance of a frailty state, but it remains mostly uncharacterised. There is also increasing evidence that inflammation is important in the development of sarcopenia and frailty. This thesis investigates the relationship between frailty, sarcopenia, immunesenescence and inflammageing, in particular characterising neutrophil function in frail older adults. The introduction therefore, proceeds to discuss: neutrophil function and muscle homeostasis in health with particular reference to the PI3kinase/Akt pathway, immunesenescence and inflammageing in frailty and, proposed relationships between frailty, sarcopenia, immunesenescence, inflammageing and the PI3kinase/Akt pathway.

1.7 Neutrophil function in health

Neutrophils are an essential component of the innate immune system providing a response to pathogens or injury once recruited. They are also the most abundant leukocyte and comprise 60-70% of the complete leukocyte population (46).

Neutrophils are granulocytes and are produced in the bone marrow from progenitor and precursor cells they then mature in the bone marrow before being released into the circulatory system (47) (48). The process of maturation is regulated by cytokines, for example G-CSF; upregulation of cytokines during periods of infection results in increased numbers of neutrophils in the circulation. Mature neutrophils leaving the bone marrow are quiescent and are activated on contact with cytokines and other regulatory proteins. A neutrophil's half-life is believed to be just 8-12 hours unless activated (49, 50). In a healthy host neutrophils exist entirely in the circulatory system present in two populations, the circulating pool, neutrophils free to circulate within the bloodstream and the marginated pool, neutrophils that roll along the endothelium of small vessels through certain organs (49). They are able to indefinitely switch populations. The number present within each population is tightly regulated resulting in a neutrophilia as a response to infection (50). Neutrophils enter tissue in response to infection or injury (51). Once within the tissue they migrate to the target site, recruit and activate other cells of the immune system providing a near immediate response to invading pathogens or injury (52). Neutrophils are either removed from the tissue by macrophages or undergo reverse transmigration back in to the circulation (53-55). Neutrophils that remain quiescent throughout their life-span return to the marginated pool and undergo apoptosis and phagocytosis to prevent damage to host tissues.

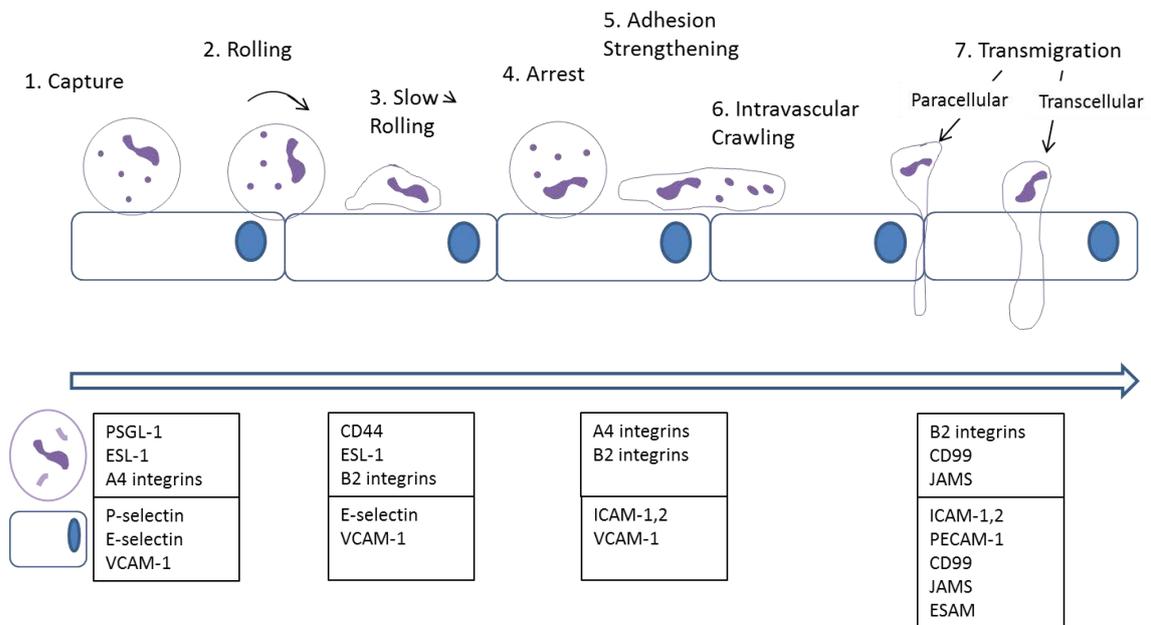
The neutrophil has a variety of functions which enable it to effectively provide an appropriate immune response. Neutrophils produce a variety of anti-microbial peptides including: cationic peptides and proteins that bind to microbial membranes, enzymes and, proteins that deprive microorganisms of essential nutrients (56). They also secrete reactive oxygen species(ROS)(57). The peptides and ROS cause damage to biomolecules which result in metabolic defects in the pathogen (and also collateral host tissue). Neutrophils are capable of phagocytosis and through a receptor mediated process of internalisation remove both pathogens and debris from host tissue (56). During an active form of neutrophil death they extrude neutrophil extracellular traps(NETs)(56). The NETs are comprised of proteins and chromatin which is capable of binding, disarming and killing pathogens extracellularly independently of phagocytosis(58). The neutrophil does not provide a completely targeted response and consequently causes collateral damage to host tissues. Neutrophils are also involved within immune cross-talk and produce cytokines and chemokines to attract a further immune response (56). The initial response is to recruit further neutrophils via IL-8 (59). They are also able to attract monocytes, macrophages, dendritic cells, natural killer cells and lymphocytes. Secretion of IL-1 β and TNF α induce other cells within the host tissue to produce chemoattractants (60, 61). Importantly neutrophils are also central to the resolution of inflammation. Apoptosis of the neutrophil and phagocytosis of the dead neutrophils by macrophages initiates the release of anti-inflammatory IL-10 and TGF β (62).

1.7.1 Neutrophil migration

The primary function of neutrophil to migrate to the source of infection or injury and provide first response is dependent, in part, upon its ability to move from the circulatory

system to the affected tissue. This process relies on cell surface adhesion molecules which bind the neutrophil to the endothelium and then allow it to migrate through the endothelium to reach the affected tissue. This process has a number of steps with various cell surface adhesion molecules becoming vital at different points (63). This process is described in Figure 1.3.

Figure 1.3 – Neutrophil adhesion cascade



Neutrophil adhesion cascade. Depiction of neutrophil and endothelial interaction including the adhesion molecules responsible for mitigating the different stages during transmigration of neutrophils through the endothelium.

The capture of neutrophils to the endothelium is mediated by L-selectin, P-selectin and E-selectin on the endothelial cell interacting with P-selectin glycoprotein 1 (PSGL1) on the neutrophil (63-65). Blood flowing through the circulatory system creates a 'sheer stress' environment which supports the adhesion of these molecules (66). PSGL1, which is present on all neutrophils, will bind to all three selectins. E-selectin and P-selectin are both expressed by inflamed endothelial cells. The binding of L-selectin to PSGL1 prompts adherent neutrophils to facilitate secondary capture of further neutrophils (63, 67). The binding of P-selectin to PSGL-1 results in upregulation of expression of $\beta 2$ integrins on the surface of the neutrophils. (See Table 1.1 for more in depth description of the role of the cell surface receptors.) The upregulation of $\beta 2$ integrins promotes L-selectin downregulation and $\beta 2$ integrins become the predominant signalling and adhesion molecule. This induces a change in the neutrophil and allows it to reach its full signalling potential (46).

Once the neutrophil has engaged with the endothelium it starts to roll slowly at a speed of 40 $\mu\text{m}/\text{sec}$ along the endothelial surface (68). This is facilitated by selectin binding and new bonds are formed before old bonds are released to prevent the neutrophil from floating away in the circulatory system. Slow rolling only occurs when the neutrophil comes into contact with inflamed endothelium. It is defined as a speed below 5 $\mu\text{m}/\text{sec}$ (68). The deceleration occurs due to the increased number of E-selectins expressed on the surface of the endothelium which increases the number of potential binding sites for PSGL-1. During rolling and slow rolling MAC-1 and LFA-1 expressed on the neutrophils become important for further neutrophil and endothelial cell interactions (68).

Arrest of the neutrophil rolling occurs following exposure of the endothelial cells to inflammatory cytokines which induces them to express adhesion molecules and synthesise chemokines on luminal surfaces. LFA-1 and MAC-1 bind to ICAM-1 and VCAM-1 expressed by the endothelial cells. LFA-1 displays increased avidity following stimulation. Chemokines on the luminal surfaces bind to glycol-protein coupled receptors (GPCRs) and induce conformational changes of ligands almost instantaneously (63). Signalling both, inside-out and outside-in, occurs which modulates the affinity of the integrins for the ligand (69). Strengthening of the neutrophil adhesion occurs with ligand induced integrin clustering which causes a conformational change and causes rapid arrest of neutrophil rolling under 'sheer stress'.

Following adhesion the neutrophil crawls along the endothelium. This is Mac-1 and ICAM-1 dependent and is important physiologically as it allows the neutrophil to find a suitable place to transmigrate through the endothelium (70, 71).

To reach the affected tissue neutrophils need to migrate from the circulatory system across the endothelium. This occurs via a process called transmigration either transcellular, through the endothelial cell, or paracellular, between the endothelial cells (63). Paracellular migration through the endothelial cells takes between two to five minutes but penetrating the endothelial to cell basement membrane takes much longer, five to fifteen minutes (63). Paracellular migration is triggered by luminal chemoattractants and endothelial cell adhesion molecules engagement with neutrophil ligands (72). A trans migratory cup, a projection of endothelial cells rich in ICAM-1 and VCAM-1 is formed to help with migration. The transcellular route, which is taken by the

minority of cells and under certain conditions such as inflammation, is rapid and takes less than one minute. This is believed to be facilitated by a vesiculo-vacular organelle (73, 74).

Multiple neutrophil adhesion molecules are expressed on the cell surface during activation. Table 1.1 describes and explains some of the roles of the cell surface receptors and their involvement in neutrophil adhesion and transmigration.

Table 1.1 - The role of the cell surface receptors and their role in neutrophil adhesion and transmigration

Cell Surface Receptor	Description	Role
CD11a	A subunit of LFA-1 LFA-1 = membrane glycoprotein	Cell to cell adhesion by interaction with ICAM-1. Also binds to ICAM-2 and ICAM-3 (70, 71)
CD11b	A subunit of Mac-1 Mac-1 = membrane glycoprotein	Cell to cell adhesion by interaction with ICAM-1 Also binds to C3bi and extracellular matrix proteins (70, 71)
CD18	B2 integrin. Binds with CD11a to produce LFA-1 Binds with CD11b to produce Mac-1	Marker of activation in neutrophils (70, 71)
CD16	Fcγ RIII, low affinity receptor for IgG	Increased abundance with neutrophil priming Constantly shed by activated neutrophils. Activates secretion of ROS (57)
CD29	B1 chain. Glycoprotein. B chain of VLA5	Cell to cell adhesion (75)
CD49f	A subunit. Binds with CD29 to form VLA-4	VLA-4 binds to laminin. Involved in adhesion and chemotaxis. (63)
CD62L	L-selectin	Upregulated by inflammatory stimuli. Activates CD18 function. Involved in activation, and interstitial migration (76, 77)
CD63	Transmembrane superfamily of protein	Stored in cytoplasmic granules and exported to cell surface on activation. Increases the cell surface expression of Mac-1 (63)
A9β1	Integrin	Role in stabilising neutrophil adhesion. Binds to VCAM-1 (63)

Table describing and explaining the role of some of the important cell surface receptors and their role in neutrophil adhesion and transmigration.

Neutrophils leave the bone marrow in a quiescent state and circulate through the blood stream awaiting activation or apoptosis. A quiescent neutrophil is in an un-activated state but is capable of becoming both primed and activated (78). A primed neutrophil produces a response, which can be up to ten times larger, than a quiescent neutrophil on exposure to a stimulant (79). Therefore, in some situations it is advantageous to prime neutrophils before they are stimulated. Quiescent, primed and activated neutrophils are often described using a traffic light analogue – red, orange and green. This depicts the readiness of primed neutrophils to produce a response.

Quiescent neutrophils are primed after exposure to certain cytokines (49), activated endothelial cells (80) or bacterial products (81). The underlying cell signalling mechanisms are complex and not fully characterised but primarily involve PI3K activation and PtdIns (3,4,5) P3 generation (82). Due to the diversity of the agents that can induce priming it is likely there are multiple intracellular mechanisms. This theory is affirmed by the differences in the intensity of response and variable time courses of the manifestations of priming depending on the agent used (80, 81, 83).

Primed neutrophils are structurally and functionally different to quiescent neutrophils. The differences, which are discussed below, change the behaviour of the neutrophil and its behaviour becomes closer in nature to that of a macrophage.

Primed neutrophils have enhanced oxidative burst on exposure to a secondary stimulus compared to quiescent cells. Oxidative burst in neutrophils is characterised by the production of superoxide, hydrogen peroxide and hypochlorous acids. Free oxidative radicals are catalysed by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase

and myeloperoxidase (84). The enhanced respiratory burst witnessed in priming occurs due to the translocation and assembly of cytosolic components of NADPH oxidase enzymes (85). The state of a neutrophil, quiescent, primed or activated, is determined by their amount of oxidative burst.

Primed neutrophils undergo a change in shape with a stiffer central region compared to the leading edge and tail region (86). This allows easier passage through the endothelium which is fundamental to their ability to reach the site of pathogen or injury. This change in shape also results in increased sequestration in the pulmonary capillary bed which is important to their function in vivo (87, 88). Primed neutrophils express neutrophil integrins in increased numbers and shed CD62L to enhance their adhesion to endothelium (89). The primed neutrophils also undergo some degranulation and lipid mediator release but this is modest when compared to the respiratory burst (90).

Priming of neutrophils inhibits the apoptosis of the cell. LPS and GM-CSF, in particular, delay the onset of programmed cell death whereas TNF α shows a bimodal distribution (91).

There is evidence from both in vivo and in vitro experiments that neutrophils can be de-primed and revert back to a quiescent state (83, 92-94). In vivo there is some evidence that this is related to the pulmonary vasculature but it can also occur without anatomical help in vitro. In vivo experiments have utilised mathematical modelling and reviewed the proportion of primed neutrophils entering the pulmonary and leaving the pulmonary vasculature (92-94). In vitro experiments have demonstrated that priming and de-priming of neutrophils occurs at different rates dependent on the priming agent. Platelet

activation factor (PAF) has been shown to produce a maximal priming effect after ten minutes of stimulation whereas priming with TNF α takes longer with a maximal priming effect seen at thirty minutes. The same experiments have also demonstrated a difference in time to de-priming, with a rapid course seen in PAF stimulation and a plateau effect then de-priming occurring with TNF α . Once a neutrophil has returned to the quiescent state it is then possible to re-prime the neutrophil and this has been demonstrated in vitro (83).

1.8 PI3Kinase

Fundamental to both neutrophil function and muscle homeostasis is the phosphoinositide 3-kinase Akt (PI3K/Akt) pathway.

1.8.1 PI3Kinase Family

PI3K are a family of structurally related enzymes divided into three distinct functional classes on the basis of the protein domain structure and associated regulatory subunits (95). There are eight different mammalian isoforms of PI3K (96). All classes of PI3K phosphorylate 3' position hydroxyl of the inositol group to synthesise three distinct phosphoinositides (97). The downstream effects of the phosphoinositides are broadly divided into localised membrane remodelling, for example the GTPases Rac1 and Rho1 which coordinate dynamic organisation of the actin cytoskeleton and membrane localised signalling, for example the AKT/mTORC1 pathway (97). Class 1 PI3K are involved in cell signalling, class 2 PI3K in both cell signalling and membrane trafficking and class 3 PI3K in membrane trafficking. Negative feedback for the PI3K family takes the form of SH2

domain-containing inositol phosphatase 1 (SHIP) and phosphatase and tensin homologue (PTEN)(98). SHIP and PTEN convert PIP_3 back to PIP_2 (99).

Within each class there are different isoforms denoted by different catalytic or regulatory subunits. Class 1 has 4 different catalytic subunits: p110 α , p110 β , p110 γ and p110 δ and five different regulatory subunits: p85 α , p85 β , p55 α , p101 and p84 (97). Whilst the catalytic subunit generates phosphoinositide the regulatory subunit acts to both transport the catalytic subunit around the cell and to provide negative regulation of the catalytic subunit (97). Class 1 can be further divided into class 1A, PI3K α , PI3K β and PI3K δ and class 1B, PI3K γ (97). Class 1A isoforms are catalytic subunits p110 α , p110 β and p110 δ bound to the regulatory subunits p85 α , p85 β or p55 γ (97). These isoforms can be recruited by a phosphorylated tyrosine kinase receptor (97). Recruitment by a phosphorylated tyrosine kinase receptor causes a class 1A PI3K to translocate from the cytosol to the inner leaflet of the plasma membrane only here it has access to PIP_2 (97). Recruitment also initiates a conformational change to p85 regulatory subunit which negates its inhibitory effect on the catalytic subunit (97). The Class 1B isoforms are the catalytic subunit p110 γ bound to either the regulatory subunit p101 or p84 (97). These isoforms are activated by the heterotrimeric G protein coupled receptor(GPCR)(97). Chemokines bind to GCPRs and therefore class 1B isoforms provide the primary response to chemokines (100). Class 1 PI3K elicit signalling responses which regulate cell growth, proliferation, survival metabolism and autophagy (101) and cortical F-actin dynamics fundamental to both chemotaxis and phagocytosis (102, 103). The class 1 isoforms share overlapping but distinct functions. The p110 γ and p110 δ isoforms are mainly restricted

to functions within immune cells but p110 α and p110 β are more ubiquitously expressed (97).

1.8.2 PI3Kinase pathway in neutrophils

The PI3K/Akt pathway is crucial to the majority of the functions of the neutrophil but different isoforms are essential for different functions: chemotaxis, phagocytosis and reactive oxygen species production (ROS)(104). P110 δ has the greatest proportion of PI3K isoforms within neutrophils (δ 90%, γ 2%, β 4%, α 4%)(105). PI3K within an inactive neutrophil is evenly spread throughout the cell on surface signalling PI3K localises to the cell's leading edge (105). Neutrophils contain approximately 25 phosphoinositide effector molecules and AKT is the key effector (105).

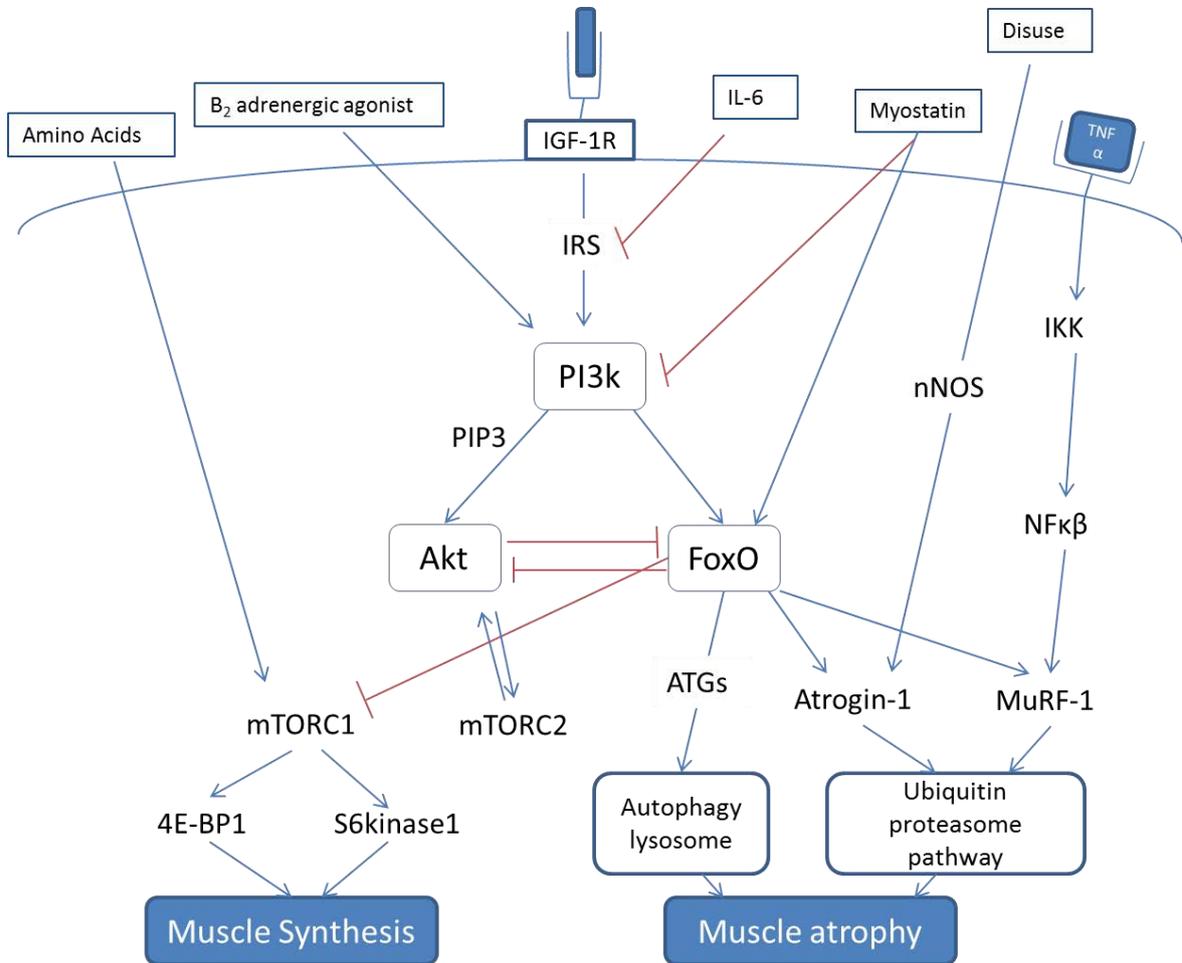
Chemotaxis, the accurate migration of a cell along a chemoattractant gradient, occurs following the activation of class one isoforms of PI3K by chemoattractants. The class 1 isoforms p110 γ and p110 δ are the most important in chemotaxis with p110 γ providing a chemotactic response to brief stimuli whereas p110 δ provides a delayed and less intense response to longer stimuli (105). Similar to the PI3K pathway within muscles this induces PI3K to produce PIP3 (106). PIP3 via phosphorylation of Akt then initiates a pathway to produce actin polymerisation which pushes the cell forwards (107, 108). A gradient of PIP3 is maintained within the cell by the negative regulatory effect of PTEN and SHIP1 and leads to actomyosin contractility allowing the release of the cell at the rear propelling the cell forward (106).

Phagocytosis of pathogens by neutrophils is also dependent upon the PI3K pathway although this is often considered to be regulated by an alternative class of PI3K. The activation of PI3K by association with tyrosine phosphorylated adaptor proteins allows PI3K to form PIP3 which is fundamental in the rearrangement of the actin cytoskeleton and the subsequent formation of pseudopodia(109). The fundamental role of PI3K in phagocytosis of large particles has been demonstrated in practice with PI3K inhibitors (110). The PI3K/Akt pathway is also central to the production of reactive oxygen species and apoptosis by the downstream Akt substrate BCL-2 antagonist of cell death (BAD) (111) in addition to a role within cytokine release (112). Reduced oxidative burst and an anti-apoptotic effect has been demonstrated in PI3K p110 γ knock out mice (113).

1.9 Muscle

Sarcopenia is the result of the complicated interplay between muscle size and strength and physical function. Whilst the role of 'muscle quality' is increasingly evident as fundamental to the syndrome of sarcopenia muscle size is a pre-requisite to the diagnosis. Muscle size is determined by the complex relationship between muscle synthesis and muscle degradation. Our current understanding of the regulation of muscle mass is predominately based on data from animal studies, with much less known of the key regulatory processes in human muscle. Studies in rodents suggest that muscle synthesis is intrinsically linked with muscle atrophy via the PI3k-Akt pathway which is central to both processes (114). The role of the PI3k-Akt pathway in muscle homeostasis is depicted in Figure 1.4 and described in section 1.9.1.

Figure 1.4 – PI3kinase/Akt pathway in muscle



The PI3kinase/Akt pathway in muscle. Stimulation of the IGF-1R upregulates PI3k which in turn generates PIP3 and this activates Akt which promotes mTOR and protein synthesis.

1.9.1 PI3Kinase pathway in muscle homeostasis

Homeostasis of muscle mass is the balance between muscle synthesis and muscle degradation. Muscle degradation is a term which broadly describes the loss of muscle via both reduction in muscle fibre size (atrophy) and reduction in the number of muscle

fibres (hypoplasia). Hypoplasia is believed to occur secondary to motor neurone death and muscle fibre denervation and atrophy secondary to the proteolytic pathways (13). Of the four major proteolytic pathways present in muscle cells two, autophagy lysosomal and ubiquitin proteasome pathway, have PI3K/Akt signalling central to their functional ability (115).

Muscle synthesis is activated by insulin-like growth factor 1 (IGF-1) binding to IGF-1 receptor which triggers the activation of its internal tyrosine kinase and results in autophosphorylation (114). This generates a docking site for insulin receptor substrate (IRS) which once docked is phosphorylated by the IGF-1 receptor (114). The phosphorylated IRS acts as a further docking site to recruit and activate PI3K (114).

Activated PI3K phosphorylates cell membrane phospholipids and generates phosphoinositide 3,4,5 triphosphate (PIP3)(114). PIP3 acts as a docking and phosphorylation site for Akt (114). The activated Akt indirectly promotes the mammalian target of rapamycin (mTOR) which in turn phosphorylates S6 kinase and other factors which promote protein synthesis via translation initiation and elongation (114). There are multiple modulators of the pathway both intrinsic and extrinsic, up and down regulating. Extrinsic regulators: amino acids can directly activate mTORC1 (116, 117) whereas beta adrenergic agents (118) and Wnt7a both upregulate PI3K/Akt pathway (119). Intrinsic regulators include S6 kinase 1 inhibiting IRS (120) and mTORC2 upregulating Akt (121).

Muscle atrophy via both the ubiquitin proteasome pathway and autophagy lysosomes is centrally regulated by forkhead box proteins (FoxO). FoxO when present in the nucleus

induces the ubiquitin E3 ligases atrogin-1 (MAFbx) and muscle RING finger protein-1 (MuRF-1) (122) which cause myofibril degradation via the ubiquitin proteasome pathway. FoxO also regulates ATG genes which promote mitochondrial degradation via autophagy (123). As expected there are multiple modulators of these pathways and arguably the most important is the effect of Akt on FoxO: activated Akt phosphorylates FoxO and transports it from the nucleus to the cytoplasm preventing it from inducing either E3 ligases or ATG genes (124-126). Myostatin, via the action of Smad 2 and 3, upregulates FoxO (127) and muscle disuse produces neuronal nitric oxide synthase (nNOS) which enhances FoxO3 mediated transcription of E3 ligases (128). TNF α directly upregulates the NF κ B pathway via IKK and produces MuRF-1 which causes myofibril degradation via the ubiquitin proteasome pathway (129, 130).

1.9.2 Muscle homeostasis in sarcopenia

The majority of studies investigating sarcopenia in humans have suggested that loss of muscle mass is primarily driven by a blunted synthetic response to both feeding and exercise, termed anabolic resistance (131-133). Moreover, there is little data to suggest that atrogenes or ubiquitin proteasomal degradative pathways are enhanced in older adults, indeed in the fasted state (when protein breakdown is at its highest) there is no difference in protein turnover or signalling through synthetic pathways such as Akt between young and old adults (134). The contribution of each pathway towards atrophy in sarcopenia remains unclarified with conflicting evidence from various human and mammal model studies (135). The ubiquitin proteasome pathway is known to be activated in disuse, fasting and denervation as well as inflammatory conditions such as cancer and COPD (13). Disuse, denervation and inflammation are all central to

sarcopenia so despite varying experimental data it is difficult to discount the effect of the ubiquitin proteasome pathway in sarcopenia. The measurement of a 1.5 to 2 fold increase in mRNA levels of atrogenes (ubiquitin E3 ligases) in sarcopenic muscles, in comparison to the ten-fold increase seen in other catabolic conditions (136), conforms with the idea that sarcopenia is an insidious syndrome rather than a rapid catabolic state.

Anabolic and catabolic pathways may be relevant in periods of extreme inactivity, such as bed rest, and in chronic inflammatory states, such as chronic obstructive pulmonary disease (COPD). In a recent 5 day bed rest study in well characterised healthy young and old subjects, leg lean mass and strength were reduced only in the old subjects. Both subject groups had blunted mTORC1 signalling and increased MURF1 expression in skeletal muscle after bed rest, but only the older group had reduced amino acid induced protein synthetic rates and increased atroгене expression (137). A study investigating the effect of resistance training in participants with COPD, a chronic inflammatory condition, demonstrated higher levels of MAFbx and MURF1 protein expression (atrogenes) and increased phosphorylation of p70s kinases (138). These studies suggest a decrease in muscle synthesis and an increase in muscle degradation (dysregulated muscle homeostasis) is seen with inactivity and chronic inflammatory conditions.

Patients with sepsis are subject to both periods of extreme inactivity and a pro-inflammatory state and could therefore be considered a model of accelerated ageing (139). Following sepsis and admission to critical care 70-100% of patients reported prolonged weakness (140) which is due both to muscle loss and changes in muscle cell

functionality. This group deserves further study for a link between inflammation and sarcopenia.

1.9.3 PI3kinase pathway in sarcopenia

The importance of the PI3K/Akt/FoxO pathway in muscle homeostasis was discussed in section 1.9.1 and has been demonstrated in mice models. Transgenic mice overexpressing Akt in skeletal muscle displayed muscle hypertrophy (114) and after denervation maintained their muscle bulk suggesting they underwent less atrophy (141). Conversely Akt 1 and Akt 2 knock out mice display a severe growth deficiency and evidence of muscle atrophy similar to that seen in IGF-1 receptor deficient mice (142). Transgenic mice overexpressing FoxO had reduced muscle mass and had a significantly decreased number of type 1 and 2 fibres compared to the wild type. Interestingly they also displayed reduced spontaneous activity (143).

Myoblasts deficient in the PI3kinase catalytic subunit p110 α isoform had decreased activation of AKT and myoblasts deficient in p110 β had increased activation of AKT (144) suggesting different roles for the PI3K isoforms within muscle synthesis. Long term dosing of healthy animals with PI3K isoform inhibitors did not cause weight loss but mice dosed with either pan PI3K inhibitors or p110 α inhibitors displayed impaired weight gain compared to controls (145). Different concentration of PI3K isoforms exist in muscle stimulated with insulin dependent on birth weight (146) suggesting lasting environmental factors affecting muscle synthesis.

1.10 Immunesenescence and inflammaging

The role played by the immune system in frailty and sarcopenia is believed to be fundamental in the development and maintenance of both a frailty and sarcopenic state, but it remains mostly uncharacterised.

1.10.1 Overview of immunesenescence and inflammaging

The decline in immune function with age, termed immunesenescence, is well documented and includes increased susceptibility to infections, reduced vaccination responses in older adults and increased risk of chronic inflammatory diseases such as Rheumatoid Arthritis (147-149). Examples of changes to the immune system with age are reported in Table 1.2. The changes displayed by neutrophils with ageing are discussed in greater depth in section 1.10.2.

Immunesenescence includes *inflammaging*, the presence of a low-grade chronic systemic pro-inflammatory state with age (150, 151). Inflammaging is characterised by increased levels (typically 2-4 fold those seen in healthy young subjects), of pro-inflammatory cytokines such as interleukin 1 β (IL-1 β), interleukin 6 (IL-6) and tissue necrosis factor alpha (TNF α) as well as c-reactive protein (CRP), and a reduced serum level of anti-inflammatory cytokines including interleukin 10 (IL-10) (150) and IL-1ra. The factors driving inflammaging are multiple and can include increased output of pro-inflammatory cytokines by resting monocytes (152-154), reduced IL-10 production by regulatory lymphocytes (155), increased adiposity leading to production of pro-inflammatory adipokines such as leptin and reduced anti-inflammatory adipokines such as adiponectin (156), reduced physical activity with age (157), senescent cells which build up with age and secrete pro-inflammatory cytokines (158).

Table 1.2 - Changes to the adaptive and innate immune system with age

	Immune Cell	Immune Cell Changes
Adaptive	B Cells	<ul style="list-style-type: none"> • Reduction of the proportion of naïve B cells despite no changes to absolute number leading to a reduction in the diversity of the B cell repertoire (159, 160)
	T Cells	<ul style="list-style-type: none"> • Involution of the thymus and the subsequent reduction in number of naïve T cells leaving the thymus (43, 44) • Contracted repertoire of both CD4+ and CD8+ T cells (161, 162)
Innate	Monocytes	<ul style="list-style-type: none"> • Increased absolute number (47) • Reduction in cytokine production (48)
	Macrophages	<ul style="list-style-type: none"> • Decrease in function
	Natural Killer Cells	<ul style="list-style-type: none"> • Increase in absolute number • Reduction in production of cytokines and chemokines on activation
	Dendritic Cells	<ul style="list-style-type: none"> • Reduction in function

Examples of changes to the adaptive and innate immune system with age.

1.10.2 Neutrophil dysfunction in ageing

In healthy older adults the function of the neutrophil is altered. Neutrophil migration is impaired by reduction in the velocity, or chemotaxis, and persistence of movement (163).

This is hypothesised to result in both an increased time for the neutrophil to reach the site of infection and more importantly increased obligate host tissue damage (164).

Neutrophils from older adults also demonstrate impaired phagocytosis (165), impaired apoptosis (165) and impaired actin polymerisation (50). The accumulation of these

defects dictates that a neutrophil in an older adult takes longer to reach its target,

damages more of the host tissues and on reaching the target has impaired ability to

destroy the pathogens. Poor neutrophil function is present in chronic inflammatory

diseases, such as chronic obstructive pulmonary disease (COPD), and it is suggested that

frailty and sarcopenia, which are associated with inflammaging, will display even poorer neutrophil function than healthy older adults (166).

1.10.3 Models

There has been little fundamental mechanistic research investigating the immune system in frailty in its truest sense. Therefore, it is necessary to consider models of frailty and draw conclusions where possible. Some of the models of frailty that have been utilised in research are discussed below.

Population based studies have demonstrated that the incidence of infection and subsequent mortality is higher in populations of frail people. Nursing homes represent a population where frailty can be assumed without direct measurement. The prevalence of pneumonia in a nursing home population is thirty times higher than the general population (167, 168). Population based studies have identified that frailty is associated with inflammaging and immunosenescence (169-172). As HIV could lead to the same characteristics of immunosenescence in older individuals, the identification of a subpopulation of HIV patients with frailty has led to comparison cohort studies between HIV patients with frailty and those without (173). These studies focus upon the adaptive immune system and T cells in particular. Conversely, centenarians have been employed as a model of successful ageing (174, 175).

Sarcopenia has been suggested as a human model of physical frailty. It is a more tangible concept than frailty and is easier to utilise in a research setting. It has been proposed it could be either the physical component or a precursor syndrome to frailty.

Murine models have been developed to investigate sarcopenia and frailty using either cell signalling pathway knock out models or accelerated ageing models. The pathogen free IL-10 homozygous deletion mouse displays changes in muscle fibre size, growth and the proportions of type 1 and 2 muscle fibres (176). In addition to these changes accelerated ageing models also display additional features of ageing such as osteoporosis and alopecia. A valid murine frailty index has been developed to improve murine frailty research (177). However, defining frailty in mice is problematic and the index relies heavily on measuring body composition and, haemodynamic and metabolic parameters; this does not reflect a global review of frailty.

This mouse model develops increased muscle weakness and decreased activity levels. Murine models offer an alternative investigative approach to frailty and sarcopenia and could be utilised for work that is difficult or unethical to complete in humans for example: muscle work, response to pathogens, cognitive function investigation. These models offer an excellent and unique opportunity to study frailty in vivo but lack the sophistication and subtleties of frailty in humans. Frailty in humans is a condition which is affected by both the dysregulation of multitudes of internal biological factors and external factors such as environmental, political and social conditions. This complex dysregulation of multiple biological systems and the interplay between the individual and their wider environment is impossible to replicate in a single gene knock out mouse model.

1.10.4 Inflammageing, frailty and sarcopenia

Inflammageing is a state of discrete chronic inflammation. A positive association has been demonstrated between IL-6 and CRP with frailty on multiple occasions in different

frail populations (169-171, 178). High levels of IL-6 predict disability and mortality, which are significant outcomes within frailty, in older community dwelling adults (179-181). Peripheral blood mononuclear cells (PBMCs) from frail older adults produce greater amounts of IL-6 on stimulation compared to non-frail counterparts (182).

Inflammatory and immunological biomarkers of frailty have been used in a variety of settings to predict poor outcomes (183, 184) The Biomarker Frailty Index incorporates markers of inflammation and immunesenescence as well as haematological and genetic markers. This model is associated with mortality and at low levels of frailty is better than the frailty index at predicting mortality (183).

Investigation of the individual components of sarcopenia and frailty has demonstrated both an association with inflammaging and inflammaging predating a loss of physical function. Cross-sectional studies have demonstrated an association of high levels of IL-6 and TNF α with low muscle mass and strength (185) and low levels of CRP with high grip strength (186). Longitudinal studies have shown that higher levels of IL-6 predict reduction in fat free mass, a hallmark of sarcopenia (187). Higher levels of IL-6 and CRP at baseline equate to a two to threefold greater risk of losing more than 40% of grip strength over a 3-year follow up (188). TNF α mRNA and protein levels are higher in frail older adults. It was also demonstrated that this could be reversed with an exercise program, which correlated with an increase in muscle strength (189).

In centenarians IL-6 -174 GG genotype, associated with higher plasma levels of IL-6, is under represented and IL-10 -1082 CC, associated with higher plasma levels of IL-10, are over-represented (174).

The direct actions of IL-6 on pathways of muscle breakdown and synthesis are yet to be fully characterised but there is some evidence that suggests it can act via the ubiquitin proteasome pathway. IL-6 is associated with increased ubiquitin protein and mRNA (190), E3 ligase protein and mRNA (191) and proteasome activity (192). In addition IL-6 can induce insulin resistance which suppresses Akt and muscle synthesis (193, 194). The literature also suggests that down-regulation of IL-10 could cause frailty and sarcopenia by the effects on muscle quality and the subsequent effect on physical activity and weakness.

The frailty murine model, pathogen free IL-10 homozygous deletion mouse, has increased levels of IL-6 at 50 weeks and phenotypically displays increased muscle weakness and decreased activity levels in comparison to the wild type (195). In murine models the exogenous administration of TNF α results in anorexia and weight loss in the mouse via the leptin pathway (196, 197). The up-regulation of leptin and the resultant anorexia causes decreased muscle synthesis due to reduced amino acids availability for protein synthesis (198). TNF α can also directly result in weight loss (199). TNF α activation of the transcription factor nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) results in myofibre atrophy and activation of the ubiquitin-proteasome pathway (129, 130, 200). Similar to IL-6 TNF α induces insulin resistance which suppresses the Akt-mTOR pathway of protein synthesis (201). This suggests that TNF α could be contributing to the development of frailty and sarcopenia via a number of pathways, direct muscle breakdown, reduction in muscle synthesis and weight loss.

The limited data available demonstrates that frailty is associated with a state of chronic inflammation. There is also some evidence that inflammaging predates a diagnosis of frailty suggesting a causative role but without rigorous longitudinal investigation of frailty and inflammation the relationship between the conditions is uncharacterised.

1.10.5 Innate immunity, frailty and sarcopenia

A small number of studies have demonstrated a dysregulation of the innate immune system in frailty. Frail adults have raised white cell and neutrophil count (within normal limits). This remains significantly elevated even after adjustment for common confounders (202-204). Raised neutrophils are also associated with low levels of physical activity and frailty (205). High white cell count can predict frailty at a ten year follow up (206). An association has been demonstrated between frailty and the upregulation of monocytic expression of CXCL 10 gene which codes for a potent pro-inflammatory chemokine (207). A single study has investigated the function of innate immune cells in a defined frail older adult population; this demonstrated that nursing home residents in comparison to community dwelling older adults had significantly elevated pattern recognition receptors TLR 4, reduced adhesion markers CD11b and CD18 and, reduced production of chemokines and cytokines CCL4, CCL20 and IL-8 both at baseline and stimulation (208).

1.10.6 Cellular mechanisms contributing to neutrophil dysfunction in frailty

Older adults and frail older adults both have higher levels of circulating cytokines with increased values seen in a frail population compared to a healthy older population (169, 170, 195). The increased levels of circulating cytokines in older and frail older adults can be hypothesised to result in a continual priming and de-priming cycle with neutrophils

spending less time as quiescent cells when compared to neutrophils from healthy younger adults. This hypothesis is supported by evidence that there is increased spontaneous ROS production in neutrophils from older adults (209) and from frail older adults (210); there is also an increased superoxide anion concentration within neutrophils from older adults. The proposed continual cycle of priming and de-priming is hypothesised to be detrimental to both the immune function and internal tissues causing further damage and perpetuating the cycle. There is evidence from multiple chronic inflammatory diseases that activated neutrophils cause tissue damage via release of tissue damaging molecules, molecules that promote inflammation and cytokines (211). A surrogate marker for neutrophil degranulation $\text{A}\alpha\text{-Val}^{360}$ has been validated as a sensitive measure of systemic neutrophil proteinase activity (212) and this has been demonstrated to be raised in a healthy older adult population (163).

Multiple studies have demonstrated that neutrophils from older subjects have poorer function and this contributes to immunosenescence. In part this could be related to the neutrophils ability to respond to priming. Neutrophils from younger subjects primed with GM-CSF demonstrated enhanced chemotaxis but neutrophils from older adults primed with GM-CSF did not show enhanced capabilities (213). A separate study showed that superoxide anion levels were lower after priming in older neutrophils compared to younger neutrophils at earlier time-points. At the later time-point of 48 hours the older neutrophils had higher superoxide anion levels (214). This suggests a difference in response to priming with a decreased maximal response and delayed de-priming contributing to increased tissue damage and poorer immune response.

The number of circulating neutrophils is raised in the frailty state (210, 215) but the mechanisms and characteristics of this relative neutrophilia are unknown. Increased release of neutrophils from the bone marrow in response to an inflammaging state would result in increased immature neutrophils but a functional deficit in the clearing mechanism of aged neutrophils would result in a shift in the neutrophil population towards senescent neutrophils. Senescent neutrophils upregulate CXCR4 and downregulate CXCR2 on their cell surface (216).

Verschoor demonstrated that there are an increased proportion of immature band neutrophils in frail older adults; immature neutrophils are released in response to infection or inflammation (210). The study also showed an increased frequency of HLA-DR expression in neutrophils from frail older adults which is associated with tissue infiltrating neutrophils and suggests an increased proportion of neutrophils undergoing reverse trans-endothelial migration (210). This may represent the unbalancing of homeostasis; to maintain the number of neutrophils with the best functional response an increased number of immature neutrophils and senescent neutrophils are required.

The integrin CD11b which is fundamental in the process of neutrophil adhesion to the endothelial cell wall has been studied both in a frail older adult population with conflicting results. CD11b was raised in nursing home residents identified as frail using a CFS and this was associated with increasing TNF α concentrations in serum (210).

Conversely a different study investigating CD11b in nursing home residents found it to be lower than healthy older adults (208). Analysis of the reported methods does not offer any explanation for the witnessed differences. The studies were conducted in similar

populations using similar methods. The most striking difference is that only the first study verified frailty in its study sample. However, there is a very high prevalence of frailty in nursing home populations suggesting both study populations were frail.

Neutrophils are central to the healing process in muscle damage and research has demonstrated in a number of models of muscle injury that neutrophils are recruited to the site of the injury within a couple of hours (217-219). However, it is believed that in addition to a role in healing neutrophils also cause secondary damage to healthy muscle and it has been shown that muscle damage is reduced when functioning neutrophils are prevented from migrating to the damaged tissue (220, 221). The original studies used an ischaemia and reperfusion model but subsequent studies have utilised a muscle stretch injury model and directly visualised the muscle damage under the microscope (222). As muscle ages it becomes more susceptible to injury and as a person ages dysregulation of entire systems, for example balance and cognition, result in a greater number of injuries. It can be postulated that muscle injury in an older person in a state of inflammaging would cause secondary damage to healthy muscle by aberrantly migrating neutrophils resulting in myocyte apoptosis, loss of muscle fibres and their replacement by fat. This loss of muscle quality and mass could result in the functional losses of physical weakness and slowness of sarcopenia.

1.10.7 Adaptive immunity and frailty

A small number of studies have described the dysregulation of the adaptive immune system in frailty, but the results have been conflicting

A prospective review of response to vaccination showed a reduction in adaptive immune function in frail individuals (223). However, the study did not attempt to investigate the individual roles of T and B cells in poor response to vaccination. Initial work into frailty in HIV patients demonstrated higher levels of frailty in HIV positive patients compared to non-HIV infected age matched controls (224) and subsequently that CD4+ T cell count can predict the development of frailty in HIV positive men independent of HAART and plasma viral load (173). Research into T cell subset expression in frailty has revealed conflicting results, particularly in the investigation of CD4:CD8 ratio which has both been positively (215, 225) and inversely associated with frailty (226, 227). The association of CMV with frailty is also unclear. Frailty has been associated with higher titres of CMV IgG (228, 229) and also higher levels of CD8+CD28- T cells (225, 227). CD8+CD28- T cells are highly associated with seropositivity to persistent infections such as CMV. However, it has also been reported that CMV infection does not appear to determine the age-related increase in serum markers of inflammation (230) and, furthermore the extremely high prevalence of CMV in all older adults make all these data difficult to interpret. Only one study has investigated the B cells of a frail population and showed that with increasing age the diversity of the B cell population decreases and this correlates with poor health (231).

1.11 Relationships between frailty, sarcopenia, immunosenescence and inflammaging

To date there is limited research describing a direct relationship between frailty, sarcopenia and immunosenescence or inflammaging. However, initial results have demonstrated an association of frailty and sarcopenia with a state of chronic inflammation and dysregulation of both the innate and adaptive immune system. It has also established that immunosenescence and inflammaging predate a diagnosis of frailty

suggesting a causative role. Figure 1.5 demonstrates a theoretical pathophysiological model linking frailty, sarcopenia and inflammaging.

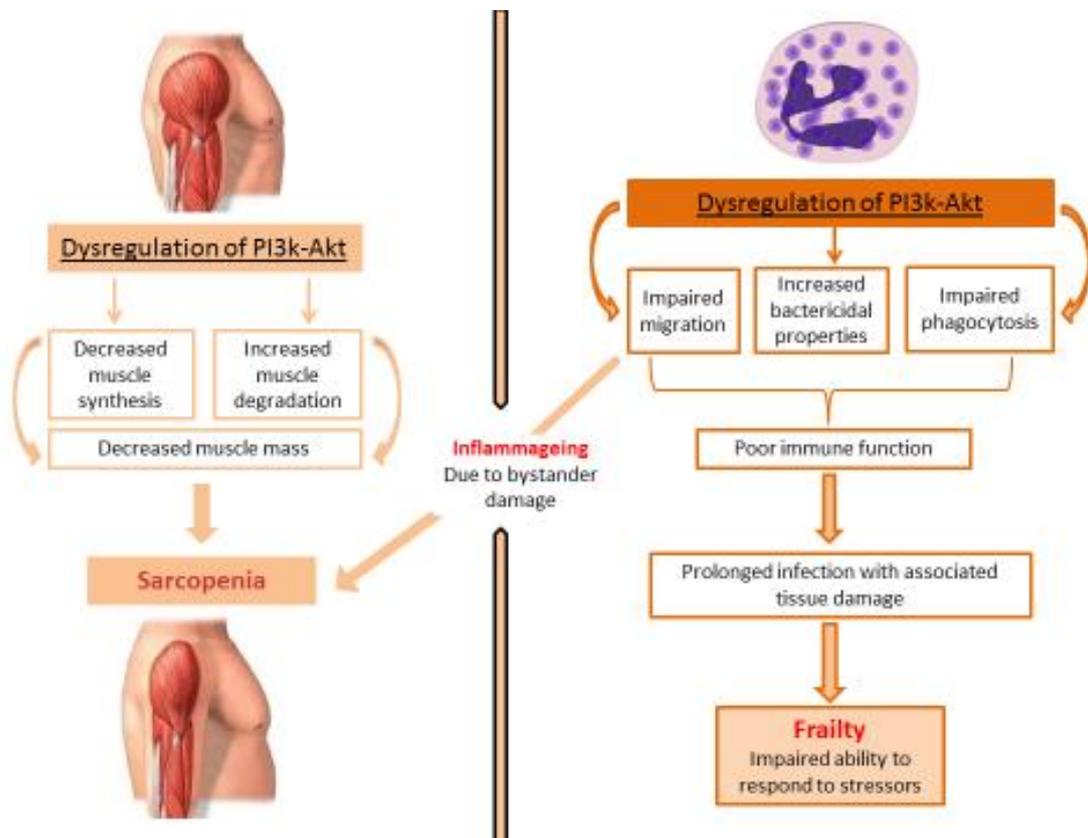
1.12 PI3kinase/Akt pathway, immunesenescence, frailty and sarcopenia

Dysregulation of the PI3K/Akt pathway with ageing could be a fundamental mechanism via which inflammaging, sarcopenia and ultimately frailty occurs and could explain some of the complex interplay between the three conditions. The central role the pathway plays in muscle synthesis and atrophy via various pathways is likely to contribute to the muscle loss experienced in sarcopenia. Studies have already demonstrated that neutrophils from PI3K γ deficient mice have impaired migration and respiratory burst (232). Not only does this result in a poorer immune response to a pathogen with the associated increased localised and systemic damage but results in increased localised tissue damage (233). The PI3K/Akt pathway is also fundamental to the removal of primed neutrophils from circulation via apoptosis (213). Dysregulation of the PI3K/Akt pathway in this instance will allow neutrophil death by necrosis with the subsequent release of tissue damaging proteases (57). The delay in neutrophil death will propagate the ongoing functions of the neutrophil including release of cytokines and chemokines, in particular TNF α and IL-6 (57). These cytokines, fundamental to the process of inflammaging, will both exacerbate the increased muscle breakdown via the NF κ B ubiquitin proteasome pathway and dysregulate the PI3K/Akt/FoxO pathway of protein synthesis and breakdown further via the inhibitory effect of cytokines on the IGF-1 initiated pathways. The cytokines will also act to recruit further neutrophils potentiating a vicious circle of forward feedback.

Recent data from our group has identified the mechanism underlying reduced chemotaxis in neutrophils from old donors. Inaccurate chemotaxis was associated with

reduced activation of PI3K in response to chemoattractants. This reduced signalling was found to be due to constitutive PI3K signalling and selective pharmacological inhibition of PI3K γ or PI3K δ restored neutrophil migratory accuracy (163). Moreover, the reduced neutrophil chemotaxis was associated with increased systemic inflammation in the older donors, most likely due to the tissue damage that occurs during neutrophil migration. However, there was no consideration in this study of the effect of frailty on neutrophil function. It is plausible that the dysregulation of PI3K already demonstrated in the neutrophils of healthy older adults could be occurring concurrently with the dysregulation of PI3K/Akt/FoxO pathway in muscle. The dysregulation could be either, driving sarcopenia and immunosenescence which then descends into frailty and inflammaging, or as a direct consequence of immunosenescence and sarcopenia. Figure 1.6 demonstrates the relationship between dysregulation of PI3k-Akt pathway in neutrophils and muscle during sarcopenia and frailty.

Figure 1.6 - Relationships between dysregulation of PI3k-Akt pathway in neutrophils and muscle during sarcopenia and frailty



Proposed relationships between the dysregulation of PI3kinase-Akt pathway in neutrophils and sarcopenia during sarcopenia and frailty.

The identification of the PI3K/Akt pathway as a probable contributor to sarcopenia and immunosenescence is particularly relevant as there is potential for modification of this pathway resulting in improvement of function. This has been successfully demonstrated using PI3K inhibitors during the chemotaxis of neutrophils of a healthy older cohort (163). Statins have also been demonstrated to improve neutrophil migration in vitro in milder infections (234). Statins reduce the production of all products down-stream of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) including isoprenoid. Isoprenoid is

utilised by cells for post-translational modification and activation of small GTP-ases such as Ras and Rho (235). These molecules are essential in a number of immune cell functions such as adhesion and migration and importantly are also down-stream of the PI3kinase-Akt pathway (235). Adjuvant statins were associated with survival benefit in hospitalised patients with community acquired pneumonia including a cohort of older adults (236). Statins target a downstream effector of PI3kinase and may offer a therapeutic option for immunesenescence in frailty. Angiotensin converting enzyme inhibitors (ACEi) are another potential therapeutic intervention for sarcopenia and immunesenescence. Prospective cohort studies have shown that women taking ACEi, in comparison to no anti-hypertensives and different anti-hypertensives, had improved muscle strength and walking speed (237). Suggesting that an ACEi can attenuate muscle loss and quality in an older population. This effect is attributed to the effect angiotensin has on reducing local IGF-1 production and impaired insulin signalling and consequently the downstream effects on PI3K/Akt pathway (238). Similarly an immune-modulatory role for ACEi has been demonstrated with evidence from large clinical trials (239).

1.13 Conclusions

The historical difficulties of definition, measurement and modelling in both sarcopenia and frailty in addition to the fundamental difficulties of investigating complex, multi-factorial syndromes has led to a lack of information regarding the pathophysiological causes of frailty and sarcopenia. Whilst there is some limited research into associations there is very little investigating more complex pathways attempting to link the two syndromes. The suggested link of increased systemic inflammation could explain the relationship between sarcopenia and immunesenescence, with aberrant neutrophil

migration potentially contributing to inflammaging and tissue damage associated with the sarcopenia of frailty. Further research on the cell pathways connecting inflammaging, sarcopenia and frailty would help to better understand these conditions and progress to potential novel therapies for frailty and sarcopenia which could include modulation of PI3k to correct neutrophil chemotaxis.

The historical difficulties of definition, measurement and modelling in both sarcopenia and frailty in addition to the fundamental difficulties of investigating complex, multi-factorial syndromes has led to a lack of information regarding the pathophysiological causes of frailty and sarcopenia. Whilst there is some limited research into associations there is practically none investigating more complex pathways attempting to link the two syndromes. The suggested link of a dysregulated PI3K/Akt pathway can elegantly explain the relationship between sarcopenia and immunesenescence and how it can spiral into frailty.

1.14 Structure of this thesis

The studies presented in this thesis were performed to examine some of the relationships between frailty, sarcopenia, immunesenescence and inflammaging and in particular consider the involvement of the intracellular pathway, PI3kinase-Akt pathway.

Chapter 3 presents a new ultrasound scanning protocol and criteria for the identification of low muscle mass in sarcopenia. This was necessary due to the absence in the literature of recommended criteria for the identification of low muscle mass using ultrasound.

Chapter 4 reports on the feasibility of muscle biopsy in healthy and frail older adults using a percutaneous needle muscle biopsy technique.

Chapter 5 examines one, neutrophil migration towards chemoattractants and two, the changes to chemotaxis that occur following: incubation with PI3kinase inhibitors, incubation with pooled plasma from healthy and frail older adults, priming with TNF α . It also investigates potential mechanisms for witnessed differences including: receptor expression, reactive oxygen species and neutrophil state.

Chapter 6 describes frailty within each of the groups and examines the relationships between different definitions of frailty and sarcopenia. It also investigates the relationship of both sarcopenia and frailty with inflammation.

2. Methods

CLINICAL METHODS

2.1 Participants

All studies reported in this thesis were approved by the Solihull ethics committee

(15/WM/0002). Participants were recruited to three groups: healthy young adults,

healthy older adults and frail older adults. 40 participants were recruited to the healthy

young and older adult groups and 37 participants were recruited to the frail older adult

group. The criteria for recruitment are described in table 2.1. All participants needed to

be able to provide written informed consent.

Table 2.1 - Criteria for recruitment

Group	Age	Clinical Criteria
Healthy Young Adults	18-35	1. Able to provide informed written consent. 2. Absence of
Healthy Older Adults	>65	<ul style="list-style-type: none"> • Significant respiratory symptoms including breathlessness and cough • Diagnosis of chronic disease including Diabetes, COPD, Asthma, Interstitial Lung disease, Bronchiectasis • Haematological or solid organ malignancy • Immunosuppressive medications
Frail Older Adults	>65	1. Able to provide informed written consent 2. Identified as frail on Frailty Index (FI> 0.2) 3. Absence of
		<ul style="list-style-type: none"> • Haematological or solid organ malignancy • Immunosuppressive medications

Table describing the clinical criteria for recruitment to each group: healthy young adults, healthy older adults and frail older adults.

2.2 Recruitment

The healthy young adults were recruited from the University of Birmingham using approved advertisement. The healthy older adults were recruited from the ‘1000 Elders’ database.

The frail older adults were recruited from three sources. The method of recruitment and involvement within the study for the frail older adult participants is described in Figure 2.1.

2.1.

Figure 2.1 - Frail older adult participants: recruitment and involvement in study

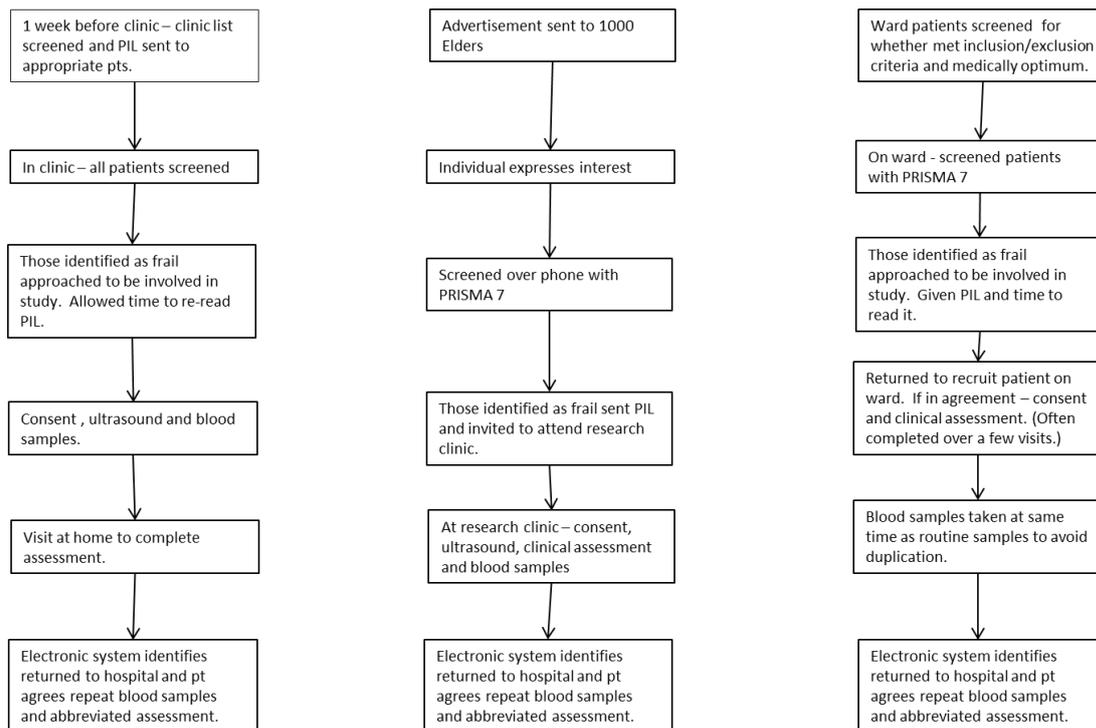


Figure describing the three routes of recruitment of frail older adults to the study. From left to right: participants recruited from a general geriatrics clinic, participants recruited from the 1000 Elders group and participants recruited from medical wards (all medically optimum with no evidence of inter-current infection).

2.3 Clinical Characterisation

The identification and diagnosis of frailty requires rigorous assessment and information from multiple aspects of a participant's life encompassing the biological, psychological, social and functional. Copies of all the assessments can be found in the appendix within the Case Report Form (CRF) or additional documents.

2.3.1 Cognitive Assessments

Two methods of cognitive assessment were selected: Addenbrook's Cognitive Examination III (ACE-III) (240) and Stroop Colour and Word Test (241).

The ACE-III tests five domains: attention, memory, fluency, language and visuo-spatial. It has good validity and reproducibility for a relatively short test (administration of the tests take approximately 15 minutes) (240). The Stroop Colour and Word Test is a psychological test developed to test ability to process information and respond appropriately (242).

2.3.2 Nutritional Assessments

The mini Nutritional Assessment was chosen to assess nutritional status; poor nutrition has been shown to contribute to frailty (243). The assessment comprises of six questions and is considered a screening tool for malnutrition..

2.3.3 Physical Function Assessments

Physical function was assessed by the Short Physical Performance Battery (SPPB)(244).

The SPPB comprises of three tasks evaluating different aspects of physical performance.

The SPPB has been modified for use in the clinical study as the original protocol includes a

2.44 metre walk course; for simplification and reduction of duplicity time over a 4 metre

walk course will be calculated and extrapolated to a 2.44 metre walk course.

2.3.4 Assessments of Independence

The Katz ADL was used to assess basic activities of daily living and Lawton IADL to assess independent living skills. These assessments are extensively used both clinically and in research and can be used in a community, hospitalised or clinic population (245, 246).

The Katz and Lawton assessments are brief questionnaires evaluating important activities of independence.

2.4 Frailty Identification

There is currently no consensus for a gold standard model for diagnosing frailty either in clinical practice or research. Therefore, due to the uncertainties in frailty research this study has included six frailty identification tools as described in table 2.2. All assessments are included within the CRF in the appendix.

Table 2.2 -Summary of frailty identification methods

	Tests	Cut Points for Frailty
Frailty Identification	PRISMA 7 (14)	= > 3
	Gait speed (14)	< 0.8m/sec
	Clinical Frailty Scale (15)	= > 5
	Edmonton Frail Scale (18)	= > 12 (6-11 Vulnerable)
Diagnosis in Research	Frailty Phenotype (5)	= > 3
	Frailty Index (7)	> 0.2

Table summarising the different frailty identification methods included within the study's comprehensive geriatric assessment.

2.4.1 Frailty Identification Methods

The gait speed is a good indicator of physical function and is utilised in other assessments present in the study: diagnosis of sarcopenia, short physical performance battery and diagnosis of frailty by Fried Phenotype (14). PRISMA 7, in contrast to gait speed reflects

multiple domains, mobility, social support and health, and is therefore more akin to the Frailty Index rather than the Frailty Phenotype (14). The Edmonton Frail Scale (EFS) is also a multi-dimensional approach to the assessment of frailty whereas the Clinical Frailty Scale (CFS) is based on a clinician's global assessment of an individual with standardised criteria.

2.4.2 Frailty Diagnosis in Research

Frailty Phenotype and Frailty Index have both been included as tools to identify frailty in research. The Frailty Phenotype criteria are described in table 2.3 and the variables included in Frailty Index are described in table 2.4. The Frailty Phenotype describes physical characteristics identifying a frail individual; individuals positive in two criteria are considered pre-frail and those positive in three criteria are considered frail (5). The Frailty Index is a compound measure incorporating multiple criteria (7). Traditionally frailty measured by the Frailty Index is considered a continuous variable rather than a present or absent phenomenon; however, a score greater than 0.2 has been described as being diagnostic of a degree of frailty (247).

Table 2.3 - Criteria for Fried frailty phenotype

Frailty Phenotype	Fried Criteria	Study Criteria
Weight Loss	Unintentional weight loss of 10 lbs (4.5kg) in the last year	Standard parameters from original paper (5).
Exhaustion	How often did you feel in the last week that: a) Everything was an effort b) Could not get going 0-rarely, 1-some of the time, 2-moderate amount of time, 3-most of the time	Standard parameters from original paper (5). (Modified from CES-D scale.)
Weakness	Grip strength in the lowest 20% after adjustment for gender and BMI.	Standard parameters from original paper (5).
Slowness	Walk speed in the slowest 20% after adjustment for gender and height	Calculated speeds from original paper applied to a 4 metre rather than 15 foot walk course (5).
Low Activity	In the lowest 20% of calories expended per week using Minnesota Leisure Time Physical Activity Questionnaire	Parameter as measured in Frailty Intervention Trial (248). (Positive for all three - No weight bearing physical activity, more than 4 hours spent sitting per day, short walk once per month or less.)

Table explaining the original criteria included in the Fried frailty phenotype and how these have been modified for this study. The statement ‘standard parameters from original paper’ pertains to the quoted cut points from the original Fried paper not the creation of new cut points using the lowest 20% of the study population.

Table 2.4 - Variables included in Frailty Index

Domain	FI Variable	Rockwood Variable	Scoring Criteria
Medical	Stroke	Yes	Y=1, N=0
	Myocardial Infarction	Yes	Y=1, N=0
	Congestive Cardiac Failure	Yes	Y=1, N=0
	Diabetes Mellitus	Yes	Y=1, N=0
	COPD	Yes	Y=1, N=0
	Cancer	Yes	Y=1, N=0
	Polypharmacy	No	>10=1, 4-10=0.5, <4=0
	Continence	No	Partially/totally incontinent=1, Lose control of urine when don't want to =0.5, No concerns with continence = 0
	Self-reported health	Yes	1,2 = 1, 3=0.5, 4,5= 0
	Self-reported weight loss – in the last year have you lost more than 4.5kg?	Yes	Y=1, Clothing is looser = 0.5, N=0
	BMI	Yes	<18.5 or >30=1, 25-30=0.5
	Physician assessment of CFS	No	7,8=1, 6=0.75, 5=0.5, 4=0.25, 1,2,3=0
	Food intake	No	Severe decrease=1, Moderate decrease=0.5, No decrease=0
Psychological	Self-reported low mood - do you often feel sad or depressed?	Yes	Y=1, N=0
Independence	Bathing	Yes	Needs help =1, Independent=0 (use Katz scoring system but reversed)
	Dressing	Yes	Needs help =1, Independent=0 (use Katz scoring system but reversed)
	Transferring	Yes	Needs help =1, Independent=0 (use Katz scoring system but reversed)
	Feeding	Yes	Needs help =1, Independent=0 (use Katz scoring system but reversed)

Independence	Toileting	Yes	Needs help =1, Independent=0 (use Katz scoring system but reversed)
	Finances	Yes	Needs help =1, Independent=0 (use Lawton scoring system but reversed)
	Shopping	Yes	Needs help =1, Independent=0 (use Lawton scoring system but reversed)
	Housework	Yes	Needs help =1, Independent=0 (use Lawton scoring system but reversed)
	Meal Preparation	Yes	Needs help =1, Independent=0 (use Lawton scoring system but reversed)
	Medications	Yes	Needs help =1, Independent=0 (use Lawton scoring system but reversed)
Cognition	Addenbrooke's Cognitive Examination	Yes	<82=1, >82=0
Physical Function	Walk Speed	Yes	Frailty phenotype 'slowness' positive=1, <0.8=0.5, >0.8=0
	Falls in last year	No	Y=1, N=0
	Everything is an effort	Yes	Most of the time =1, Moderate amount of time =0.75, Some of the time =0.5, None of the time =0
	Trouble getting going	Yes	Most of the time =1, Moderate amount of time =0.75, Some of the time =0.5, None of the time =0
	Handgrip	Yes	Lowest 20% for gender/BMI = 1, Highest 80% for gender/BMI =0

Table describing the variables included in the Frailty Index and the criteria for scoring. All variables summed together and divided by 30 to give a compound continuous measure of frailty.

2.4.3 Frailty Diagnosis in Clinical Practice

The Clinical Frailty Scale (CFS) (15) and the Edmonton Frail Scale (EFS) (18) were both included as identification tools as the CFS is more likely to be used by geriatricians and the EFS by other practitioners.

The CFS, is based upon a comprehensive geriatric assessment (CGA) and only really appropriate for use by a geriatrician or persons trained in the use of CGA. The CGA includes an assessment of multiple domains: medical, psychological, social circumstances, functional capacity and environment.

The EFS is a quick method of diagnosis which does not require the clinician to have any experience in CGA and was originally intended for general practitioners (18). In theory this is more akin to the Frailty Index than the Frailty Phenotype and includes assessment of several domains: cognition, general health, functional independence, social support. The EFS has been slightly modified for this study, again to avoid duplicity. Functional performance in the original paper was assessed by a timed get up and goes but this has been substituted for a SPPB another assessment of functional performance.

2.5 Sarcopenia

A diagnosis of sarcopenia requires measurement of muscle mass, muscle strength and physical function. Table 2.5 summarises the tools and equipment utilised to identify sarcopenia as well as the criteria for a positive diagnosis.

Table 2.5 – Summary of diagnosis of sarcopenia

Component of sarcopenia	Method of identification	Equipment	Criteria for diagnosis
Muscle Mass	Muscle Thickness by ultrasound	Acuson Antares Premium Edition (Siemens) Voluson-i (GE Healthcare)	BATT – Women < 38.55mm Men < 54.36mm
Muscle Strength	Grip Strength	Calibrated Takei analogue hand grip dynamometer using the NIH toolbox protocol	Women: BMI ≤ 23 ≤ 17 kg BMI 23.1–26 ≤ 17.3 kg BMI 26.1–29 ≤ 18 kg BMI > 29 ≤ 21 kg Men: BMI ≤ 24 ≤ 29 kg BMI 24.1–26 ≤ 30 kg BMI 26.1–28 ≤ 30 kg BMI > 28 ≤ 32 kg
Physical Function	Gait Speed – 4 metre walk course at average speed		<0.8 m/sec

Table summarising the methods of diagnosis for each different component of sarcopenia including method of identification, equipment and criteria for diagnosis.

2.6 Ultrasound as a diagnostic tool in sarcopenia

2.6.1 Participant protocols

Depending on the participant there were three different protocols; the protocols are explained in greater detail below in table 2.6. The muscle thicknesses measured by the different sonographers and using different machines were not measured independently of each other.

Table 2.6 – Protocols adhered to within study subsections

	Protocol 1	Protocol 2	Protocol 3
Sonographer	DW	DW <u>and</u> HS	HS <u>and</u> HM
Ultrasound Machine	Accuson static machine <u>or</u> Voluson portable machine depending on location of participant	Accuson static machine <u>and</u> Voluson portable machine	Accuson static machine
Muscle measurements	Rectus femoris, vastus intermedius and anterior thigh subcutaneous tissue bilaterally	Rectus femoris, vastus intermedius and anterior thigh subcutaneous tissue bilaterally <u>and</u> Biceps brachii and forearm flexors bilaterally	Rectus femoris, vastus intermedius and anterior thigh subcutaneous tissue bilaterally <u>and</u> Biceps brachii and forearm flexors bilaterally

Table describing the three protocols used with the study sub-section including the sonographer, muscle thicknesses measured and the ultrasound machines (manufacturers and models given below).

2.6.2 Equipment

Quantitative ultrasound scanning and image capture were completed using two different diagnostic sonography machine Acuson Antares Premium Edition (Siemens) and Voluson-i (GE Healthcare). These machines use slightly different software settings were chosen to closely match. The MSK factory setting was used on the Siemens Acuson and this corresponded well to the ‘Muscle’ factory setting of the Voluson-i scanner. Both ultrasound machines used a gain of -3dB and a magnification factor of x1. A depth of 5-7cm could be achieved on both ultrasound machines to ensure all tissues scanned were fully depicted on the screen.

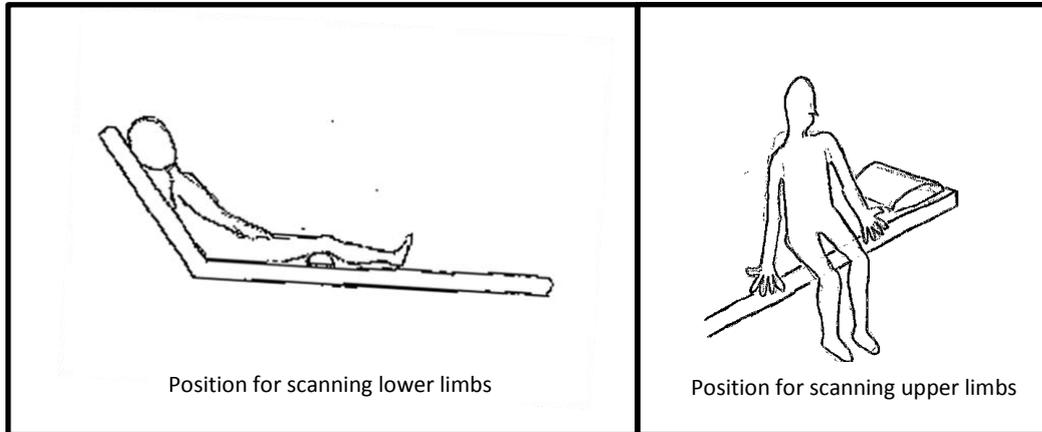
Multi-frequency transducers were used with each machine, however the frequency of each probe was standardised throughout the study. A Vf7-3 probe was used with the Siemens Acuson scanner; this is able to produce a frequency between 3-7MHz and therefore, achieve acceptable penetration in the anterior thigh. This probe was used in its 7.27MHz linear array capacity. An SP10-16-RS probe was used with the Voluson-i scanner and is able to produce a frequency between 7-18MHz. SP10-16-RS is a wide band, multi-frequency linear transducer. This probe was used in its 15-16MHz capacity in the Muscle factory setting mode. Contact gel (Aquasonic 100, Parker Laboratories, Inc., USA) was applied to the skin to minimise distortion of images from excess compression of the skin and subcutaneous tissue.

2.6.3 Participant position

The anterior thigh was measured with participants sitting at an angle greater than 45 degrees with knees resting comfortably in extension near the natural resting position of 10–20°. A purpose designed cushion placed under the knee was used for positioning and participants were instructed to relax their muscles during the examination.

The arm was measured with participants sat on the edge of a couch with their arms hanging freely at rest by their sides in the anatomical position. Figure 2.2 depicts the scanning positions.

Figure 2.2 - Participant position for scanning



Hand drawn figure depicting the positioning of participants for both upper and lower limb ultrasound scanning.

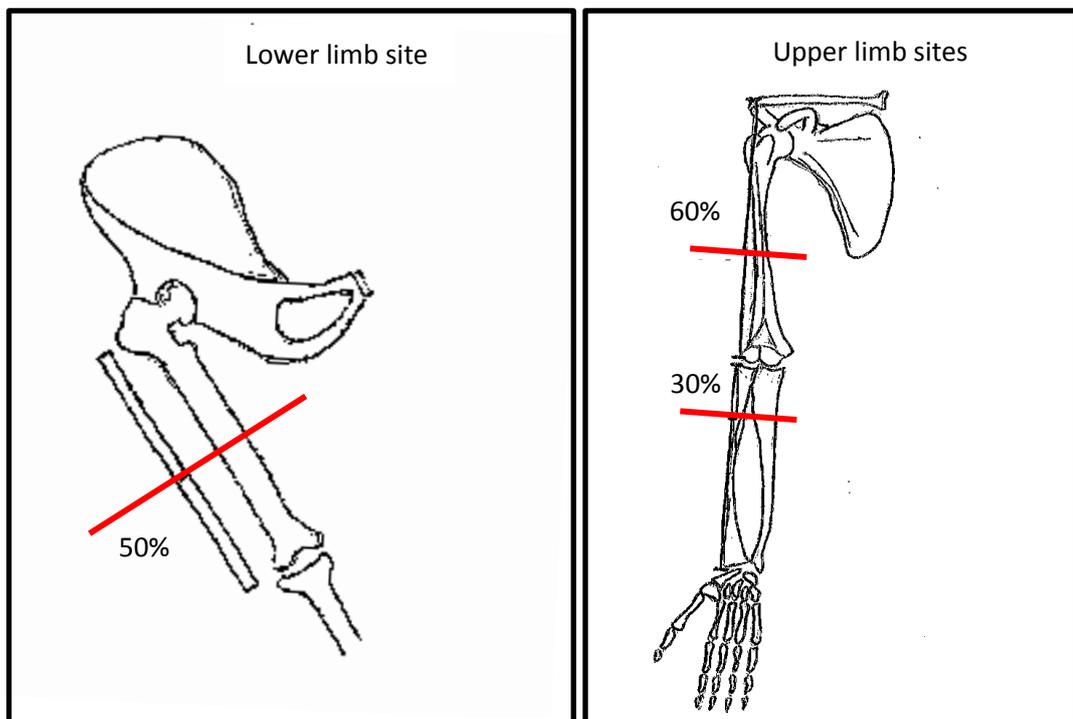
2.6.4 Sites for ultrasound measurement

The identification of locations to be scanned was informed from previous work (249, 250) but modified for inclusion in this study. A pen mark was made at the locations described below and extended transversely to identify the anterior surface location. The rectus femoris, vastus intermedius and subcutaneous tissue of the thigh were all captured in the same image. This image was captured at 50% of the femur length (greater trochanter to the lateral condyle of the femur) on the anterior surface of the thigh. Figure 2.3 for pictorial depiction. In some participants it was impossible to capture complete images of both muscles and the subcutaneous tissue due to greater muscle or fat bulk in their thigh. In these participants an alternative probe with a lower frequency was used to gain sufficient depth to image all the muscles.

The biceps brachii image was captured at 60% proximal humerus length (acromial process of the scapula and the lateral epicondyle of the humerus) on the anterior surface of the upper limb (participant in anatomical position).

The anterior forearm image was captured at 30% proximal radius length (styloid process and the head of the radius) on the anterior surface of the upper limb (participant in anatomical position).

Figure 2.3 - Sites of ultrasound measurement



Hand drawn figure depicting the anatomical sites and landmarks for ultrasound measurements in the lower and upper limb.

2.6.5 Ultrasound measurements

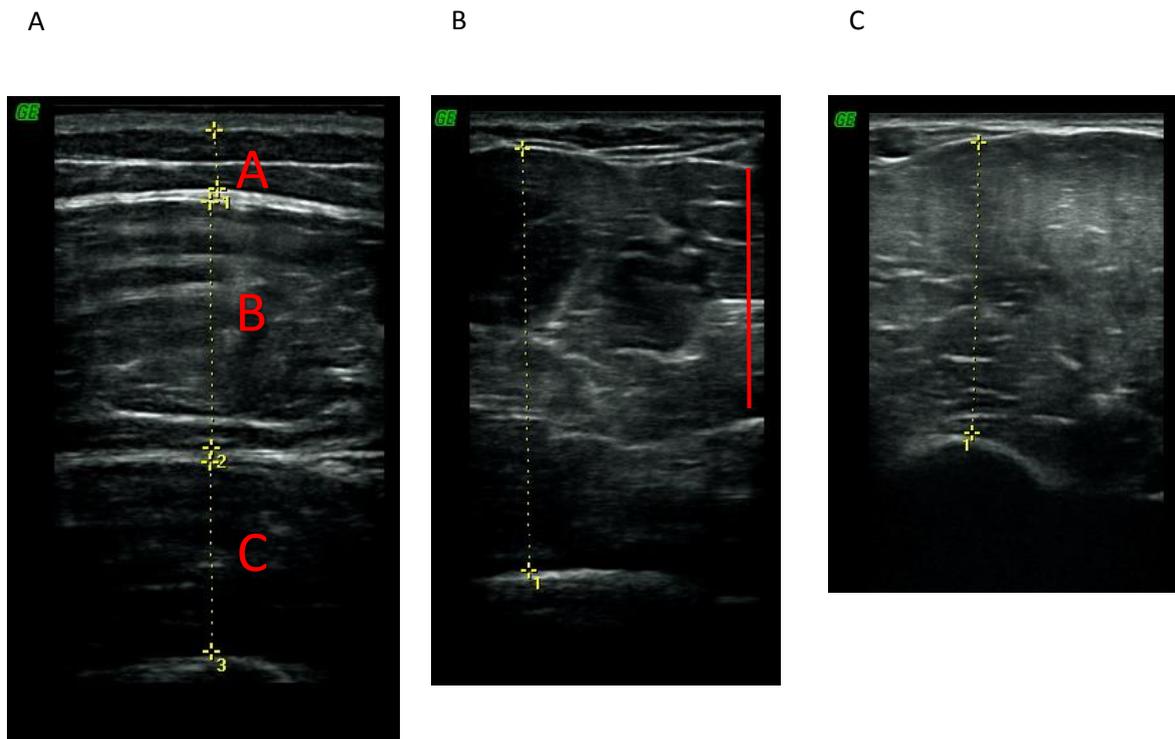
The depths of the muscles were measured in millimetres on freeze frame images using the ultrasound machine software. These images were also saved and exported for grayscale analysis. All images were scanned in the transverse plane (251). Care was taken to avoid exerting excessive pressure on the imaged tissue. In this position three images were captured bilaterally. Maximal muscle thickness was measured as the distance between the superficial and deep fascia at the widest distance. (Figure 2.4) Images were repeated if there was poor concordance between the values and the average value used in analysis. The images were saved and exported for further analysis.

The boundaries used for muscle depth measurements were as follows (superficial → deep):

- Anterior Subcutaneous Tissue: skin-fat interface to fat-fascia interface with rectus femoris
- Rectus femoris: fat-fascia interface to deep fascia-fascia interface with vastus intermedius
- Vastus intermedius: fascia-fascia interface with rectus femoris to fascia-bone interface with femur
- Anterior forearm: fat-fascia interface to muscle/fascia-bone interface with ulna
- Bicep brachii: fat-fascia interface to muscle/fascia-bone interface with humerus

Where applicable, fascia was not included in the depth measurements (e.g., measurement of rectus femoris would begin deep to the superficial fascia and end superficial to the deep fascial boundary).

Figure 2.4 - Sites of muscle measurements



A – Sites of depth measurements in anterior thigh. Depth measurements appear as broken yellow line. Letters correspond to the different tissue measurements. A – subcutaneous tissue, B – rectus femoris, C – vastus intermedius

B – Sites of muscle depth measurement in anterior forearm. Depth measurement of anterior forearm appears as broken yellow line. Depth of forearm muscle to ulna appears as red solid line (unrecorded measurement).

C – Sites of muscle depth measurement in proximal upper arm. Depth measurement of biceps brachii appears as broken yellow line.

2.6.6 Ultrasound echogenicity

To assess for intramuscular adipose tissue exported ultrasound images were analysed on Image J (Version 1.51, US National Institute of Health, USA) using an adapted protocol from a recent paper (252). The greyscale value was obtained by defining a region of interest for analysis. The rectangular marquee tool was used to define a region of interest within the fascial borders of the rectus femoris. Due to the potential differences in echogenicity secondary to the depth of tissue only the rectus femoris was analysed. The captured images had been optimised for muscle measurement and in some instances there are areas of loss of contact. Any areas of loss of contact were excluded from the region of interest. The largest rectangle possible was used due to the observed heterogeneity of muscle quality within participants. The mean echo intensity of this region was calculated (black = 0, white = 255). The values from all available images were averaged.

LABORATORY METHODS

2.7 Blood samples

Blood samples were all taken peripherally using the BD vacutainer® Safety-Lok™ system of blood collection. Blood was collected in sterile vacutainers (BD biosciences). All blood samples were processed within 30 minutes of collection. The type of vacutainer used for collection was dependent on the experiments to be performed, as described in table 2.7.

Table 2.7 - Table describing types of vacutainers used for each experiment

Colour of tube	Additive	Experiments
Green	Lithium heparin	Isolation of neutrophils Chemotaxis Neutrophil phenotyping Western Blotting IL-8 and fMLP expression Plasma samples
Red	Nil	Serum samples

Table describing the vacutainers used for each experiment.

2.8 Reagents

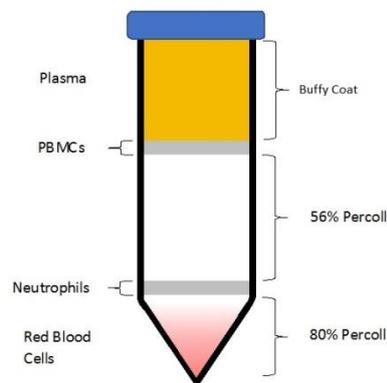
Unless described otherwise RPMI was supplemented with GPS (L-Glutamin, 100 units of penicillin and 0.1mg streptomycin per ml).

2.9 Isolation of neutrophils

Neutrophil Isolation was carried out using a Percoll gradient method (253), for rapid separation of minimally activated neutrophils (254). Peripheral blood, collected in lithium heparin vacutainers, was transferred to 50ml Falcon tubes. 2% dextran (Pharmacosmos) was added to the blood at a ratio of one to six (dextran: blood) and gently inverted. This was then incubated for 30 minutes generating leukocyte rich plasma, layered above the sedimented red blood cells. The plasma layer was removed

and layered onto a Percoll density gradient (pH 8.5-9.5; GE Healthcare), 80% Percoll (2.5ml) under 56% Percoll (5ml). The gradients were spun for 20 minutes at 280g in a freestanding centrifuge (Jouan C3i Centrifuge, DJB Labcare) with no break or acceleration at 20^oC. (Figure 2.5)

Figure 2.5 - Resultant layers in Percoll density gradient following spin in centrifuge



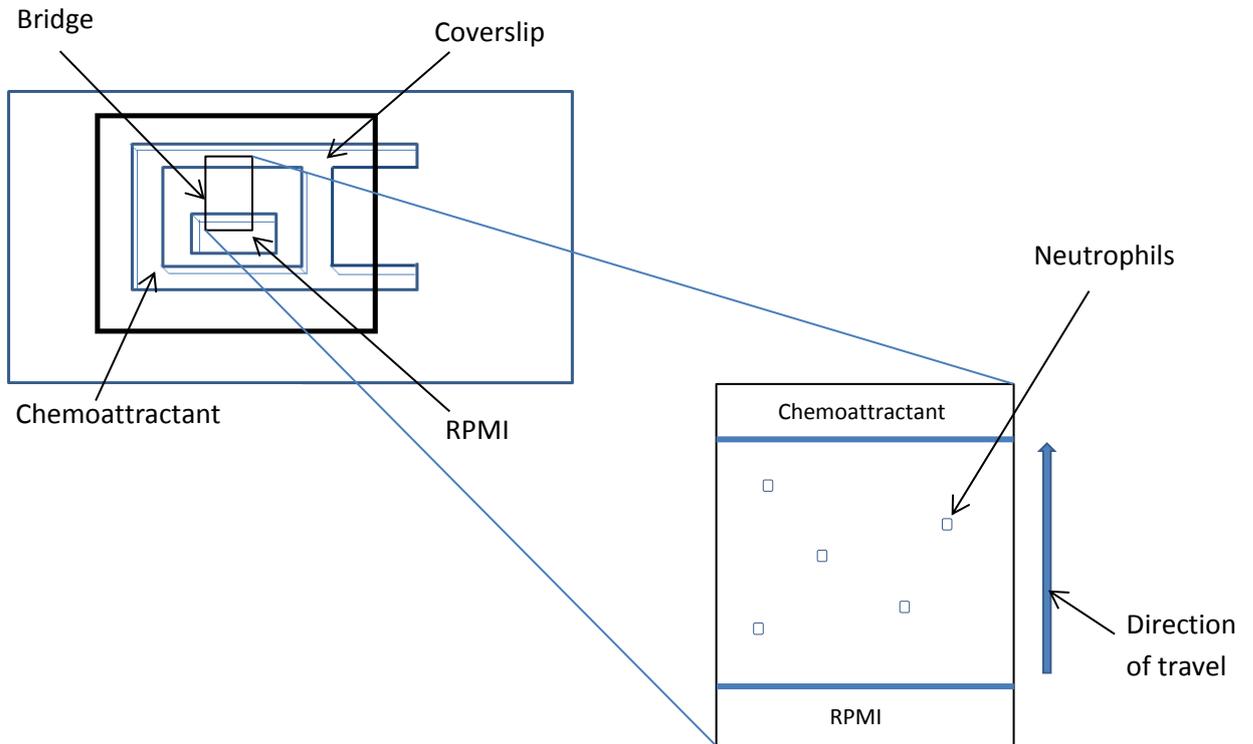
Resultant layers in a Percoll density gradient following spin in centrifuge for 20 minutes at 280g.

Following centrifuge the neutrophils, resident at the 80-56% interface, were removed with a Pasteur pipette and transferred to sterile RPMI-1640 media (Sigma Aldrich). This was pelleted by spinning in a centrifuge at 350g for 10 minutes at 20^oC (Jouan C3i Centrifuge, DJB Labcare). Following centrifugation the supernatant was discarded and the cells resuspended in RPMI-1640 media (Sigma Aldrich). Purity was determined by cyto-spin, regularly greater than 95%, and viability was determined by trypan blue exclusion, regularly greater than 98%.

2.10 Migratory accuracy of neutrophils

1.125% bovine albumin (Life Technologies) was added to neutrophils at a concentration of 2.0×10^6 . Coverslips (22x22mm, VWR International) were cleaned by acid washing (0.4M H_2SO_4) and then coated with 7.5% bovine albumin fraction (Life Technologies). 400 μ l of neutrophil suspension at 2×10^6 was added dropwise to the cleaned and coated coverslips and left for 20 minutes. Concurrently the Insall Chamber (Weber Scientific International Ltd, Teddington) was washed with RPMI-1640 media (Sigma Aldrich) and loaded with RPMI-1640 media (Sigma Aldrich). After 20 minutes the excess fluid from the coverslip was removed by simple inversion. The coverslip was then immediately inverted over the Insall Chamber. The RPMI-1640 was removed from the Insall Chamber using blotting paper and replaced with either RPMI-1640, the vehicle control, or 10 nM fMLP (Sigma Aldrich) or 100 nM IL-8 (R&D), chemoattractants. (Figure 2.6) The gradient was allowed to develop over at least a minute before commencing video microscopy. The area of the slide imaged was consistent throughout all the experiments.

Figure 2.6 – Insall chamber with inverted coverslip



Insall chamber with inverted coverslip. Neutrophils adhered to the bovine serum albumin on the coverslip during period of incubation. Coverslip inverted over both wells initially filled with RPMI. RPMI in outer 'A' shaped well removed with blotting paper and replaced with chemoattractant to establish a gradient over the bridge for the neutrophils to migrate towards.

Chemotaxis was assessed using real-time video microscopy. Images were captured by a Leica DMI6000B with DFC360FX camera which captured 36 frames over a 12 minute period with a 20 second delay between frames. The images were analysed using Image J software (Wayne Rasband, NIH, Bethesda). The slice remover plug-in was used to produce a series of 18 images from the original 36 exported from the microscope. The manual tracking plug-in was used to track the movement of ten randomly selected neutrophils per experiment. These were randomly chosen using the grid function on

Image J and a random number generator (RANDOM.ORG). Only neutrophils deemed to be migrating by morphology and those that did not collide with other neutrophils were analysed. Neutrophils were deemed to be migrating if there was a leading edge visible, there was movement of more than half a cell length and there was no visible pinning of part of the neutrophil that might prevent full migration. Four parameters were assessed: chemokinesis, chemotaxis, directional persistence and chemotactic index.

Chemokinesis, also described as the speed of a cell, is the distance travelled by a cell between two frames measured in $\mu\text{m}/\text{min}$. This is the non-specific movement of the cell in any direction.

Chemotaxis of the neutrophil is the migrational speed of the cell in a consistent direction towards the chemoattractant source also measured in $\mu\text{m}/\text{min}$. This is a measure of the speed of migration towards a chemoattractant source.

Directional persistence is a measure of continuity of cell orientation over time. It is calculated by the cosine of an angle between directions in consecutive frames and is expressed on a scale of 0 to 1. This determines how frequently and rapidly the cells change direction during migration. The closer the cell's direction is to a straight line the higher the persistence.

Chemotactic index is a measure of accuracy of a cell's directional orientation. It is calculated by the cosine of the angle between the cell's direction and the orientation of the chemoattractant gradient. It is expressed on a scale of -1 to 1. It is an overall measure of how accurate and how rapid a cells is in migrating towards a chemoattractant source.

2.11 Chemotaxis of neutrophils incubated with PI3K inhibitors

To investigate the effect of PI3K inhibition on neutrophil migration neutrophils were incubated with selective inhibitors of PI3K isoforms α , β , γ and δ . Stock solutions were prepared using dimethyl sulfoxide (DMSO, Sigma Aldrich) further dilutions to achieve the half maximal inhibitory concentration (enzymatic IC₅₀) were completed with RPMI.

Neutrophils isolated at 2×10^6 were incubated with the different PI3K inhibitors for 40 minutes (20 minutes in eppendorf and 20 minutes on coverslip). (Table 2.8) DMSO was also included in the chemotaxis assay as an appropriate vehicle control. Neutrophils incubated with PI3K inhibitors or DMSO were then migrated towards 100nM IL-8 as described above.

Table 2.8 – Concentration, names and manufacturer of PI3K inhibitors

Isoform	Inhibitor Name	Concentration/IC ₅₀
P110 α	PIK-75 (Chemie Tek; CT-PIK750)	7.8nM
P110 β	TGX-221 (Selleck Chemicals; S1169)	10nM
P110 γ	AS-252424 (Selleck Chemicals; S2671)	33nM
P110 δ	CAL-101 (Selleck Chemicals; S2226)	65nM

Inhibitor names, manufacturers and concentrations used in experiments for the PI3kinase inhibitors. Quoted concentrations are enzymatic IC₅₀s.

2.12 Chemotaxis of primed neutrophils over a time-course of increasing rest period

Neutrophils were isolated at the concentration of 1×10^6 and split between two 50ml Falcon tubes: primed neutrophils and vehicle control. $\text{TNF}\alpha$ (Sigma Aldrich) at 1.15nM concentration was added to the neutrophils in the 'primed neutrophils' falcon tube. The primed neutrophils were then incubated for 15 minutes on a rollator. During the incubation of the primed neutrophils the vehicle control neutrophils were washed (350g for 10 mins at 20°C). Once the vehicle control neutrophils had been washed they were re-suspended in RPMI-1640 media (Sigma Aldrich) at a concentration of 2×10^6 and chemotaxis continued as described above towards 10nM fMLP. Following the 15 minute incubation the primed neutrophils were washed (350g for 10 mins at 20°C) and then re-suspended in RPMI-1640 media (Sigma Aldrich) at a concentration of 2×10^6 . The primed neutrophils were then migrated towards 10nM fMLP following a time-course of increasing rest period (see table 2.9 for time-points). The 50ml falcon with the primed neutrophils remained on the rollator during the rest period. Figure 2.7 describes the process in pictorial form.

Table 2.9 - Timings for the time-course of primed neutrophils

Neutrophils	Wash Timing	Time neutrophils applied to coverslip	Time neutrophils applied to slide
VC	0-10	15	35
15	15-25	30	50
15+15	15-25	45	65
15+30	15-25	60	80
15+45	15-25	75	95

Timings for the timecourse of neutrophils primed with $\text{TNF}\alpha$ prior to migration towards fMLP.

Figure 2.7 - Time-course of primed neutrophils

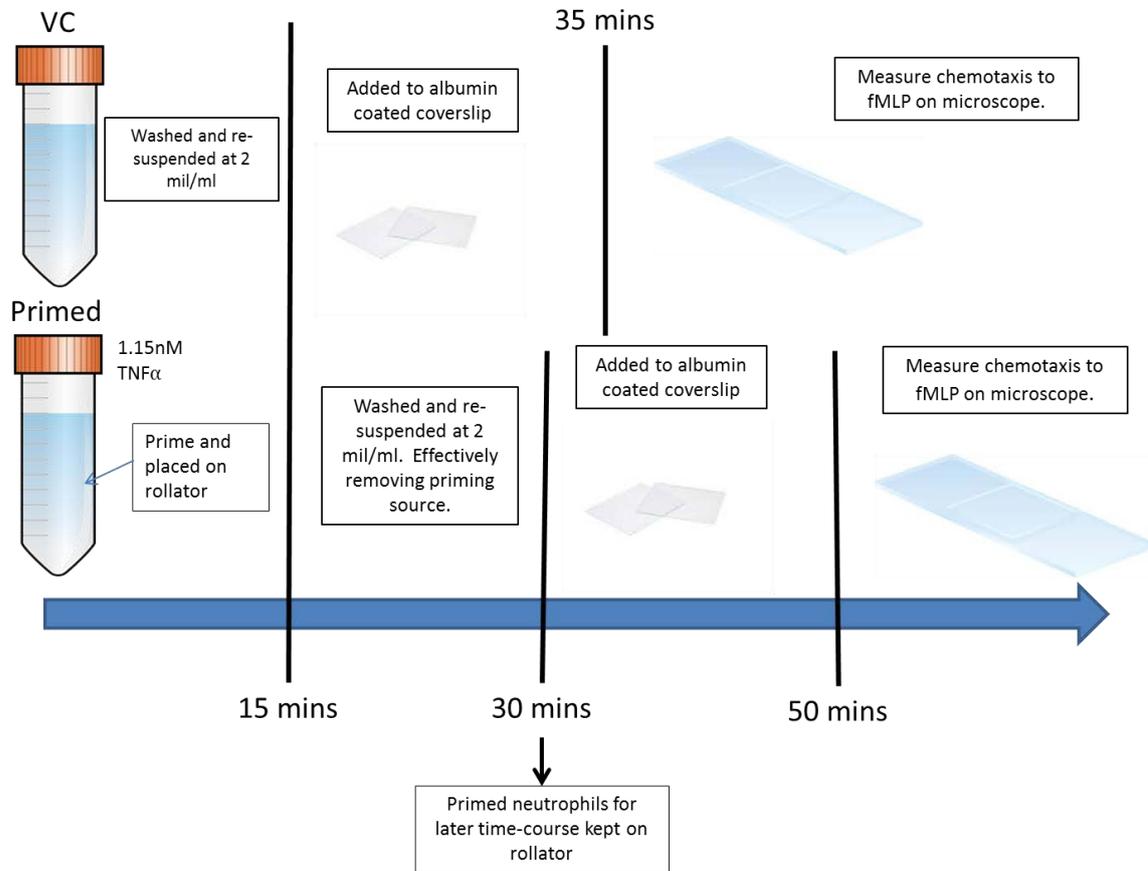


Diagram explaining the timecourse of neutrophils primed with TNF α prior to migration towards fMLP.

2.13 Chemotaxis of healthy young adult's neutrophils towards pooled plasma

Healthy young adult's neutrophils were bathed in either RPMI-1640 (Sigma Aldrich), pooled plasma from healthy older adults or pooled plasma from a frail older adults to assess migration following incubation with pooled plasma. Plasma samples from 18 healthy older adults were combined to create pooled plasma of healthy older adults and plasma samples from 18 frail older adults were combined to create pooled plasma of frail older adults. Isolated neutrophils at the concentration 4×10^6 were incubated with either

RPMI-1640, pooled plasma from healthy older adults or pooled plasma from a frail older adult for a period of 40 minutes. The neutrophils were then pelleted in a centrifuge (400g for 10 minutes at 20^oc) and resuspended in RPMI-1640 at 2 x10⁶. Chemotaxis was performed as described above towards RPMI and IL-8.

2.14 Neutrophil phenotyping

To determine whether neutrophils from frail older participants display a different phenotype to healthy older participants and healthy younger participants the expression of different surface markers was quantified by flow cytometry. To reduce the risk of activation during the process neutrophils were not isolated from whole blood and instead whole blood was used and the neutrophil population gated for analysis. For each set of antibodies a stimulated and un-stimulated test was performed. IgG1 and IgG2 isotype matched irrelevant antibodies were also used at the same concentration to control for non-specific binding.

Whole blood was collected in a lithium heparin vacutainer and pelleted by centrifuge at 250g for 10 minutes. The plasma layer was removed from the centrifuged blood by Pasteur pipette. 2mls of the autologous plasma (AP) was added to 2% phosphate buffer solution (PBS)(Gibco)/bovine serum albumin (BSA)(Fisher Scientific) to make 2% PBS/BSA + 5% AP. The pellet of erythrocytes was re-suspended and the plasma volume initially removed was replaced with the same volume of 2%PBS/BSA + 5%AP. 100µl of red blood cells re-suspended in 2% PBS/BSA + 5% AP was pipetted into each FACS tube.

10nM fMLP (Sigma Aldrich) diluted in 2% PBS/BSA + 5% AP was added to the stimulated condition FACS tubes and incubated for 5 minutes at room temperature. Following the

incubation all FACS tubes were placed on ice and the antibodies added as follows. For each group a stimulated and unstimulated test were performed. Following the addition of antibodies all FACS tubes were then incubated for 20 minutes, on ice, in the dark to allow for antibody/receptor binding.

Table 2.10 - Antibody and isotype concentrations and manufacturer

Stimulated and Unstimulated Tests				Isotype Tests			
Group	Antibody	Manufacturer	Concentration (µg/µl)	Group	Antibody	Manufacturer	Concentration (µg/µl)
Group 1	CD16 – PE-Cy7	ebioscience, San Diego, Ca	0.03/5	Group 1	mIgG1 - PE-Cy7	Biolegend, San Diego, Ca	0.03/5
	CD49F - FITC	Biolegend, San Diego, Ca	1/5		rlgG2a - FITC	Biolegend, San Diego, Ca	1/5
	PE anti-human Integrin α9β1	Biolegend, San Diego, Ca	1/5		mIgG1 - PE	Biolegend, San Diego, Ca	1/5
	APC anti-human CD29	Biolegend, San Diego, Ca	1/5		mIgG1 - APC	Biolegend, San Diego, Ca	1/5
Group 2	CD16 - PE-Cy7	ebioscience, San Diego, Ca	0.03/5	Group 2	mIgG1 - PE-Cy7	Biolegend, San Diego, Ca	0.03/5
	CD11b - PE	R+D, Mineapolis, Mn	0.5/20		mIgG1 - APC	Biolegend, San Diego, Ca	1/5
	CD11a - FITC	R+D, Mineapolis, Mn	1.2/40		mIgG2b - PE	Southern Biotech, Birmingham, Al	0.5/20
	CD18 – APC	Biolegend, San Diego, Ca	0.5/3.33		mIgG1 - FITC	Southern Biotech, Birmingham, Al	0.2/2.5
Group 3	CD16 - PE-Cy7	ebioscience, San Diego, Ca	0.03/5	Group 3	mIgG1-PE - Cy7	Biolegend, San Diego, Ca	0.03/5
	CD11b – PE	R+D, Mineapolis, Mn	0.5/20		mIgG2b - PE	Southern Biotech, Birmingham, Al	0.5/20
	FITC anti-human CD11b	Biolegend, San Diego, Ca	2/5		mIgG1 - FITC	Southern Biotech, Birmingham, Al	2/5
Group 4	CD16 - PE-Cy7	ebioscience, San Diego, Ca	0.03/5	Group 4	mIgG1 - PE-Cy7	Biolegend, San Diego, Ca	0.03/5
	APC anti-human CD26L	ebioscience, San Diego, Ca	0.3/3		mIgG1 - PE	Biolegend, San Diego, Ca	1/5
	CD63 - PE	Invitrogen, Frederick, Md	0.5/5		mIgG1 - APC	Biolegend, San Diego, Ca	1/5
	CD181 - FITC	ebioscience, San Diego, Ca	0.4/4		mIgG2b - FITC	Biolegend, San Diego, Ca	2

Stimulated and Unstimulated Tests				Isotype Tests			
Group	Antibody	Manufacturer	Concentration (µg/µl)	Group	Antibody	Manufacturer	Concentration (µg/µl)
Group 5	CD16 PE-Cy7	ebioscience, San Diego, Ca	0.03/5	Group 5	mIgG1 - PE-Cy7	Biolegend, San Diego, Ca	0.03/5
	CD184 – APC	ebioscience, San Diego, Ca	0.25/5		mIgG2a - APC	Biolegend, San Diego, Ca	1.25
	CD182 – PE	ebioscience, San Diego, Ca	0.25/5		mIgG1 - PE	Biolegend, San Diego, Ca	1.25
	CD66b - FITC	ebioscience, San Diego, Ca	0.3/3		mIgM - FITC	Biolegend, San Diego, Ca	1.5

Antibody and isotype concentrations and manufacturers. Antibodies given as the groups analysed within.

The cells were then washed at 250g, 4^oc for 5 minutes in 2% PBS/BSA + 5% AP using a centrifuge (Jouan C3i Centrifuge, DJB Labcare). The supernatant was carefully removed from each FACS tube with a Pasteur pipette. 2mls of lysis solution, 1 FACS lyse (BD Biosciences, Oxford UK): 9 ddH₂O, was added to each FACS tube to remove the erythrocytes. The cells with lysis solution were incubated for 10 minutes at room temperature. The cells were then washed twice at 250g, 4^oc for 5 minutes; the second time with 2% PBS/BSA + 5% AP and then re-suspended in 100µl of 2% PBS/BSA + 5% AP.

The samples were immediately analysed on a BD Accuri C6 Flow Cytometer (BD Biosciences). 10,000 cells were analysed in each sample and the neutrophil population was identified by assessing forward and sideways light scattering. Forward scatter equates to cell size and side scatter to cell granularity. Gating was used to ensure only signals from the neutrophil population were analysed. Neutrophil fluorescence from those labelled with specific receptor antibodies were determined relative to those labelled with the analogous non-specific antibody by subtraction of the isotype Median Fluorescence Intensity (MFI) value from that of the receptor antibody.

2.15 Neutrophil Phenotyping by CD16 and CD62L

To consider the effect of cell maturity samples subject to condition 4 which included exposure to both the antibodies, CD16 and CD62L, were gated using the isotypes as a negative control. The baseline sample without exposure to stimulation was used as a comparison.

2.16 Western Blots

To determine whether the expression of pAKT is reduced and delayed in frail older adults Western blots were performed.

2.16.1 Preparation of Western lysates

1 ml of isolated neutrophils at a concentration of 5×10^6 were incubated at 37°C in a petri dish (35mm diameter) for 20 minutes. The supernatant was carefully removed and 100nM IL-8 added for a period of stimulation: 20 sec, 40 sec or 60 sec. For baseline comparison this step was omitted. Following stimulation the supernatant was removed and the petri dish placed on ice. 150 μ l radioimmunoprecipitation assay (RIPA) buffer (see table 2.11 for reagents, concentrations and suppliers) was immediately added to terminate all reactions; the process of dissociation was enhanced with a cell scraper. The supernatant containing the intracellular protein was removed into an Eppendorf, vortexed and kept on ice throughout the experiment. Before storing the lysates at -80°C the protein concentration was determined with a Bicinchoninic acid (BCA) assay.

Table 2.11 - RIPA buffer

Reagent	Concentration	Supplier
PhosStop	Diluted 1 tablet: 10mls RIPA buffer (Sigma Aldrich)	Roche
Protein Inhibitor Cocktail	Diluted 1:10	Sigma Aldrich
Phenylmethanesulfonylfluoride	50mM	Sigma Aldrich
Sodium Fluoride	10mM	Sigma Aldrich
B-glycerophosphate	500mM	Sigma Aldrich
Sodium Vanadate	10mM	Sigma Aldrich

The reagents within RIPA buffer, their concentrations and suppliers.

2.16.2 BCA Assay

A commercially available BCA assay (Thermo-fisher) was used to perform protein concentration determination. Albumin Standard was diluted in accordance with

instructions to provide a range of BCA concentrations from 0 to 2000µg/ml. 12.5µl of each BSA concentration standards and 12.5µl of each sample were pipetted into separate wells in a flat bottomed 96 well plate. 87.5µl of BCA working reagent (50 parts BCA reagent B: 1 part BCA reagent A) was added to each well. Standards and samples were completed in duplicate to improve accuracy. The 96 well plate was incubated in the dark at 37^oC for 30 minutes and immediately analysed using a plate reader and corresponding Gen 5 software (BioTek). The data was exported to Microsoft excel for determination of protein concentration.

2.16.3 Western Blot

The protein concentration of lysates was adjusted to enable loading of 40µg protein per lane. SDS sample treatment buffer (containing 0.05M Trizma, 2% SDS, glycerol and 10% β-mercaptoethanol) was added. Proteins were separated using a 10% acrylamide gel and then transferred onto a nitrocellulose membrane using wet transfer. Blots were blocked with 5% BSA before probing overnight at 4^oC with 1:750 pAKT Thr308 ((D25E6) XP[®] Rabbit mAB #13038, Cell Signalling Technologies), 1:2000 pAKT Ser473 ((D9E) XP[®] Rabbit mAB #4060, Cell Signalling Technologies) or 1:4000 β-Actin (mouse mAb, clone AC-15, Sigma UK) primary antibodies and appropriate horseradish peroxidase conjugated secondary antibodies. Western blots were visualised using enhanced chemiluminescence (Amersham ECL-Prime, GE healthcare) and Chemidoc[™] MP (BioRad, UK). Density of the protein was assessed using Image Lab 5.2.1 software (BioRad, UK).

2.17 Oxidative burst

The oxidative burst protocol was devised to investigate whether neutrophils primed for the chemotaxis assay were stimulated rather than primed. Neutrophils were isolated as

described above at the concentration of 2×10^6 and 500 μ l of isolated neutrophils were pipetted into individual eppendorfs.

1.15nM (10ng/ml) TNF α was added to the isolated neutrophils primed conditions and vortexed. Following a 15 minutes period of priming the neutrophils were washed at 250g, 4^{oc} for 5 minutes in a centrifuge (Jouan C3i Centrifuge, DJB Labcare) and then resuspended in RPMI. The neutrophils were then stimulated with 5 μ l of 1 μ M fMLP for 25 minutes. Following 10 minutes of stimulation with fMLP 2.8 μ l of 30mM dihydrorhodamine-123 (DHR) or 2.8 μ l DMSO was added to the Eppendorf and were incubated for 15 minutes. On completion of 25 minutes of stimulation and the simultaneous 15 minutes of incubation with DHR all reactions were terminated with incubation on ice for 10 minutes. The neutrophils were then washed twice at 250g, 4^{oc} for 5 minutes using a centrifuge (Jouan C3i Centrifuge, DJB Labcare) and re-suspended in 50 μ l of FACS fixing solution.

The samples were then immediately analysed on a BD Accuri C6 Flow Cytometer (BD Biosciences). 10,000 cells were analysed in each sample and the neutrophil population was identified by assessing forward and sideways light scattering. Forward scatter equates to cell size and side scatter to cell granularity. Gating was used to ensure only signals from the neutrophil population were analysed. Neutrophil fluorescence from those labelled with DHR was determined relative to those labelled with DMSO. The Median Fluorescence Intensity (MFI) value was quoted.

2.18 Collection and storage of serum and plasma samples

Blood was collected using the BD vacutainer system, green vacutainers for plasma and red vacutainers for serum. The samples were rested for 30 minutes and then pelleted in

a centrifuge at room temperature (plasma 438g for 8 minutes, serum 1539g for 10 minutes). The serum and plasma were then removed using a pipette and stored at -80^{0c}.

2.19 Determination of inflammatory profile

To determine the inflammatory profile cytokine concentrations were measured using multiplex technology and hsCRP was measured using ELISA technology; both protocols were completed according to the manufacturer’s instructions (Bio-Plex Pro Human Cytokine Standard 27-Plex, Bio-Rad; hsCRP kit, IBL International). Table 2.12 displays the cytokines measured with the Human Cytokine Standard 27-Plex.

Table 2.12 – Human Cytokine Standard 27-Plex

IL-1β	IL-10	IFN-γ
IL-1ra	IL-12p	IP-10
IL-2	IL-13	MCP-1
IL-4	IL-15	MIP-1α
IL-5	IL-17A	MIP-1β
IL-6	Eotaxin	PDGF-BB
IL-7	Basic FGF	RANTES
IL-8	G-CSF	TNFα
IL-9	GM-CSF	VEGF

Cytokines measured using the human cytokine standard 27-Plex from Bio-Rad.

Briefly, wells within a 96 well plate were pre-wet with wash solution. 50μl of a 1X antibody bead stock for the aforementioned cytokines was added to each well of the assay plate. Following a 30 second incubation at room temperature the plate was

washed twice with 100µl of wash solution. 50µl of each serum sample, prepared to a 1 in 4 dilution (25µl serum sample: 75µl of Bio-Plex sample diluent), or standard solution was added to the relevant wells and the plate incubated in the dark for 30 minutes at room temperature on an orbital shaker (speed 800-900 rpm; Grant Instruments Limited, Cambridge, UK). Post incubation, the plate was washed three times with 100µl wash buffer and 25µl of 1x detection antibodies was added to all the wells. The plate was then incubated in the dark for 30 minutes at room temperature on an orbital shaker (speed 800-900 rpm). The plate was then washed three times with 100µl of wash buffer and 50µl of 1X streptavidin-RPE was added to all the wells. This was followed by a 10 minute incubation at room temperature on an orbital shaker (speed 800-900 rpm) and three further washes with 100µl wash buffer. The antibody beads were resuspended in 125µl of assay buffer. Cytokine concentrations were then analysed using a Luminex® 100TM instrument (Luminex® Corporation, Austin, Texas, USA).

To determine the hs-CRP concentration of the samples the serum samples were first diluted to 1 in 1000 with the supplied sample diluent. 100µl of diluted serum sample or standard solution was added to the relevant wells in a 96 well plate and the plate was then incubated in the dark for 30 minutes at room temperature on an orbital shaker (speed 800-900 rpm; Grant Instruments Limited, Cambridge, UK). Post incubation, the plate was washed three times with 100µl wash buffer. Following the wash step 100µl of conjugate solution was added to each well and the incubation and wash step repeated as described above. 100µl of chromogen solution was then added to each well and the plate was then incubated in the dark for 10 minutes at room temperature on an orbital shaker (speed 800-900 rpm). Following the incubation 50µl of stopping solution was added to

each well and immediately analysed using a plate reader and corresponding Gen 5 software (BioTek).

2.20 Determination of neutrophil elastase activity

Neutrophil elastase activity was determined by quantification of the amount of elastase-specific fibrinogen breakdown product, A α Val³⁶⁰ within the previously stored serum samples (see section 3.8) using a competition ELISA as previously described (212).

Neutrophil elastase activity was determined on the 18 frailest older individuals and 18 healthiest older individuals as determined by frailty index.

Briefly, rabbit ant-sera raised against free carboxyl group of A α Val³⁶⁰ was incubated with plasma samples at 1:2500 dilution overnight at 4°C. The samples were pipetted into a 96 well tissue culture plate pre-coated with neutrophil elastase cleaved fibrinogen. Free A α Val³⁶⁰ within the samples binds to the degraded fibrinogen and this is detected and subsequently quantified with a europin-conjugated anti-rabbit IgG secondary antibody. All samples were completed in duplicate and an average of the two readings used in further analysis.

2.21 Statistics

Statistical analyses were performed using the SPSS statistical program (version 22.0 Chicago, USA). Data were tested for normality using the Shapiro-Wilk or Kolmogorov Smirnov test and the appropriate parametric (ANOVA, t-test – independent or paired) or non-parametric test (Kruskal-Wallis, Mann-Whitney) was used to compare the groups. Relationships between variables were examined with a Pearson rank correlation test (normally distributed data) or Spearman's correlation test (non-normally distributed data). A p value < 0.05 was considered to be statistically significant where appropriate Bonferroni correction was applied. The reliability of ultrasound measurements was

tested with the intraclass-correlation coefficient and interpreted using the Koo and Li criteria (<0.5 = poor reliability, 0.5-0.75 = moderate reliability, 0.75-0.9 = good reliability, >0.9 = excellent reliability)(255).

3. Ultrasound as a diagnostic tool for the identification of low muscle mass and poor muscle quality in sarcopenia.

3.1 Introduction

Sarcopenia is an under recognised condition which is rarely diagnosed in clinical practice despite poor associated outcomes and evidence of available treatment with benefit (11).

A barrier to recognition in clinical practice is the diagnosis of low muscle mass (an absolute criterion of sarcopenia). Traditional methods such as DXA and CT or MRI are

both expensive and impractical in a frail community based population. Ultrasound is a portable diagnostic tool that could be utilised to diagnose low muscle mass. However,

there are no standardised diagnostic criteria for low muscle mass measured by

ultrasound and criteria quoted for more traditional diagnostic tools, for example dual-energy x-ray absorptiometry (DXA), are not translatable to ultrasound measurements.

Guidance from the international working groups on sarcopenia suggest that low muscle

mass in older adults should be defined as less than two standard deviations from the

mean of a healthy younger population, similar to diagnostic criteria for osteoporosis (11).

The quality of muscle is increasingly recognised as important to physical function as

quantity (256). Recent advances suggest that increased volumes of intramuscular

adipose tissue correlates with poor physical function (257). The current gold standard

diagnostic method of measuring low muscle mass DXA does not provide an assessment of

muscle quality. Ultrasound echogenicity expressed as echo intensity has been previously

utilised in the diagnosis of muscular dystrophy and assessment of muscle quality in

sarcopenia (257-259). Analysis of muscle biopsies has demonstrated that increased

ultrasound echogenicity is due to intramuscular adipose tissue rather than fibrosis within

the muscle (260).

This chapter aims to:

- Present a simple scanning protocol that can be utilised in a clinical setting.
- Determine whether ultrasound is an accurate diagnostic technique in the identification of sarcopenia considering inter-rater, intra-user, inter-instrument reliability.
- Provide the criteria for the diagnosis of low muscle mass in sarcopenia using the suggested simple scanning protocol
- Determine the importance of reviewing muscle quality in older adults.

Results

3.2 Participant Characteristics

3.2.1 Reference population

113 young adult participants, 59 male and 54 female, were assessed by ultrasound to form the reference population. Demographic data are displayed in Table 3.1. The calculated criteria for identification of low muscle mass derived from the appendicular lean muscle mass/ ht^2 (two standard deviations below the mean of a healthy young population) measured by BIA in the reference population are 5.6 kg/m^2 for women and 7.39 kg/m^2 for men. These values are almost identical to criteria previously quoted in the literature (5.5 , 5.67 and 5.67 kg/m^2 in women and 7.26 , 7.25 and 7.23 kg/m^2 in men) from much larger sample sizes and utilising a variety of assessment methods (two standard deviations below the mean of a healthy young population and lowest gender specific 20% in older adults as the benchmarks) (11). This suggests that the sample is representative of a healthy young adult population and the proposed criterion for the diagnosis of low muscle mass in older adults, bilateral anterior thigh thickness (BATT), has been created from an appropriate reference population.

3.2.2 Healthy older adult and frail older adult populations

40 healthy older adults and 31 frail older adults were scanned to form the comparison populations; demographic data are displayed in Table 3.1. The two groups were anthropologically similar with no significant difference in age, height, weight or BMI (median data shown for all data). The healthy older and frail older men were very similar in age, but the frail older women were older than the healthy older women. This was a non-significant difference, $p=0.174$. There was very little difference in the height of the frail older men compared to the healthy older men, HO 1.77m and FO 1.75m , but there

was a trend of the frail older women being shorter than their healthy older counterparts, HO 1.60m and FO 1.54m. Again this was not significant, $p=0.062$. The frail older men and women were lighter than the healthy older adults: HO men 73.1kg, FO men 69.5kg, $p=0.220$, HO women 62.0kg, FO women 59.0kg, $p=0.624$. Despite differences in weight there was no difference in BMI between the healthy older and frail older adults: HO men 23.3, FO men 23.1, $p=0.621$, HO women 24.3, FO women 25.5, $p=1.00$. As expected there was a significant difference in frailty index: HO men 0.03, FO men 0.40, $p<0.001$, HO women 0.05, FO women 0.41, $p<0.001$.

Table 3.1 – Demographics of healthy younger adult reference population and the comparison populations: healthy older adults and frail older adults

		Healthy Young Adults		Healthy Older Adults		Frail Older Adults		Groups		HO and FO	
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Proportion (N) (%)		52.2% (59)	47.8% (54)	32.5% (13)	67.5% (27)	45.2% (14)	51.6% (17)				
Age (years)		24.0 +/- 9.0	23.0 +/- 5.0	76.0 +/- 11.0	71.0 +/- 8.0	77.5 +/- 13.0	85.5 +/- 15.0	p<0.001	p<0.001	P=1.00	P=0.174
Height (m)		1.78 +/- 0.00	1.66 +/- 0.00	1.77 +/- 0.12	1.60 +/- 0.08	1.75 +/- 0.10	1.54 +/- 0.04	p=0.081	p<0.001		P=0.062
Weight (kg)		80.5 +/- 17.0	61.3 +/- 10.0	73.1 +/- 22.8	62.0 +/- 11.8	69.5 +/- 26.5	59.0 +/- 18.9	p=0.220	p=0.624		
BMI (kg/m²)		24.3 +/- 4.0	21.7 +/- 4.0	23.3 +/- 4.4	24.3 +/- 4.4	23.1 +/- 6.5	25.5 +/- 5.7	p=0.621	p<0.001		p=1.000
Appendicular Lean Muscle Mass/ht² (kg/m²)		8.97 (8.73- 9.20) (45 data sets)	6.94 (6.71- 7.16) (37 data sets)	N/A	N/A	N/A	N/A				
Frailty Index		0.02 +/- 0.02 (20 data sets)	0.00 +/- 0.02 (20 data sets)	0.03 +/- 0.06	0.05 +/- 0.07	0.40 +/- 0.19	0.41 +/- 0.15	p<0.001	p<0.001	p<0.001	p<0.001
Exercise	Exceeds	51.2% (21)	38.3% (13)	N/A	N/A	N/A	N/A				
	Meets	22.0% (9)	32.4% (11)								
	Doesn't meet	26.8% (11)	29.4% (10)								

Data sets: Healthy Young (HY) – 113, Healthy Old (HO) -40, Frail Old (FO) – 31.
Median +/- IQR given except appendicular lean muscle mass where mean and CI are given. Categorical data given as percentage of total population with raw number in brackets. Appendicular lean muscle mass - data shown from smaller subset of 45 men and 37 women. Exercise (adheres to government recommendations for exercise and physical activity) - data shown from smaller subset of 41 men and 34 women. N/A – data not available. Statistical difference analysed with Kruskal-Wallis with post-hoc pairwise analysis with Bonferroni correction.

Female – Age: HY and HO not ND, FO ND, Height: HY, HO and FO all ND, Weight: HY, HO and FO all ND, BMI: HY and HO not ND, FO ND, Appendicular lean muscle mass: HY ND, FI: HY and HO not ND, FO ND.

Male – Age: HY not ND, HO and FO ND, Height: HY, HO ND, FO not ND, Weight: HY and HO ND, FO not ND, BMI: HY ND, HO and FO not ND, Appendicular lean muscle mass: HY ND, FI: HY and HO not ND, FO ND.

3.3 Ultrasound as a diagnostic tool

3.3.1 Ultrasound is a reproducible diagnostic tool for the measurement of muscle thickness

Lay sonographers were able to reliably reproduce the same measurements on the same individuals. The intra-class correlation coefficient of 304 muscle thicknesses in 38 healthy young individuals (eight muscle thicknesses each individual) measured by both sonographer one and sonographer two was 0.972 (0.966-0.978). The intra-class correlation coefficient of 210 muscle thickness in 35 different healthy young individuals (six muscle thicknesses each individual) measured by sonographer two and sonographer three was 0.992 (0.989-0.995). The muscle thicknesses considered here are averages of three replicates.

There was also excellent agreement (using Koo and Li's criteria for interpreting ICC) between measurements taken on two different instruments. The intra-class correlation coefficient of 312 muscle thicknesses in 39 healthy young individuals measured by one sonographer on two different ultrasound machines was 0.989 (0.986-0.991).

The bilateral variability and intra-rater variability are not strictly measures of repeatability because of both the short period of time between the image replicates and the natural or pathological variations in muscle size bilaterally. However, the excellent agreement for all the sonographers for both bilateral variability and intra-rater variability suggests that ultrasound imaging is repeatable. The intra-class correlation coefficient of: 312 replicates of muscle thicknesses measured in 39 individuals by sonographer one was 0.992, 210 replicates of muscle thicknesses measured in 35 individuals by sonographer two was

0.982 and 210 replicates of muscle thicknesses measured in 35 individuals by sonographer three was 0.997. The intra-class correlation coefficient of: 308 muscle thicknesses in 77 individuals was 0.992, 105 muscle thicknesses in 35 individuals was 0.981 and 105 muscle thicknesses in 35 individuals was 0.982. The muscle thicknesses considered in bilateral variability are averages of three replicates and the muscle thicknesses considered in intra-rater variability are the replicates before averaging. (Table 3.2.)

Considering the repeatability of ultrasound as a diagnostic tool for healthy and frail older adults bilateral variability is less useful because of possible pathological differences such as a previous fractured hip. However, there is excellent agreement in intra-rater variability in frail older adults and good agreement in intra-rater variability in healthy older adults. The intra-class correlation coefficient of: 160 muscle thicknesses in 80 healthy older individuals was 0.867 and 124 muscle thicknesses in 31 frail older individuals was 0.928. (Table 3.3.)

These data suggest that ultrasound determination of muscle thickness is repeatable in a healthy young, healthy older and frail older adult population. It is also reproducible by lay sonographers with minimal training in healthy young adults. Ultrasound as a diagnostic tool could be used for the identification of sarcopenia in both research and clinical settings with minimal training. (Table 3.2)

3.3.2 Ultrasound could be used as a research tool for measurement of echogenicity

Lay sonographers were unable to reproduce the same echogenicity values in the same individuals with consistency. The intra-class correlation coefficient of measurements of echogenicity from 74 individuals analysed by one analyser from images performed by sonographer one and sonographer two was 0.559 (0.179-0.924). The intra-class correlation coefficient of measurements of echogenicity from 70 individuals analysed by one analyser from images performed by sonographer two and sonographer three was 0.794 (0.180-0.847). This was also the case when comparing one sonographer taking images on different ultrasound machines. The intra-class correlation coefficient of measurements of echogenicity from 74 individuals analysed by one analyser from images performed by one sonographer on two different machines was 0.305 (-0.085-0.597). The low levels of reproducibility suggest using the current protocol for the identification of high ultrasound echogenicity would not be accurate enough to use as a diagnostic tool in clinical practice.

However, different analysers analysing the same exported image showed excellent agreement. The intra-class correlation coefficient of measurements of echogenicity on the same 160 images analysed by different analysers was 0.924 (0.843-0.957). There was also good agreement between bilateral measurements and intra-rater variability suggesting higher levels of repeatability than reproducibility. The intra-class correlation coefficient of intra-rater variability: 262 replicates of muscle echogenicity measured by sonographer one was 0.895, 70 replicates of muscle echogenicity measured by sonographer two was 0.799 and 70 replicates of muscle echogenicity measured by

sonographer three was 0.768. The intra-class correlation coefficient of bilateral variability: 142 images of muscle echogenicity measured by sonographer one was 0.891, 35 images of muscle echogenicity measured by sonographer two was 0.862 and 35 images of muscle echogenicity measured by sonographer three was 0.820. The muscle echogenicity considered in bilateral variability are averages of three replicates and the muscle echogenicity considered in intra-rater variability are the replicates before averaging. (Table 3.2)

Strasser et al commented on the large variability in ultrasound echogenicity measurements witnessed in older adults when considering inter-subject variance in a young and old population (250). However, the variability between replicates of echogenicity in healthy older adults and frail older adults in this study was as good, or even better than healthy younger adults; intra-class correlation coefficients: sonographer one for healthy young was 0.895, for healthy old was 0.912 and for frail old was 0.889.

These data suggest that whilst ultrasound determination of muscle echogenicity using the described protocol would not be useful in a clinical setting, in a research setting where the sonographer and machine can be standardised this could be an adequate method of determining muscle quality which is both easier and cheaper than the current gold standard.

Table 3.2 – Inter-rater, intra-rater, inter-instrument, inter-analyser and bilateral variability for muscle depths and ultrasound echogenicity using ultrasound for reference healthy young adult population

Inter-rater Variability			
	Sonographer 1 v Sonographer 2		Sonographer 2 v Sonographer 3
Muscle Depth	0.906* (0.880-0.927)		0.992* (0.989-0.995)
Ultrasound echogenicity	0.559 (0.179-0.757)		0.794 (0.180-0.924)
Inter-instrument Variability			
	Machine 1 v Machine 2		
Muscle Depth	0.992* (0.990-0.993)		
Ultrasound echogenicity	0.305 (-0.085-0.597)		
Inter-analyser Variability			
	Analyser 1 v Analyser 2		
Ultrasound echogenicity	0.923* (0.839-0.957)		
Intra-rater Variability			
	Sonographer 1	Sonographer 2	Sonographer 3
Muscle Depth	0.992* (0.990-0.993)	0.982* (0.980-0.985)	0.997* (0.996-0.997)
Ultrasound echogenicity	0.895 (0.873-0.914)	0.799 (0.717-0.863)	0.768 (0.676-0.842)
Bilateral Variability			
	Sonographer 1	Sonographer 2	Sonographer 3
Muscle Depth	0.992* (0.990-0.993)	0.981* (0.974-0.988)	0.982* (0.974-0.988)
Ultrasound echogenicity	0.891 (0.851-0.920)	0.862 (0.746-0.928)	0.820 (0.674-0.905)

Table shows the variability in measurements between different: sonographers, instruments, analysers and the variability within measurements from different sonographers and comparing measurements from the right to the left leg. Measurements for inter-rater, inter-instrument, inter-analyser and bilateral variability are the mean of three to four replicates. Intra-rater measurements are variability within the three to four replicates. Numbers given are intra-class coefficient, 2-way mixed, absolute agreement and single measures. Interpreting ICC using Koo and Li criteria: >0.9 = excellent reliability, 0.75-0.9 = good reliability, 0.5-0.75 = moderate reliability and <0.5 = poor reliability. Values starred indicate those of excellent reliability. Below are the number of measurements included in ICC statistical test for each criteria.

Inter-rater variability. The variability between one sonographer and another when taking measurements from the same individual. Sonographer 1 v Sonographer 2: muscle depth – 304, ultrasound echogenicity - 74. Sonographer 2 v Sonographer 3: muscle depth - 210, ultrasound echogenicity - 70. Inter-instrument variability. The variability between accuson static machine and voluson portable machine when measuring the same individual. Machine 1 v Machine 2: muscle depth - 312, ultrasound echogenicity -74 . Inter-analyser variability. The variability in calculated ultrasound echogenicity values from analyser 1 and analyser 2 when calculating ultrasound echogenicity using Image J on images created by one sonographer. Analyser 1 v Analyser 2 = 160. Intra-rater variability. The variability in replicates for one individual by one sonographer. Sonographer 1: muscle depth – 312, ultrasound echogenicity – 261, Sonographer 2: muscle depth – 210, ultrasound echogenicity – 68, Sonographer 3: muscle depth – 210, ultrasound echogenicity – 70. Bilateral variability. The variability between right and left measurements. Sonographer 1: muscle depth – 308, ultrasound echogenicity – 142, Sonographer 2: muscle depth – 105, ultrasound echogenicity – 35, Sonographer 3: muscle depth – 105, ultrasound echogenicity – 35.

Table 3.3 - Intra-rater variability for anterior thigh muscle depths and ultrasound echogenicity for healthy older adult and frail older adult population

Bilateral Variability		
	Healthy Older Adult	Frail Older Adult
Muscle depth	0.711 (0.581-0.806)	0.737 (0.593-0.835)
Ultrasound echogenicity	0.842 (0.682-0.922)	0.724 (0.386-0.891)
Intra-rater Variability		
	Healthy Older Adult	Frail Older Adult
Muscle depth	0.867 (0.807-0.913)	0.928* (0.889-0.995)
Ultrasound echogenicity	0.912* (0.859-0.948)	0.889 (0.8.09-0.941)

Table shows the variability when comparing measurements from the right to the left and variability within the replicates. Measurements for bilateral variability are mean of three replicates. Numbers given are intra-class coefficient, 2-way mixed, absolute agreement and single measures. Interpreting ICC using Koo and Li criteria: >0.9 = excellent reliability, 0.75-0.9 = good reliability, 0.5-0.75 = moderate reliability and <0.5 = poor reliability. Values starred indicate those of excellent reliability. Below are the number of measurements included in ICC statistical test for each criteria.

Bilateral variability. The variability between right and left thigh measurements. Healthy Older Adult: muscle depth – 80, ultrasound echogenicity – 31, Frail Older Adult: muscle depth – 58, ultrasound echogenicity – 17.

Intra-rater variability. The variability within replicates: Healthy Older Adult: muscle depth – 160, ultrasound echogenicity – 44, Frail Older Adult: muscle depth – 124, ultrasound echogenicity – 31.

3.4 Identification of low muscle mass in older adults

3.4.1 Bilateral Anterior Thigh Thickness

The mean bilateral anterior thigh thickness (BATT) for healthy young women was 60.64 mm and for healthy young men was 75.78 mm. Both datasets were normally distributed (male dataset 3 outliers excluded). The standard deviation was 11.1 mm and 10.71 mm for women and men respectively. Therefore, the cut off using the BATT for low muscle mass identification in older adults was 38.53 mm in women and 54.36 mm in men in this population. (Table 3.4)

In a community based healthy older adult population using the EWGSOP classification of sarcopenia (11) with the BATT criteria for identification of low muscle mass: 51.3% (20/39) were identified as having low muscle mass but only 2.6% (1/39) were diagnosed as having sarcopenia. (Figure 3.1)

In a frail older adult population using the EWGSOP classification of sarcopenia with the BATT criteria for identification of low muscle mass: 93.5% (29/31) were identified as having low muscle mass, 80.6% (25/31) were then further classified as having severe sarcopenia and 9.7% (3/31) were classified as having sarcopenia. (Figure 3.1) For further discussion on sarcopenia prevalence and the relationship with frailty please see chapter six.

The BATT correlated with physical markers of performance, namely both grip strength and walk speed. Adjusted hand grip BATT for women, $R=0.750$, $p<0.001$. Adjusted hand

grip and BATT for men, $R=0.619$, $p<0.001$, both assessed by Spearman's correlation coefficient two-tailed test. Walk time and BATT for women, $R=-0.599$, $p<0.001$. Walk time and BATT for men, $R=-0.324$, $p=0.003$. (Figure 3.2). The regression line was different for men and women, there was an increased gradient of the regression line for the male population; the same increase in muscle size for both genders resulted in smaller gains to muscle strength for women.

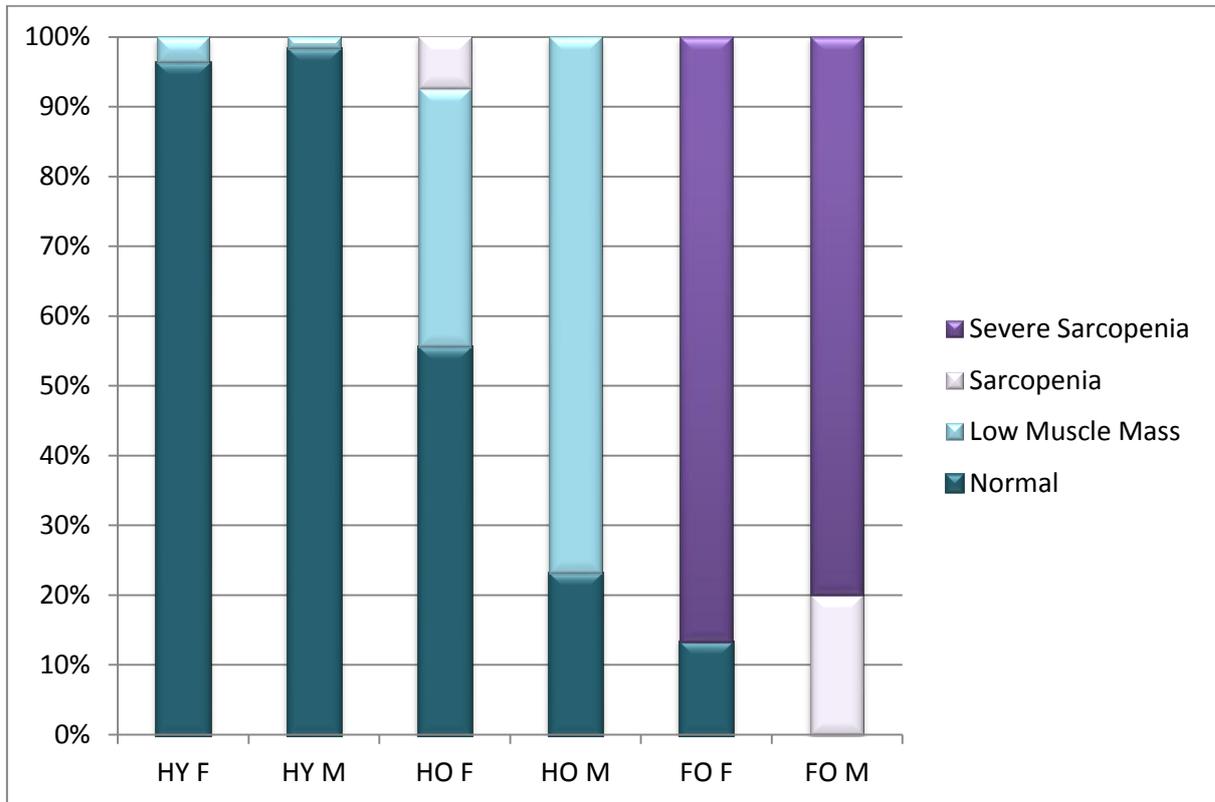
3.4.2 Forearm thickness

Forearm thickness was measured in a subset of the healthy younger adult population ($N=77$). The mean forearm thickness of the right arm was 34.54 mm for HY women ($N=35$) and 43.52 mm for men ($N=42$). Both datasets were normally distributed. The standard deviation was 2.87 mm and 3.06 mm for women and men respectively.

Therefore, the criteria for low muscle mass using forearm thickness for low muscle mass identification in sarcopenia in older adults is 28.8 mm in women and 37.4 mm in men.

(Table 3.4)

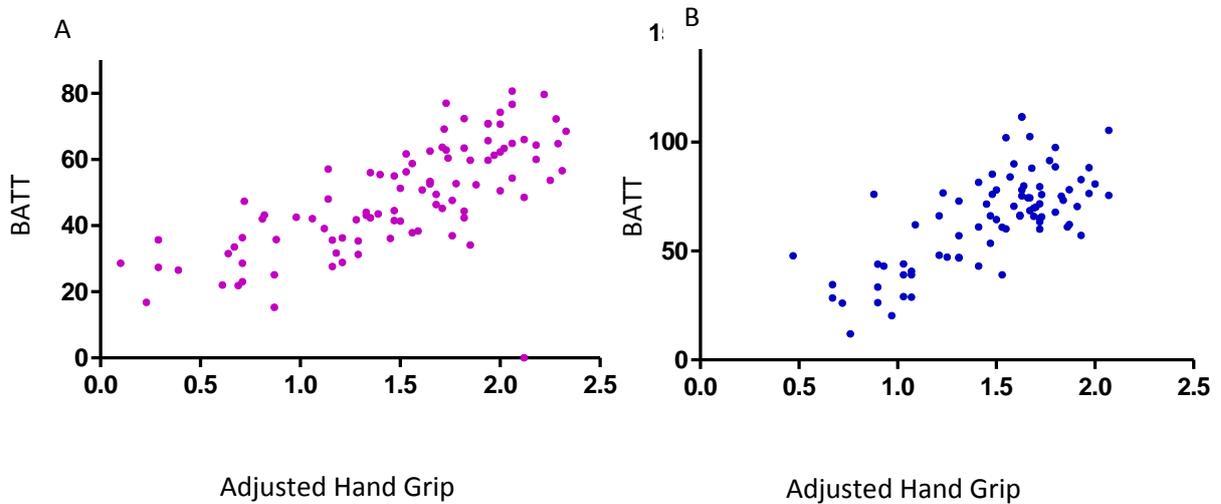
Figure 3.1 – Prevalence of sarcopenia within each cohort



Prevalence of sarcopenia within each cohort expressed as percentage of total number of individuals within the cohort. Low muscle mass defined as BATT less than 38.55mm for women and 54.36 for men. Sarcopenia defined as BATT less than 38.55mm for women and 54.36 for men and either gait speed of less than 0.8 m/sec or grip strength in highest performing hand less than the cut off for lowest 20% for gender and BMI in over 65s. Severe sarcopenia defined as BATT less than 38.55mm for women and 54.36 for men and gait speed of less than 0.8m/sec and grip strength in highest performing hand less than the cut off for lowest 20% for gender and BMI in over 65 population.

N = HY F - 54, HY M - 59, HO F - 26, HO M - 13, FO F 15, FO M - 10

Figure 3.2 – Correlation of grip strength with BATT



Correlation of grip strength with BATT in A – women and B – men. Hand grip adjusted for gender and BMI (fold change of cut-off low lowest 20% for gender and BMI in over 65) plotted against BATT (sum of rectus femoris depth right and left and vastus intermedius right and left). BATT is given in mm. BATT was normally distributed in women, all other data sets not normally distributed.

A – N=95. Spearman’s correlation coefficient. 2-tailed. R=0.750. P<0.001

B – N=83. Spearman’s correlation coefficient. 2-tailed. R=0.619 P<0.001

Table 3.4 - Identification of low muscle mass criteria using ultrasound

Women	BATT < 38.55
	Forearm Thickness < 28.8
Men	BATT < 54.36
	Forearm Thickness < 37.4

Table displays low muscle mass criteria for women and men. Low muscle mass criteria derived from two standard deviations below the mean in a healthy younger population. Low muscle mass criteria measured in mm.

3.5 Reliability of BATT as a measure of low muscle mass identification

To determine the effect of height on muscle thickness in our populations the BATT was correlated with muscle thickness. There was no association between height and muscle thickness in any of the three cohorts which suggests differences in height do not result in differences in muscle thickness. (Table 3.5) A height adjusted BATT (BATT (mm) /ht (m)) showed weaker correlation with muscle strength than a non-adjusted value. All data included (HY, HO and FO) for Spearman’s correlation coefficient. BATT with grip strength: $R=0.812$, $p<0.001$. Height adjusted BATT with grip strength: $R=0.737$, $p<0.001$.

Table 3.5 – Correlation of BATT with height for the three separate groups

	Male	Female
Healthy Young Adults	R = -0.74 P = 0.578	R = 0.190 P= 0.098
Healthy Older Adults	R = 0.109 P = 0.735	R = 0.064 P = 0.777
Frail Older Adults	R = -0.193 P = 0.509	R = 0.330 P = 0.351

Correlation of height measured in metres with BATT measured in mm. Data sets are all normally distributed except HY Male BATT, FO Female BATT and FO Male BATT. Data given are Spearman’s correlation coefficient.

HY: Male = 59, Female = 54. HO: Male = 12, Female = 22. FO: Male = 14, Female = 10.

3.6 Ultrasound echogenicity

'Muscle quality' (muscle strength or power per unit of muscle mass) (261) is considered to be as, or more, important as muscle mass in the development of sarcopenia and frailty. Ultrasound echogenicity has been suggested as a tool to assess muscle quality (262) but its utility in frail older adults has not been investigated.

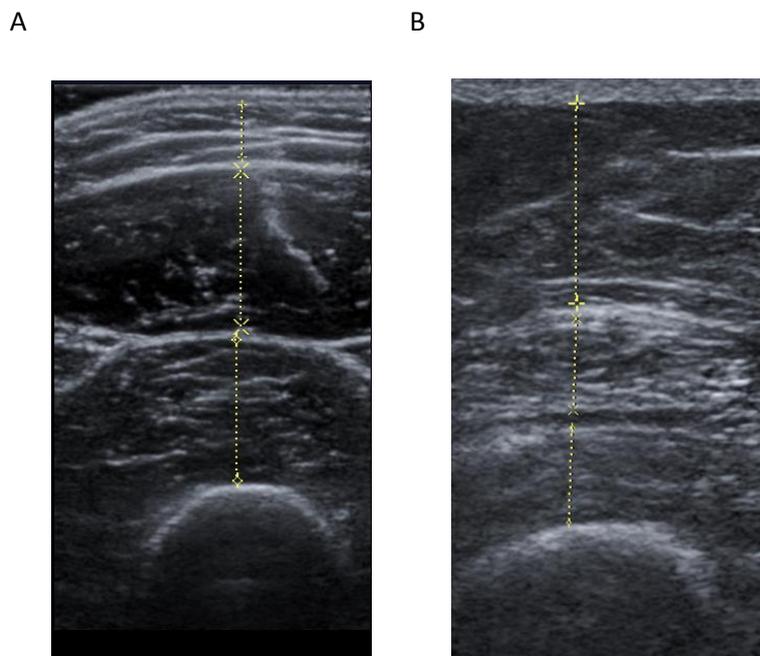
Visual interpretation of ultrasound scans suggested that there was increased ultrasound echogenicity in scans from frail older adults. The method of determination of ultrasound echogenicity is discussed in chapter two and is adapted from the method suggested by Harris-Love (257). Figure 3.3 depicts representative ultrasound images from a healthy older and frail older individual.

Ultrasound echogenicity increased with both age and frailty. Ultrasound echogenicity (values adjusted to HY population: median +/- IQR) - HY: 0.95+/-0.31, HO: 1.48+/-0.55, FO: 1.80+/-0.88. (Figure 3.4.) Ultrasound echogenicity was not related to BMI, ultrasound echogenicity and BMI: $R = 0.061$, $p = 0.465$ by Spearman's correlation coefficient. Ultrasound echogenicity was also unrelated to subcutaneous tissue depth, ultrasound echogenicity and subcutaneous tissue depth: $R = -0.060$, $p = 0.475$. However it was correlated with the physical parameters of sarcopenia, muscle strength and physical performance (Figure 3.5). Ultrasound echogenicity and adjusted hand grip strength: $R = -0.529$, $p < 0.001$ by Spearman's correlation coefficient. Ultrasound echogenicity and walk time: $R = -0.302$, $p < 0.001$ by Spearman's correlation coefficient. This effect was independent of muscle size. Ultrasound echogenicity and adjusted hand grip strength controlling for BATT: $R = -0.408$, $p < 0.001$ by Partial correlation coefficient.

Ultrasound echogenicity and walk time controlling for BATT: $R = -0.306$, $p < 0.001$ by Partial correlation coefficient.

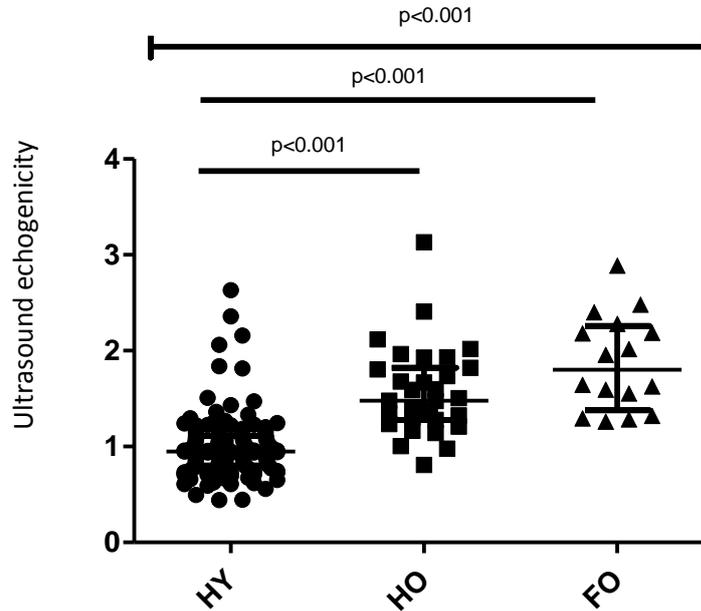
Linear regression suggests that ultrasound echogenicity has a larger effect on muscle strength than muscle size. Every increase in the fold change of ultrasound echogenicity by 0.46 resulted in a decrease in the adjusted hand grip by 0.17. (Table 3.6)

Figure 3.3 – Representative ultrasound scans of anterior thigh in a healthy young adult and frail older adult



Ultrasound scans from A – healthy younger adult B – frail older adult. Anatomical structures from top of scan to bottom – skin, subcutaneous tissue (marked by first yellow measuring line), rectus femoris (marked by second yellow measuring line), vastus intermedius (marked by third yellow measuring line), femur. It is possible to see increased ultrasound echogenicity in both rectus femoris and vastus intermedius in frail older adults compared to the healthy younger adult. Increased ultrasound echogenicity visualised as increased whiteness of muscle.

Figure 3.4 – Ultrasound echogenicity in healthy young, healthy old and frail old adults

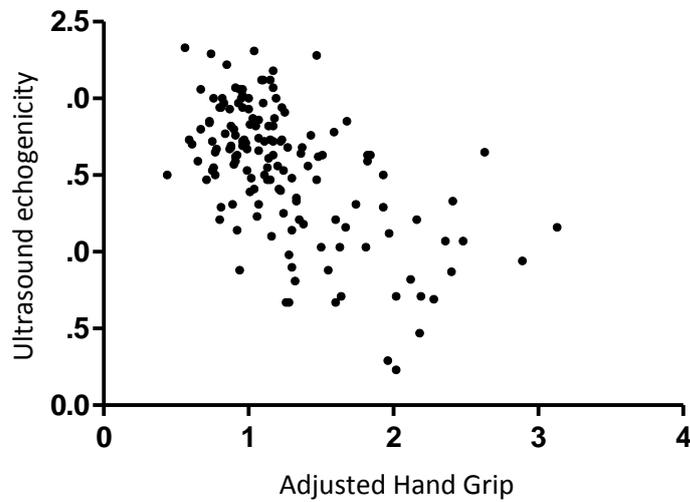


Ultrasound echogenicity of healthy young adults (HY), healthy older adults (HO) and frail older adults (FO). Ultrasound echogenicity is expressed as fold change of healthy younger adults to allow data from two different instruments to be analysed together. HY and HO not normally distributed. Data shown are median +/- IQR. HY – 99, HO – 33, FO – 16

HY – 0.95 +/- 0.31, HO – 1.48 +/- 0.55, FO – 1.80 +/- 0.88.

Kruskal-Wallis test: $p < 0.001$, post-hoc pairwise comparisons with Bonferroni correction: HY-HO $p < 0.001$, HY-FO $p < 0.001$, HO-FO $p = 0.863$

Figure 3.5 – Correlation of ultrasound echogenicity and muscle strength



Ultrasound echogenicity and adjusted hand grip strength. Ultrasound echogenicity is expressed as fold change of healthy younger adults to allow data from two different instruments to be analysed together. Hand grip adjusted for gender and BMI (fold change of cut-off low lowest 20% for gender and BMI in over 65). N=148. Neither data set was normally distributed.

Ultrasound echogenicity and adjusted hand grip. $R = -0.529$, $p < 0.001$. Spearman's correlation coefficient.

Table 3.6 - Multiple linear regression of model 1 – BATT and model 2 – BATT and ultrasound echogenicity

	B	SE B	B	Significance
Model 1				
Constant	0.880	0.093		
BATT	0.011	0.001	.54	$P < 0.001$
Model 2				
Constant	1.575	0.156		
BATT	0.007	0.002	.34	$P < 0.001$
Ultrasound echogenicity	-0.366	0.069	-.40	$P < 0.001$

Multiple regression of hand grip strength, BATT and ultrasound echogenicity.

3.7 Discussion

This chapter has reviewed ultrasound as a diagnostic technique in the identification of sarcopenia using a simple scanning protocol to produce the BATT that can be utilised in both a clinical and research setting to diagnose low muscle mass. It has also measured the BATT in a reference population, 113 in total, and proposed criteria for the identification of low muscle mass in older adults based on this reference population. It provides evidence for routinely reviewing muscle quality in sarcopenia and suggests that ultrasound echogenicity can act as an appropriate surrogate marker for muscle quality in research (where the sonographer can be standardised) but not clinical practice.

To consider the use of ultrasound as a diagnostic tool in both clinical and research settings a number of factors, such as accuracy, reliability and bias, need to be considered; most of these have been addressed in this chapter but there are flaws in the study methodology which mean the results presented in this chapter need to be interpreted with caution. The most significant limitation is the absence of a comparison of ultrasound both as a diagnostic tool and also the proposed BATT criteria to a gold standard technique. It is also important to consider the two potential roles for ultrasound, identification of low muscle mass and identification of poor muscle quality, separately due to the reported differences in reliability.

Current gold standard diagnostic methods for the identification of low muscle mass are neither easily accessible nor suitable for assessing frail older adults particularly those within the community setting. Assessment of muscle mass using MRI, CT and DXA scanners is expensive and multiple physical and cognitive barriers exist preventing a frail population from accessing these imaging techniques. The most significant barrier to

utilising these methods in a frail population is non-attendance; 12% of all NHS outpatient clinic appointments are not attended; the most frequent reasons for not attending are: forgetting (33%), not well enough to attend (15.2%), caring responsibilities (12.1%) and lack of transport (6.1%) (263, 264). These studies were not completed in an older adult population but the declared reasons for non-attendance are particularly generalisable to an older adult population. CT, MRI and to a lesser extent DXA all require comprehension and adherence to instructions and this can preclude their use in older adults with cognitive impairment. The most clinically important difference between ultrasound and the gold standard techniques is the portability of the machine allowing an immediate diagnosis and management plan to be made. This is essential in an older adult population where timely intervention is fundamental to good care and the intervention can be carefully explained to all those involved in an individual's care. In a frail older adult population those most vulnerable to the consequences of sarcopenia are those most likely to be lost to follow up due to poor mobility, difficulty accessing transport, inability to read and understand appointment letters, memory impairment and increased risk of inter-current illness. A portable method of identification of low muscle mass is particularly important in this subsection of the older adult population.

An alternative portable diagnostic technique to ultrasound is BIA but this can be unsuitable in a population with a high prevalence of co-morbidities that affect fluid balance. There has been limited validation of BIA as a technique to determine muscle mass in healthy older adults and none in frail older adults (265-268). Original BIA machines were able to diagnose fat free mass or proportion of fat within the body; newer machines extrapolate that data using set protocols to provide estimates of muscle mass

(269). A recent study has demonstrated that current protocols in use are not accurate in older adults with functional impairment, over-estimating muscle mass (270). BIA does not provide information on muscle quality but the technique is simpler and quicker than ultrasound. The role of BIA in the diagnosis of low muscle mass in a frail older adult population needs to be further investigated particularly in comparison with both ultrasound and a gold-standard method.

In section 3.2 data are presented that support the repeatability, reproducibility and accuracy of ultrasound as a diagnostic tool in the measurement of muscle depth by lay sonographers. It demonstrates that three sonographers with no prior experience in the practical application of ultrasound following a period of minimal training were able to reproduce measurements on healthy young individuals with excellent consistency. It also shows excellent consistency between measurements taken on different ultrasound instruments, namely a static and portable instrument in healthy young adults. There was also excellent consistency between the replicates from each sonographer in the healthy young and frail older adults and good consistency between the replicates from healthy older adults suggesting acceptable repeatability within all the populations.

One of the concerns of applying techniques originally investigated and refined in healthy younger adults to healthy older and frail older adults is that the degree of variability within the older population renders these techniques inaccurate (250). However, the data presented in this chapter demonstrate that the variability witnessed in the healthy older and frail older population was comparable to the variability witnessed in the healthy young population and would not affect the validity of conclusions drawn from

these data. The accuracy of the data, the degree to which the measurement of muscle thickness represents muscle mass, is not possible to calculate without comparison to a gold standard which is absent in this study. However, there is a significant correlation between muscle thickness and physical parameters indicative of muscle health such as grip strength and walk speed which suggests ultrasound determination of muscle thickness is an accurate method of measuring muscle mass. The accuracy of ultrasound in the estimation of muscle quality is discussed later in this chapter.

This chapter also proposes the BATT as a novel method for the identification of low muscle mass. The BATT is a composite measure of muscle thickness from four different muscles in the anterior thigh incorporating bilateral measurements. A composite measure including both right and left legs was considered to be more accurate than a single measure because of the degree of bilateral variability demonstrated in both the healthy and frail older adults. Lower limb measurements were included rather than upper limb measurements for two reasons: one, it has been demonstrated that older adults lose muscle preferentially from the lower limbs (271); two, loss of independence in the basic activities of daily living is primarily dependent on lower limb rather than upper limb performance (272). The raw muscle thicknesses were included in the BATT rather than a muscle thickness adjusted for height. This is similar to other ultrasound studies (249, 273) though there are a variety of methods within the literature including those which do adjust for height (274). Abe and colleagues demonstrated that adjustment for height did not improve their correlations of muscle thickness with muscle strength or physical performance, or improve the accuracy of their predictive models (275).

Anthropological measurements in younger males demonstrated there was no correlation

between muscle thickness and height (276). The data presented in this chapter also demonstrate no relationship between muscle thickness and height for all three groups and adjustment for height weakened the correlation with physical parameters. It could be argued that the BATT is a simplistic approach to determination of muscle mass as it measures a thickness, one dimension, rather than area, two dimensions or volume or weight, three dimensions. However, a recent study has demonstrated that ultrasound determination of mid-femur vastus lateralis muscle thickness was significantly correlated with both volume and cross-sectional area of the muscle measured by MRI (277).

The proposed BATT criteria for low muscle mass were derived from the healthy young reference population; gender specific, two standard deviations below the mean of the reference population. This criterion has been suggested by EWGSOP and is identical to criteria used to identify osteoporosis (11). Although the reference population is not extensive, 113 in total, 59 men and 54 women, appendicular lean muscle mass measured by BIA in our reference population is almost identical to values quoted in multiple studies with much larger sample sizes and calculated using a variety of methods (two standard deviations below the mean of a healthy young population and lowest gender specific 20% in older adults) (11). This suggests that the sample is representative of a healthy young adult population and the proposed criterion for the diagnosis of low muscle mass in older adults (BATT) has been created from an appropriate reference population. Three outliers were excluded from the male population to ensure a normal distribution of the data; calculation of low muscle mass criteria using two standard deviations below the mean would not have been possible without a normal distribution. This manipulation of the

data may have introduced bias into the results; increasing the reference population size would hopefully ameliorate the effect of outliers on the quoted BATT criteria.

It could be suggested that the reference population is not representative of an average UK younger adult population. The BMI for the reference population for men was 24.3 and for women was 21.7, whereas national averages in these age groups are 25.1 for men and 25.3 for women (278). 73% of the men and 70.4% of the women in the reference population met government recommendations for physical activity whereas the national average is only 67% of men and 55% of women (279). The increased amounts of physical activity in the reference population could result in an increased muscle mass which may not be representative of an average UK younger adult population. However, considering the term 'healthy' in the truest sense, the absence of chronic disease plus meeting target recommendations for exercise and activity, the reference population could be argued to be a more appropriate comparison population than the national average.

A further limitation of the study is that the healthy young reference population included individuals from a variety of ethnicities. The majority of the healthy young reference population was white (British, Irish, Brazilian and other European countries), 85.8%. The remainder of the healthy young reference population was Asian (9.7%), Arab (1.8%), Black African (1.8%) and Chinese (0.6%). The healthy older and frail older adults were all white either British or Irish. There are ethnic differences in skeletal muscle mass with African Americans having increased skeletal muscle mass and Asian Americans having decreased skeletal muscle mass compared to white Americans (280). Whilst the reference population is likely to be an appropriate comparison group to the healthy and

frail older adults described in this study it cannot be extrapolated to be used in other non-white ethnic groups.

The female healthy older and frail groups were significantly shorter and heavier than the healthy young adult group whereas the male healthy older and frail older groups were comparable to the healthy young adult group. This is likely secondary to both the effect of osteoporosis and osteopenia on vertebral height and nutritional deficiencies during the war and post-war rationing in childhood and young adulthood. Women are more likely to suffer from malnutrition due to the double burden of increased requirements secondary to menstruation and motherhood, and gender inequality (281). However, as discussed before height does not appear to have an effect on muscle thickness, so the BATT criteria can be appropriately applied to the female healthy older and frail older populations.

This chapter also presents the data for forearm thickness in a smaller subset of healthy young adults. Whilst this chapter does not present the rigorous comparison data for healthy older and frail older adults, the forearm thickness data was included for two reasons: one, others have demonstrated its relationship with physical performance and muscle strength (273); two, the clinical utility of the technique particularly in a community based frail population. It is recognised that the relatively small data set limits these data's use but it was included because it is hoped that further research will acknowledge the potential of forearm thickness in sarcopenia diagnosis and add to this small dataset.

The hierarchical nature of sarcopenia diagnosis, with low muscle mass an obligatory component but not the sole criteria, suggests that the identification of low muscle mass could be considered as a screening step in the diagnosis of the syndrome. As a screening tool the sensitivity should be high and this may be achieved at the detriment of the specificity. The current lack of evidence for any management of low muscle mass but conservative management would ensure that the potential negative impacts of false positives would be limited. However, this would need to be reconsidered if a pharmacological management was proven to be of benefit.

In the era of personalised medicine a diagnostic criterion for sarcopenia that includes a blanket definition of low muscle mass regardless of height, weight or body composition seems rather inelegant and potentially inaccurate. Particularly as research suggests that muscle loss with ageing occurs differently with gender and ethnicity (280) and there are generational differences in diet, lifestyle and body composition. I propose that further research should be conducted to inform criteria for diagnosis that closely mimic loss of function. Further longitudinal work is required to consider the relationship of muscle strength, muscle size and physical performance with outcomes of independence and well-being over an extended period of time.

The chapter provides evidence for routinely reviewing muscle quality in sarcopenia and furthermore suggests that ultrasound echogenicity can act as an appropriate surrogate marker for muscle quality. There was a significant difference between the echogenicity of the three groups and there appears to be a difference in echogenicity of healthy older adults and frail older adults but due to the small numbers of the two groups this cannot

be confirmed with statistical tests. This difference is independent of age. Modelling the data suggests that ultrasound echogenicity is of the same if not of more importance than muscle size in the determination of muscle strength. Whilst the groups are not large enough to draw complete conclusions there is significant evidence that ultrasound echogenicity is an excellent surrogate marker or component of muscle quality and should be included in further research studies. Higher ultrasound echogenicity values have been previously demonstrated to be associated with sarcopenia in older adults (250, 257).

Section 3.2 highlights the difficulties in measuring ultrasound echogenicity with the currently available equipment; there are limitations with the method, and measurements from different sonographers and instruments are not reproducible. However, there was good to excellent repeatability suggesting data from one sonographer on one machine can be considered reliable. While it would not be possible to standardise both the sonographer and machine in a clinical environment it is feasible that both could be standardised for research purposes. As presented here an alternative to using a single instrument is that measurements on different instruments by one sonographer can be standardised to the healthy young adult mean value. The witnessed differences in measurements between the different sonographers is hypothesised to be secondary to different scanning techniques probably related to the pressure applied with the probe, angle of incidence (the angle at which ultrasound waves encounter the anatomical structure) and volume of contact gel. Following analysis of sonographer one and sonographer two and considering differences in technique to be the probable cause for witnessed differences in ultrasound echogenicity the scanning technique for sonographer three was standardised to sonographer two. Whilst this improved the reproducibility

between the two sonographers the confidence intervals remained very large suggesting an unacceptable degree of variability. The difference between the two machines is likely to be due to discrepancies in clarity secondary to both hardware and software. The difference witnessed between the two machines is unlikely to follow an entirely linear relationship because whilst re-running the ICC analysing consistency rather than absolute agreement does improve the agreement it is still not of acceptable agreement (ICC=0.521. 0.333-0.669). A previous study has suggested a method of calibrating a form of ultrasound echogenicity which allows comparisons from different machines with both different software and hardware to be made (282). A further limitation of the method that cannot be currently standardised is the effect of different depths of overlying subcutaneous tissue on muscle echogenicity measures. Subjective assessment of the images suggested that there was increased echogenicity of the rectus femoris compared to the vastus intermedius and this was confirmed with objective assessment in a small subset of the complete sample. Echogenicity is a measure of acoustic reflectance but deep structures may appear hypoechoic because the incident ultrasound beam is weak from attenuation resulting in diminished returning echoes. Interestingly this has not been previously discussed in the literature in the context of sarcopenia or sarcopenic obesity.

It is however possible to conceptualise an ultrasound instrument that could standardise both the pressure applied through the probe and the angle of incidence plus be able to run algorithms that could standardise the effect of the overlying tissue. This could improve the accuracy of the technique and ensure that values taken by different sonographers on different instruments are comparable.

This evidence all supports the use of ultrasound as a method to identify low muscle mass in sarcopenia diagnoses. Sarcopenia is rarely formally diagnosed in clinical practice with the most significant barriers to diagnosis being availability and accessibility of DXA or other gold standard diagnostic methods for identification of low muscle mass. The relative ease of measuring the other two criteria, low muscle strength and poor physical performance, suggests that if low muscle mass could be easily identified sarcopenia would be more likely to be diagnosed. Ultrasound provides a pragmatic diagnostic tool that is: non-invasive, with no radiation exposure, and can be used within both community and hospital settings. The proposed BATT criteria are a simple method of identification of low muscle mass which have excellent correlation with physical parameters of muscle health. However, the absence of a gold standard comparison in this study precludes widespread adoption of this protocol. In addition whilst this chapter has demonstrated the importance of ultrasound echogenicity in sarcopenia and physical function it has not established an accurate or reproducible approach to its measurement. Further research on the comparison of the BATT protocol with gold standard techniques of low muscle mass identification is vital for sarcopenia identification in the clinical environment.

**4. Muscle biopsy is not a
feasible tool in a
community based geriatric
population.**

4.1 Introduction

Sarcopenia, the loss of muscle mass and physical function in older adults, and frailty, a syndrome of diminished strength, endurance, and reduced physiological function that increases the risk of adverse outcomes, are both common conditions affecting older adults (4, 11). The pathophysiology of both syndromes is complex and incompletely characterised however, muscle health is central to both conditions (283). 'Muscle quality' (muscle strength or power per unit of muscle mass) (261) is considered to be as, or more, important as muscle mass in the development of sarcopenia and frailty. In Chapter 3 the association of ultrasound echogenicity, a marker of muscle quality, with physical parameters was demonstrated. Factors affecting the quality of the muscle include: morphological characteristics of the muscle, aerobic capacity, intramuscular adipose tissue, fibrous tissue and motor units (256).

Investigators with research interests that include sarcopenia and frailty require muscle tissue to investigate factors contributing to muscle quality; due to the complexities of both syndromes animal models are unlikely to provide an adequate model for investigation although they do exist (284). Animal models which utilise the manipulation of a single gene, such as the IL-10 knock out mouse, are often considered too pathophysiologically simplistic to represent a multi-system human syndrome. The IL-10 knock out mouse models do not fully express the frailty phenotype, no decrease in physical activity or weight loss in the mouse models, so cannot be considered a complete representation of frailty. Animal models of premature ageing are more complex but whether the ageing witnessed in these models occurs by the same pathophysiological process as frailty is unknown (285).

Muscle tissue from humans can be obtained from either a percutaneous muscle biopsy or as a by-product of surgery. Percutaneous muscle biopsy is preferred for most research because muscle tissue acquired as a by-product of surgery will be by definition not from healthy older adults and it can be difficult to separate the effects of inflammatory conditions such as arthritis from the effects of sarcopenia and frailty. Percutaneous muscle biopsy encompasses both the conchotome forceps technique and needle biopsy techniques (286). Percutaneous muscle biopsy has been used with success on multiple occasions in individuals with sarcopenia (287-289) and on fewer occasions in frailty (290). A recent study in the UK reported on both the acceptability and feasibility of muscle biopsy in an older community dwelling male population and highlighted the importance of completing this type of study in a frail population (289). Anecdotal evidence has described difficulties in obtaining good muscle samples (reasonable weight sample sizes with minimal attached subcutaneous tissue) from muscle biopsy in older community dwelling women (291).

This chapter reports on the difficulties of obtaining muscle samples using a Bergstrom needle technique in a frail older adult population (292) and potential problems in obtaining reasonable muscle sample sizes in both healthy older adults and frail older adults. The chapter discusses participant factors resulting in contra-indications to muscle biopsy; these have been categorised as: safety of biopsy, ability to attend for biopsy, ability to care for wound post biopsy and anticipated high risk for complications. Examples for each category are described in table 4.1 and are specific to this sample; the examples were all present in at least one individual in the study population.

Table 4.1 – Participant factors both absolute and relative contraindications.

Participant Factors	Absolute Contra-Indications	Relative Contra-Indications
Safety of biopsy	<ul style="list-style-type: none"> • Anti-coagulation (293) • Anti-platelet (293) • Anticipated difficulties with positioning for biopsy 	
Ability to attend for a biopsy	<ul style="list-style-type: none"> • Disengagement with services/research study • Death • Started on palliative treatment • New diagnosis of cancer • Loss of capacity • Frequent attendances at hospital 	
Ability to care for wound post-biopsy		<ul style="list-style-type: none"> • Unable to complete personal care • Visual impairment
Anticipated high risk for complications	<ul style="list-style-type: none"> • Multiple falls 	

Participant factors present in the frail older adult population that are contra-indications to muscle biopsy. Absolute contra-indications cannot be ameliorated. Relative contra-indications could be ameliorated with appropriate resources.

Anti-coagulation and anti-platelet therapy are considered an absolute contraindication to muscle biopsy; clinical guidelines suggest a wash out period prior to biopsy (293). For this study all individuals on anti-coagulation or anti-platelet therapy were excluded from consideration for a muscle biopsy however, a previous study which judged individuals to be taking anti-platelets for primary prevention and low risk for cardiovascular complications paused aspirin for a period of four to seven days prior to muscle biopsy for research (294). The other contraindications are proposed on pragmatic grounds. All the

proposed contraindications occurred in the frail older individuals and were considered by two experienced geriatricians prior to the individual's exclusion from the muscle biopsy sub-study.

Muscle biopsies, particularly from the vastus intermedius, are considered an extremely safe procedure. The most common complications are intra-procedure pain and numbness of the leg post-procedure (289). The pain is frequently described as discomfort and was reported to not deter participants considering a further biopsy (289). Serious wound infections or disability have not been reported in the literature. The aftercare of a muscle biopsy site is relatively simple and most individuals are able to care for their own wound; dressing change at four days and removal of steri-strips at seven days plus avoidance of vigorous activity for 72 hours and immersion in water for 48-72 hours (293). Individuals unable to complete their own personal care due to physical disability were excluded from the study as it was considered they would be unable to adequately care for their wound site. Individuals with significant visual impairment were also excluded as ability to review the wound for signs of infection was considered essential. Although beyond the scope of this study these contraindications could be potentially ameliorated in future studies with the appropriate resources, for example daily nursing care to care for the incision post-operatively. These contra-indications which could be potentially mitigated are therefore considered relative rather than absolute.

Anticipated difficulties with positioning for the biopsy describes a number of individuals with: significant lower limb tremor, difficulties with rigidity and immobility secondary to

Parkinson's Disease that would preclude adequate positioning, severe chronic back pain that would prevent an individual from remaining in a semi-recumbent position for thirty minutes and an individual with morbid obesity where following ultrasound assessment of the thigh it was judged the Bergstrom needle window would not reach the muscle due to the increased depth of the subcutaneous tissue.

Disengagement with services describes both those individuals who became uncontactable after the first or second contact or those who were unwilling to return to the hospital for the purpose of the study. Frequent attendances at hospital describes a number of individuals who following their recruitment to the study were admitted to hospital a number of times during the subsequent year; these individuals were judged to never be well enough for muscle biopsy. Death and starting on palliative treatment within the year following recruitment were considered to be an absolute contra-indication. An individual with a new diagnosis of cancer was excluded because of the effect of cancer cachexia on muscle. There was no provision in the study ethics for inclusion of individuals who did not have capacity. All participants at the time of recruitment had capacity but a number lost their ability to consent before a muscle biopsy could be organised.

Multiple falls (more than one a month) was considered an anticipated high risk for complications due to the potential for damage of the healing biopsy site on falling and also the unknown effect of muscle biopsy on falls frequency.

Contraindications that have been previously reported in muscle biopsy studies in sarcopenic individuals but did not occur in this sample (therefore not listed) are medications that impair healing such as steroids and methotrexate (11).

This chapter aims to:

- Consider the prevalence of contraindications to muscle biopsy in both a healthy and frail older adult population
- Consider the impact of muscle thickness on the success of muscle biopsy in both healthy and frail older adults
- Determine the overall feasibility of muscle biopsy in both healthy and frail older adults

4.2 Results

40 HO and 37 FO adults were recruited to the study; their demographic data is described below. (Table 4.2)

Table 4.2 – Demographic data of recruited participants

	Healthy Older Adults	Frail Older Adults	MW
Gender	67.5% (27) Female 32.5% (13) Male	54.1% (20) Female 45.9% (17) Male	
Age	71.9 +/- 9.0	84.0 +/- 15.0	p<0.000
BMI	23.4 +/- 4.0	24.9 +/- 7.0	p=0.782
FI	0.03 +/-0.07	0.41 +/- 0.17	p<0.000

Data sets: HO – 40, FO – 37. Median +/- IQR given. Categorical data is given as percentage of total population with raw number in brackets. All data not normally distributed except FO Age and FO FI. Statistical difference analysed with Independent samples Mann-Whitney U.

4.3 Contraindications to muscle biopsy

Seven (17.5%) healthy older adults had a recognised contraindication to muscle biopsy.

All seven participants were precluded from undergoing a muscle biopsy due to the safety of biopsy; this was secondary to anti-platelet or anti-coagulant use. One of the healthy older adults in addition to taking an anti-coagulant subsequently became chronically unwell which would have prevented them from attending for a muscle biopsy within the follow up period (one year). In stark comparison only two (5.4%) of the frail older adults did not have a contraindication to muscle biopsy. (Table 4.3).

The majority of frail older adults had more than one contraindication to muscle biopsy; 12 (32.4%) had a single contraindication, 15 (40.5%) had two, 5 (13.5%) had three, 2 (5.4%) had four and 1 (2.7%) had five contraindications. The contra-indications were

grouped in to four categories: safety of muscle biopsy, inability to attend for muscle biopsy, inability to complete own personal care and anticipated high complication rates post-biopsy. Inability to attend and safety of the biopsy were the most frequent contra-indications. (Table 4.4) Figure 4.1 demonstrates both the frequency of each contra-indication and the number of participants with multiple contraindications illustrating the complexities of the individuals.

Considering the contraindications to muscle biopsy as either absolute, cannot be ameliorated, for example anti-coagulation, or relative, modifiable with appropriate resources, the number of frail older adults who could potentially undergo a muscle biopsy increased to 4 (10.8%). (Table 4.5)

Table 4.3 – Prevalence of contra-indications to muscle biopsy in healthy older and frail older populations

	No contraindications	Contraindications
Healthy Older Adults	82.5% (33)	17.5% (7)
Frail Older Adults	5.4% (2)	94.6% (35)

Prevalence of contra-indications to muscle biopsy in healthy older and frail older groups. Percentage of total number in group given with raw number in brackets. HO – 40, FO – 37.

Table 4.4 – Prevalence of the categorised contra-indications in healthy older and frail older population

	Safety	Attendance	Personal Care	Complications
Healthy Older Adults	17.5% (7)	2.5% (1)	0% (0)	0% (0)
Frail Older Adults	51.3% (19)	54.1% (20)	35.1% (13)	13.5% (5)

Prevalence of categorised contra-indications to muscle biopsy in healthy older and frail older groups. Percentage of individuals with a contraindication in each category given with raw number in brackets. HO – 40, FO – 37.

Table 4.5 – Prevalence of absolute and relative contra-indications to muscle biopsy in frail older adults

	No contra-indications	Relative contra-indications	Absolute contra-indications
Frail Older Adults	5.4% (2)	5.4% (2)	89.2% (33)

Prevalence of relative and absolute contraindications in frail older adults. FO – 37.

Figure 4.1 – Frequency of contraindications to muscle biopsy in the frail older adults

	Anti-coagulation	Anti-platelet	Positioning	Disengagement	Death	Palliative care	New cancer	Loss of capacity	Frequent attendee	Personal care	Visual impairment	Frequent falls
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												
17												
18												
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21												
22												
23												
24												
25												
26												
27												
28												
29												
30												
31												
32												
33												
34												
35												
36												
37												

Frequency of contraindications to muscle biopsy. Each row represents a single frail older individual. Each column represents the contraindication. Coloured block represents that individual having that contraindication. Number = 37.

4.4 Muscle thickness

As described in the methods section, to obtain an adequate muscle sample total muscle thickness of the vastus lateralis and underlying vastus intermedius at the site of sampling should be thicker than the needle point and window; the total depth of needle point and window of a Bergstrom needle is 17.5mm. Therefore, at a muscle thickness greater than 17.5mm a muscle sample between 25 and 140mg should be obtained which is an adequate size for most laboratory based experiments including Westerns (293). As illustrated in figure 4.2C at a muscle thickness between 9.5mm and 17.5mm a muscle sample will be obtained but it may not be of adequate size for experiments and will contain subcutaneous tissue. At a muscle thickness less than 9.5mm no muscle will be obtained using a Bergstrom needle biopsy within the biopsy sample. (Figure 4.2D.) Whilst there are no reports in the literature of directly measured muscle depths linked to failed Bergstrom needle muscle biopsies there are multiple reports of failed needle biopsies in patients with significantly wasted muscle (286, 295, 296).

Figure 4.2 – Illustration of Bergstrom needle muscle biopsy

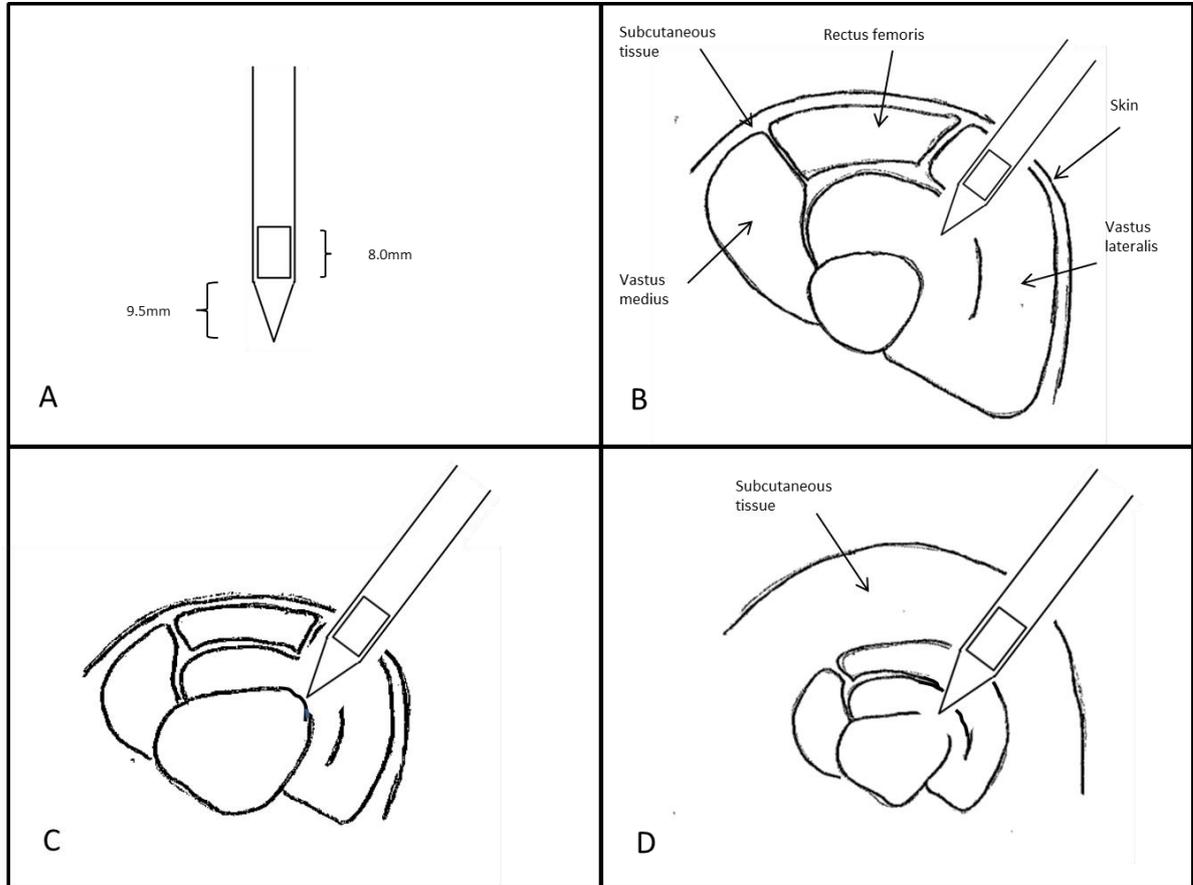


Illustration of Bergstrom needle muscle biopsy. Figure A – Bergstrom needle muscle biopsy with dimensions. Figure B – successful Bergstrom needle muscle biopsy with labelled anatomical structures. Figure C – unsuccessful Bergstrom needle muscle biopsy in individual with inadequate muscle thickness. Figure D - unsuccessful Bergstrom needle muscle biopsy in individual with inadequate muscle thickness obscured by large amount of subcutaneous tissue.

Research has demonstrated that the combined muscle thickness of rectus femoris and vastus intermedius at 50% of the femur length is the same as the muscle thickness of vastus lateralis and vastus intermedius at maximum cross-sectional area of vastus

intermedius, the location of muscle biopsy site (297). Therefore, it is possible to take the sum total muscle thickness of rectus femoris and vastus intermedius measured at 50% femur length as a surrogate measure of muscle thickness at the muscle biopsy site.

Analysing the entire samples (including those with a contraindication to muscle biopsy) there is no risk of a failed biopsy in of healthy older males but there is a risk in 40.7% of the healthy older females. The risk to the individual is that multiple passes may be required to obtain an adequately sized muscle biopsy which increases the probability of trauma and pain or an inadequate sized muscle sample is obtained which is too small for analysis and the procedure was therefore unnecessary. In the frail older adults only 18.7% of females and 21.4% of males had adequate muscle depth to ensure a successful biopsy. It would be possible in 68.8% of females and 57.1% of males to obtain some muscle from a biopsy but the sample may not be of adequate size or multiple passes might be required to obtain an adequate sample. (Table 4.6) Figure 4.3 demonstrates the spread of the data. It shows that although there is a risk of an inadequate sized muscle biopsy in 41% of healthy older females the muscle samples obtained in these individuals are likely to be of sufficient size for analysis because most of the healthy older females had muscle thicknesses closer to 17.5mm than 9.5mm. Conversely in the at risk frail older individuals the proportion of muscle thickness between 9.5 mm and 13.5 mm is much higher suggesting a real possibility of a failed muscle biopsy: 50% of the at risk females and 60% of the at risk males.

Combining the data on muscle thickness with the data presented on absolute contraindications to muscle biopsy to consider the application of muscle biopsy as a

research tool in both healthy and frail older adults demonstrates real problems with the Bergstrom needle muscle biopsy technique in frail older adults.

There were 33 healthy older adults who have no contraindications to muscle biopsy, 25 females and 8 males. All eight males had adequate muscle thickness for a Bergstrom needle biopsy. 20% (5 individuals) of the healthy older females had inadequate muscle thickness bilaterally and 20% (5 individuals) of the healthy older females had inadequate muscle thickness on a single side. Therefore, in the total sample size of 40: 82.5% (33) had no contraindications to muscle biopsy, 12.5% (5) were at risk of multiple passes or failed attempts at muscle biopsy, consequently 70% (28) of the original healthy older adult sample had no contraindications and would have sufficient muscle thickness for a successful muscle biopsy.

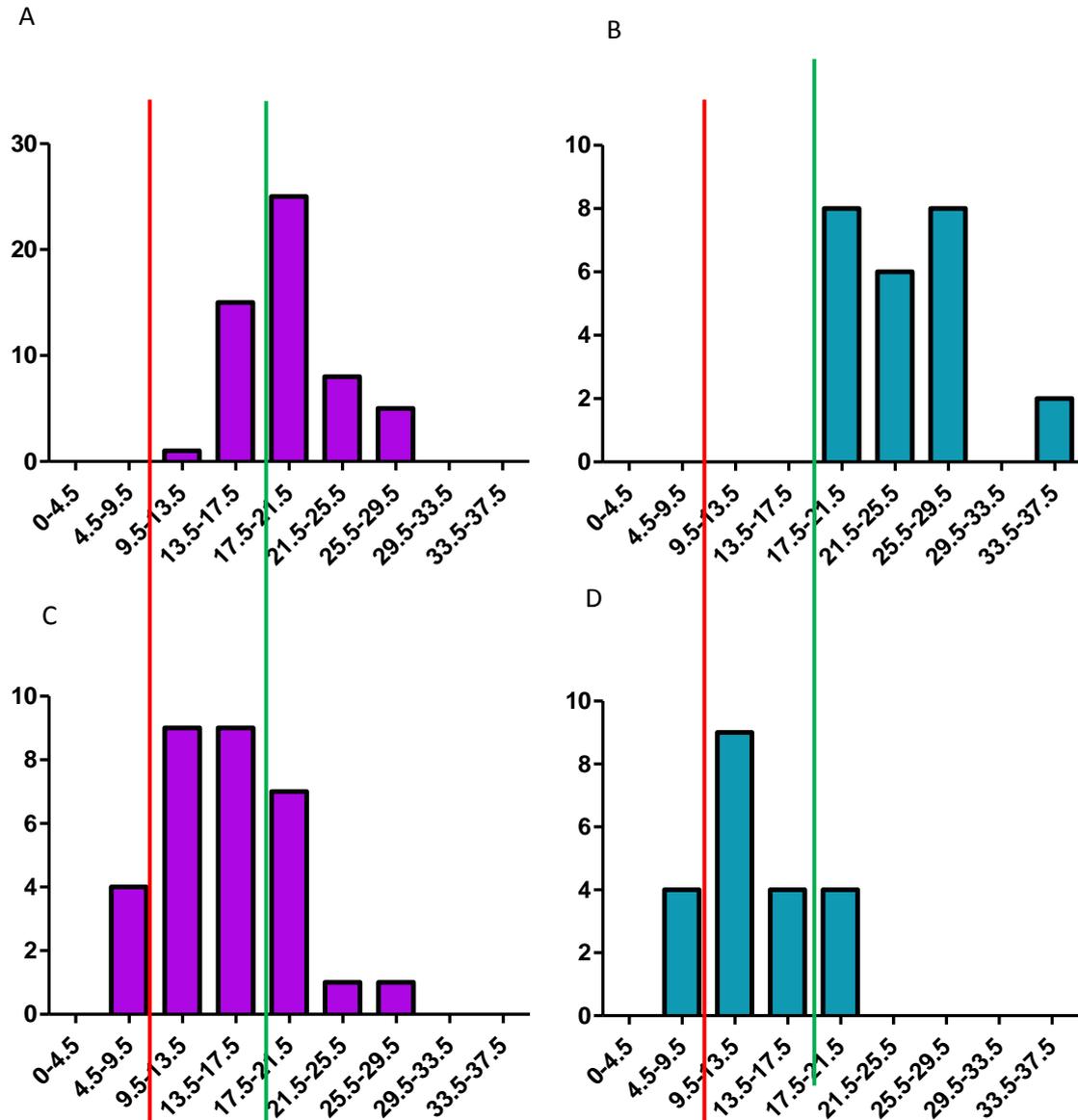
There are four frail older adults who have either relative or no contraindications to muscle biopsy: one male had sufficient muscle thickness for a successful muscle biopsy (muscle thickness right leg 22.8 mm and left leg 25.0 mm), two individuals were at risk of a failed muscle biopsy (100 year old male muscle thickness right leg 13.65 mm and left leg 12.7 mm and 85 year old female muscle thickness right leg 10.1 mm and no muscle thickness measurements for left leg due to poor image quality), one female was lost to follow up prior to ultrasound measurements (not categorised as disengagement as reasonable effort was not made by the research team to contact the patient).

Table 4.6 – Prevalence of muscle depths less than 17.5mm and 13.5mm in healthy older adult and frail older adult population

		Risk of Failed Biopsy	
		Female	Male
Healthy Older Adults	>17.5mm	59.3% (16)	100% (13)
	9.5 - 17.5mm	40.7% (11)	0% (0)
	<9.5mm	0% (0)	0% (0)
Frail Older Adults	>17.5mm	18.7% (3)	21.4% (3)
	9.5 - 17.5mm	68.8% (11)	57.1% (8)
	<9.5mm	12.5% (2)	21.4% (3)

Prevalence of muscle depths in healthy older adults and frail older adults. Numbers are percentage of total individuals within group: HO F -27, HO M – 13, FO F – 16, FO M – 14. Raw numbers in brackets. Risk of failed biopsy – muscle depth less than 17.5 or 9.5 in right and/or left leg. 17.5 mm is the depth of needle point and window and this corresponds to a complete muscle biopsy sample. 9.5 mm is the depth of needle point. Muscle depths between 9.5 and 17.5 mm correspond to an incomplete muscle biopsy sample.

Figure 4.3 – Distribution of muscle thickness



Distribution of muscle thickness. A – healthy older women. B – healthy older men. C – frail older women. D – frail older men. X axis is muscle thickness measured in mm. Y axis is frequency of individuals. Left of the red line are individuals in who no muscle would be obtained using a Bergstrom needle biopsy technique. Left of the green line are individuals who are at risk of inadequate muscle samples using a Bergstrom needle biopsy technique.

4.5 Discussion

This chapter has reviewed the feasibility of muscle biopsy in both a healthy older adult and frail older adult population. It reports on the difficulties in obtaining muscle samples in a frail older adult population and the potential difficulties with obtaining adequately sized muscle samples in a healthy older women and frail older adult population.

Section 4.2.1 describes the numerous contraindications to percutaneous muscle biopsy in frail older adults. It demonstrates that 94.6% of frail older adults had a contraindication either absolute or relative to muscle biopsy and that the majority (62.1%) had multiple contraindications. It also established that the majority of these contraindications (89.2%) were absolute with no capacity for amelioration. Whilst this data relates to muscle biopsies performed using the Bergstrom technique it is also applicable to the other percutaneous muscle biopsy techniques, conchotome forceps and microbiopsy needle techniques (286, 298). The contraindications are the same for all the techniques, although it could be argued that the incision with the microbiopsy needle technique is so small that the individual being able to perform adequate post-biopsy care would be unnecessary. Nevertheless, even if this was the case only 10.8% of the sample population would have no contraindications to muscle biopsy. These data interpreted together suggests that percutaneous muscle biopsy performed on a subset of a relatively small sample of frail older adults is not feasible.

Section 4.2.2 reviews the potential difficulties in obtaining muscle samples of a reasonable size in both healthy older adults and frail older adults using a Bergstrom needle technique. There is a risk of an inadequately sized muscle sample of 40.7% in a community based healthy older female population. In a research population undergoing

an 'unnecessary' procedure this is an unacceptable risk. In this population the risk can be easily ameliorated with ultrasound assessment of muscle depth at the biopsy site. This would raise the success rate of adequate muscle biopsy sample size from 59.3% (individuals with adequate muscle depth bilaterally) to 81.5% (individuals with adequate muscle depth on at least one side) and would also prevent individuals undergoing an unsuccessful procedures. In the healthy older male population there was no risk of a failed muscle biopsy and therefore muscle biopsies could be undertaken without prior ultrasound assessment. There is a considerable risk of a failed muscle biopsy in frail older adults, 81.3% in female and 78.5% in male populations. This risk can be ameliorated to some extent by ultrasound assessment of muscle depth at the biopsy site. This would raise the success rate of adequate muscle biopsy sample size from 18.8% in females and 21.4% in males (individuals with adequate muscle depth bilaterally) to 43.7% in females and 50% in males (individuals with adequate muscle depth on at least one side).

The data presented on the difficulties in obtaining adequate muscle samples in this chapter relates to muscle biopsies obtained using a Bergstrom needle technique. However it is possible to apply the same rationale to muscle biopsies obtained using other percutaneous muscle biopsy techniques, namely conchotome forceps and microbiopsy needle technique. The advantage of using a microbiopsy needle technique is the small gauge of the needle causing less trauma to both the muscle and overlying tissues. However, due to the narrowness of the gauge to ensure an adequate sized muscle biopsy sample the window is longer. The tip of the needle to window is 9mm which is comparable to the Bergstrom needle but the window length is 19mm over double the length of the Bergstrom needle (8mm). This amounts to a potentially

inadequately sized muscle sample in individuals with a muscle thickness of less than 28mm; in this sample population only adequate muscle biopsy samples would be obtained in 3.7% (1) of healthy older women, 23.1% (3) of healthy older men and none of the frail older adults. The conchotome forceps are an alternative to a needle biopsy technique with the advantage of the researcher being able to operate at shallower depths of muscle (293). However, this is at the expense of increased trauma to the area with an incision 5-10mm in length required to access the muscle. An alternatively proposed method for needle biopsy in individuals with low muscle mass is penetrating the muscle at a much shallower angle. The majority of needle biopsy protocols describe the needle entering the muscle perpendicularly however it has been suggested that a shallower angle will be more successful in individuals with a small muscle thickness (293, 296). However, this will disrupt more of the muscle architecture and may therefore not be appropriate technique in a research study.

The data presented in this chapter is contrary to current literature which suggests that muscle biopsy is feasible in older adults with frailty and/or sarcopenia. The paucity of negative results in published literature means that whilst muscle biopsy has been possible on occasions in these populations it is unclear whether the data presented here is an anomaly or a real threat to successful research. Appropriate study design is fundamental to the success of muscle biopsy within these populations and by exploring the differences in study design between this sample population and the successful studies potential improvements to study design will be highlighted for future research.

The Hertfordshire sarcopenia study reported on the feasibility and acceptability of muscle biopsy using the conchotome forceps method in a community dwelling male population. The study was able to perform 105 successful muscle biopsies in men of an average age of 72.4. However, 1086 men were screened and only 105 underwent a muscle biopsy. Therefore only 9.7% were either eligible or willing to have a muscle biopsy. Of these 105 men only 6% had sarcopenia as defined by EWGSOP criteria (289, 299). This study whilst successful in performing muscle biopsies on older males employed a broad screening process and included only a small percentage of individuals with sarcopenia. Alternative studies that have specifically performed muscle biopsies on sarcopenic populations employ diagnostic criteria of sarcopenia that do not include measures of muscle strength or physical performance and could therefore be considered to be criteria of low muscle mass rather than sarcopenia (287, 288). The methods utilised for defining sarcopenia included the FNIH criteria (ALM/BMI) in this sample the BMI was high (34.0) and therefore sarcopenia could be falsely reported due to high BMI (287). Muscle biopsies were obtained from 43 pre-frail and frail individuals of an average age 78. To obtain a total sample population of 62 (of which 43 received a muscle biopsy) 686 individuals were originally invited and 233 screened again employing a broad screening process before individuals were recruited to the study (290).

The chapter provides evidence that ultrasound assessment of the biopsy site prior to needle insertion should be mandatory in all frail populations and female community dwelling older adults. As part of best practice it could also be performed on male community dwelling older adults. Whilst potentially causing more trauma to the tissue the conchotome forceps method may be preferred in adults with less muscle mass as a

more reliable method of obtaining adequate muscle samples. The data presented in the chapter also demonstrates that performing muscle biopsies on a subset of a relatively small sample of frail older adults is not possible. Reviewing the study design of successful muscle biopsy studies highlights the importance of a large number of invitees (289, 290). In the UK this could be relatively easily achieved with access to general practice databases that routinely calculate the electronic frailty index (eFI) along with routine data on the use of anti-coagulants, anti-platelets and diagnosis of cognitive impairment. This screening approach to recruitment could lead to better targeting of appropriate individuals.

5. Neutrophil dysfunction in frail older adults.

5.1 Introduction

Frailty is the increased vulnerability of an individual to stressors (4, 300); its pathophysiology is uncharacterised but it is postulated that the immune system is central to both its development and propagation. There are known associations with inflammation and a dysregulated immune system which are thought to pre-date the physical manifestations of frailty (301). A raised white cell and neutrophil count have been associated with frailty along with an increase in the pro-inflammatory cytokines, IL-6 and TNF α , and the acute phase protein CRP (169-171, 178). Research has also demonstrated that baseline IL-6 and CRP are predictive of muscle strength loss at three years (302). Recent work has shown for the first time that neutrophils from frail older adults are functionally compromised; stimulated neutrophils from frail older adults produce less chemokine than neutrophils from healthy older adults (208).

Previous work by has demonstrated dysregulation in the function of neutrophils with an individual's increasing age (163). To reach interstitial pathogens neutrophils must migrate through tissue but as neutrophils migrate they cause obligate tissue damage through the release of proteinases and reactive oxygen species(303, 304); therefore it has been hypothesised that inaccurate neutrophils will cause an increased amount of tissue damage and the associated release of pro-inflammatory cytokines propagating an environment of chronic inflammation (233). The PI3K signalling pathway is central to neutrophil motility, and previous work has implicated dysregulation of this pathway in the inaccurate neutrophil migration associated with ageing. Importantly, in vitro neutrophil migratory inaccuracy can be corrected with selective PI3K inhibitors, and this provides a potential therapeutic strategy (163).

Previous investigation of immunosenescence and inflammaging has been very limited in frailty. This chapter investigates the functional ability of neutrophils from frail older adults, in particular focusing on neutrophil migration, and describes an environment of chronic inflammation.

This chapter aims to

- Investigate neutrophil migratory dynamics in healthy older and frail older adults
- Describe the difference in neutrophils from healthy older and frail older adults using cell surface markers thought to be fundamental to neutrophil adhesion, activation and senescence
- Investigate the role of PI3 kinase in migratory dynamics in neutrophils from frail older adults
- Describe a 'frail' in vivo environment and consider its effect on neutrophils

5.2 Results

Experiments were conducted on three extensively characterised groups: healthy younger adults, healthy older adults and frail older adults. Not all individuals were included in every experiment and therefore slight variations in characteristics are present. Table 5.1 describes some of the characteristics in the groups as a whole. More in depth analysis of the three groups is described in Chapter 6.

Throughout Chapter 5 the data is displayed as either:

1 – Parametric data. Mean (Confidence Intervals)

2 – Non-parametric data. Median +/- Interquartile range

Table 5.1 – Demographics of healthy young adult, healthy older adults and frail older adults.

	HY	HO	FO	I K-W	Pairwise comparison
Number	40	40	37		
Gender % (F:M)	51:49	67:33	54:46		
Age	26.0 +/- 9.8	71.0 +/- 9.0	84.0 +/- 14.8	p<0.001	HY-HO p<0.001 HY-FO p<0.001 HO-FO p<0.019
Frailty Index	0.02 +/- 0.02	0.04 +/- 0.07	0.30 +/- 0.11	p<0.001	HY-HO p<0.005 HY-FO p<0.001 HO-FO p<0.000
Adjusted grip strength	1.71 (1.61-1.81)	1.31 (1.23-1.39)	0.73 (0.64-0.82)	p<0.001	HY-HO p<0.001 HY-FO p<0.001 HO-FO p<0.001
Walk speed (m/sec)	1.62 +/- 0.00	1.26 +/- 0.00	0.33 +/- 0.00	p<0.001	HY-HO p<0.007 HY-FO p<0.001 HO-FO p<0.001
SPPB	12.0 +/- 0.0	12.0 +/- 2.0	2.0 +/- 3.0	p<0.001	HY-HO p=0.051 HY-FO p<0.001 HO-FO p<0.001

Demographics of the three different groups: HY - healthy young adults, HO - healthy older adults and FO - frail older adults. N = HY – 40, HO – 40, FO – 37. Categorical data presented as proportion of total. Age, frailty index, walk speed and short physical performance battery (SPPB) are not distributed normally. Data given as median +/- IQR. Statistical tests are Independent Kruskal-Wallis (I K-W) with pairwise comparison with Bonferroni correction. Adjusted grip strength normally distributed and data given as mean with confidence intervals. Statistical test is ANOVA with post-hoc Tukey’s for pairwise comparison. All statistically significant values appear in bold text.

Migration was assessed using 3 parameters: Average cell speed of movement ($\mu\text{m}/\text{minute}$) was measured from the distance travelled between frames, in any direction, over time (termed chemokinesis). Average cell velocity (speed in a consistent direction towards the chemo-attractant, termed chemotaxis) was measured in $\mu\text{m}/\text{minute}$. Only distance travelled in the y-direction over time was included in calculations of chemotaxis. Accuracy (termed chemotactic index) was calculated by the cosine of the angle between the cell's direction and the orientation of the chemoattractant gradient at each time frame forming a vector analysis of movement, expressed in a comparative scale (cs) ranging from -1 to 1 , where 1 represents movement directly toward the chemoattractant, and -1 represents movement directly away from the chemoattractant source in all frames.

5.3 Neutrophils from healthy and frail older adults do not have a reduction in chemokinesis.

Migratory pathways from individual cells suggested that neutrophils from frail subjects migrated more slowly and with less accuracy than those from younger subjects. Figure 5.1 shows representative neutrophil migratory pathways as tracked in one healthy young subject and one frail older subject.

There were no significant differences between the chemokinesis of isolated neutrophils to either IL-8 and fMLP for the three groups. However, there was a non-significant reduction in chemokinesis towards fMLP across healthy older adults and frail older adults. (Figure 5.2.) Chemokinesis to fMLP ($\mu\text{m}/\text{min}$): HY 4.26 ± 2.08 , HO 3.62 ± 1.71 and FO 3.24 ± 1.13 . Independent Kruskal-Wallis $p=0.131$. Chemokinesis to IL-8 ($\mu\text{m}/\text{min}$): HY 3.26 ± 1.45 , HO 3.25 ± 0.64 and FO 2.86 ± 0.83 . Independent Kruskal-Wallis $p=0.219$.

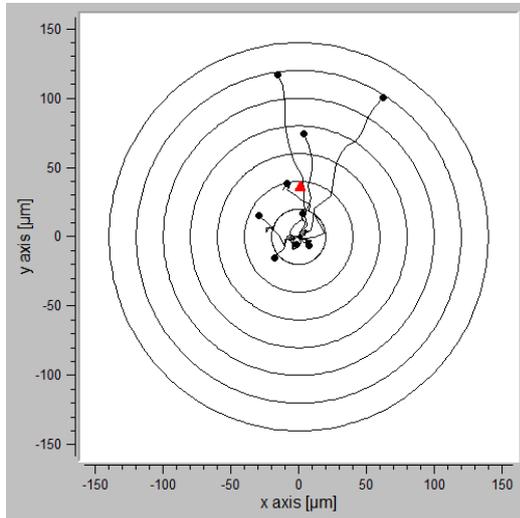
5.4 Neutrophils exhibit an age related decline in chemotaxis to a bacterial chemoattractant but a frailty related decline to an endogenous chemoattractant

Neutrophils from frail older adults showed a reduction in chemotaxis to both a bacterial chemoattractant, fMLP, and an endogenous chemoattractant, IL-8. Neutrophils from healthy older adults only showed a significant decline in migratory accuracy to the bacterial chemoattractant fMLP; there was preserved chemotaxis to IL-8. (Figure 5.3.)

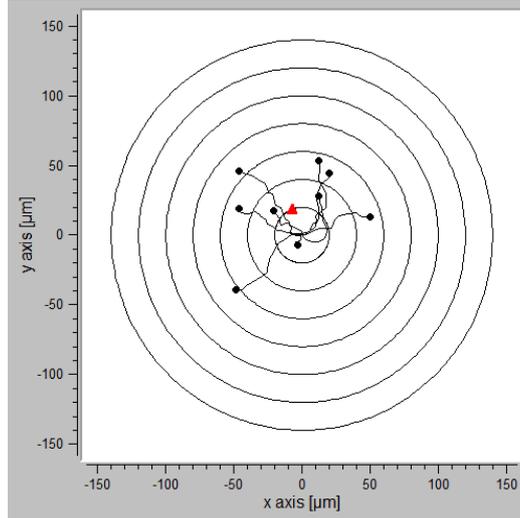
Chemotaxis to fMLP ($\mu\text{m}/\text{min}$): HY 1.68 (1.40-1.95), HO 0.78 (0.49-1.08) and FO 0.92 (0.48-1.36). ANOVA $p < 0.001$. Chemotaxis to IL-8 ($\mu\text{m}/\text{min}$): HY 1.07 (0.72-1.41), HO 0.90 (0.64-1.16) and FO 0.49 (0.26-0.73). ANOVA $p = 0.042$.

Figure 5.1 – Representative migratory pathways of neutrophils towards fMLP

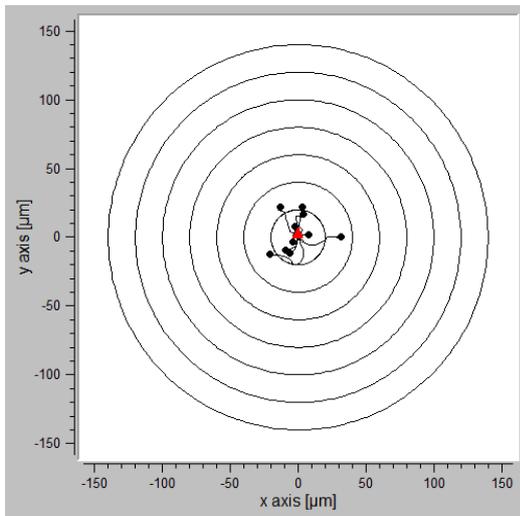
A - HY



B - HO

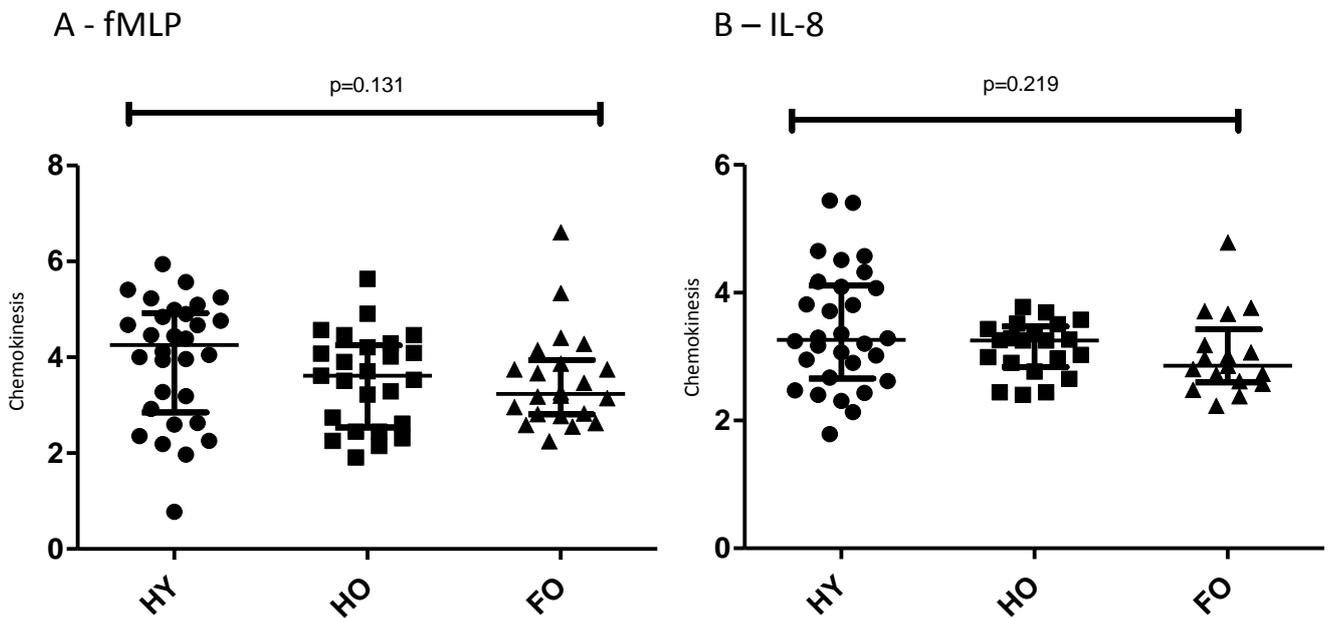


C - FO



Representative migratory pathways of neutrophils to fMLP in A – healthy young adult, B – healthy older adult and C – frail older adult. Migratory pathways of neutrophils over a 12 minute period standardised to start within the centre of the circular grid. Chemoattractant (fMLP) is at the top of the circle and control (RPMI) at the bottom of the page establishing a gradient over the region of the circular grid. Each line represents one neutrophil with the black circle marking the position of the neutrophil following 12 minutes of tracking. Ten neutrophils were tracked for each sample. The red triangle represents the average position of the tracked neutrophils. The circular lines on the grid are 20 μ m apart. A – HY: 33M, FI 0.00. B – HO: 84M, FI 0.00, C-FO:95F, FI 0.38.

Figure 5.2 - Chemokinesis of neutrophils

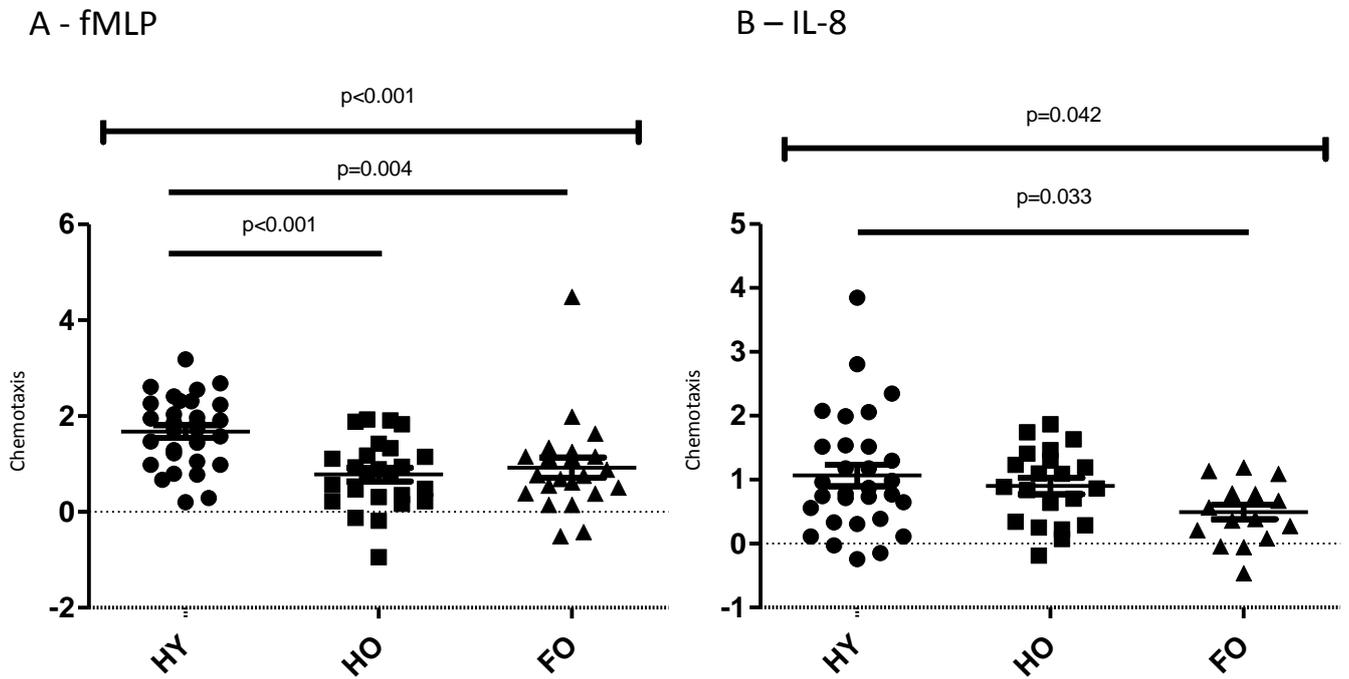


Chemokinesis of neutrophils towards A – fMLP and B – IL-8. Isolated neutrophils from healthy young adults (HY), healthy older adults (HO) and frail older adults (FO) migrated towards A -10nM fMLP and B- 100nM IL-8. Chemokinesis has been calculated and expressed as $\mu\text{m}/\text{min}$. Data sets are median+/-IQR. Chemokinesis to IL-8 not normally distributed. Chemokinesis to fMLP normally distributed.

A – N = HY-30, HO-25, FO-22. . HY - 22.9, FI 0.01. HO - 75.6, FI 0.05. FO - 84.1, FI 0.41. Independent Kruskal-Wallis p=0.131, Post-hoc Dunn's test all non-significant.

B - N = HY-30, HO-21, FO-17. HY – 22.3, FI 0.01. HO – 76.4, FI-0.06. FO – 84.1, FI 0.41. Independent Kruskal-Wallis p=0.219, Post-hoc Dunn's test all non-significant.

Figure 5.3 – Chemotaxis of neutrophils



Chemotaxis of neutrophils migrating toward A – fMLP and B – IL-8. Isolated neutrophils from healthy young adults (HY), healthy older adults (HO) and frail older adults (FO) migrated towards; A -10nM fMLP and B- 100nM IL-8. Each dot represents one participant. Chemotaxis calculated and expressed as $\mu\text{m}/\text{min}$. The line and error bars represent the mean \pm -SEM; both normally distributed.

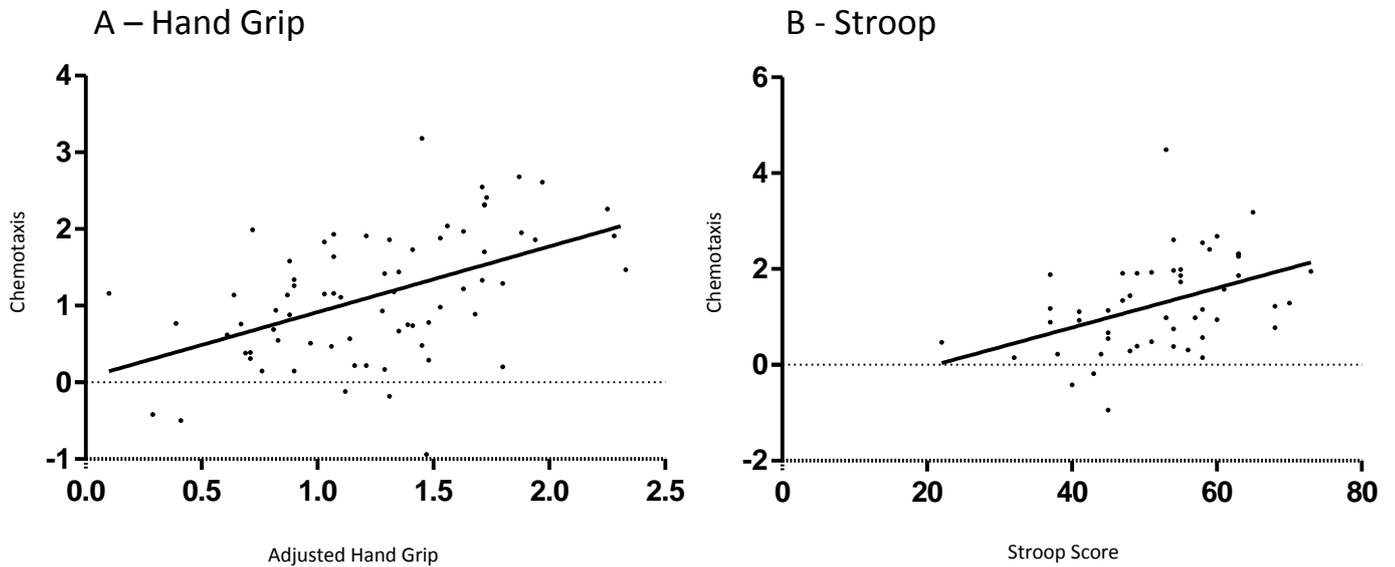
A: Numbers in each group are as follows: HY-30, HO-25, FO-22. Age and frailty index as follows: HY - 22.9, FI 0.01. HO - 75.6, FI 0.05. FO - 84.1, FI 0.41. ANOVA $p<0.001$, Post-hoc Tukey's – HY-HO $p<0.001$, HY-FO $p=0.004$, HO-FO $p=0.831$

B: Numbers in each group are as follows: HY-30, HO-21, FO-17. Age and frailty index as follows: HY – 22.3, FI 0.01. HO – 76.4, FI-0.06. FO – 84.1, FI 0.41. ANOVA $p=0.042$, Post-hoc Tukey's – HY-HO $p=0.708$, HY-FO $p=0.033$, HO-FO $p=0.212$

5.5 Neutrophil chemotaxis correlates with both physical markers of frailty and sarcopenia, and cognitive parameters; this is independent of age.

To determine whether increasing frailty was associated with decreasing chemotaxis both physical markers of frailty, namely grip strength adjusted for gender and BMI (fold change of cut-off for lowest 20% for gender and BMI in over 65) and cognitive parameters, Stroop score, were correlated with chemotaxis towards fMLP. There was a linear relationship between the markers of frailty and chemotaxis; increasing frailty in an individual was associated with decreasing migratory accuracy of their neutrophils. Chemotaxis to fMLP ($\mu\text{m}/\text{min}$) and adjusted hand grip. $R=0.492$. $p<0.001$. Chemotaxis to fMLP ($\mu\text{m}/\text{min}$) and Stroop score. $R=0.430$. $p=0.002$. (Figure 5.4.) Chemotaxis to fMLP ($\mu\text{m}/\text{min}$) and walk time. $R=-0.381$. $p=0.005$. (Pearson's correlation coefficient for all.) This relationship was independent of age and a partial correlation adjusted for age demonstrated significant correlation between both physical and cognitive markers of frailty. Chemotaxis to fMLP ($\mu\text{m}/\text{min}$) and adjusted hand grip and adjusted for age. $R=0.305$. $p=0.005$. Chemotaxis to fMLP ($\mu\text{m}/\text{min}$) and Stroop Score adjusted for age. $R=0.255$. $p=0.037$. Chemotaxis was also associated with indicators of muscle health, ultrasound echogenicity and BATT. Chemotaxis to fMLP ($\mu\text{m}/\text{min}$) and ultrasound echogenicity. $R= -0.371$, $p=0.012$. Chemotaxis to fMLP ($\mu\text{m}/\text{min}$) and adjusted BATT. $R=0.394$, $p=0.004$, (Pearson's correlation coefficient for both.)

Figure 5.4 – Correlation of neutrophil chemotaxis to fMLP with adjusted hand grip and Stroop interference score



Correlation of neutrophil chemotaxis to fMLP with A – adjusted hand grip strength and B – Stroop interference score. Isolated neutrophils from healthy young adults, healthy older adults and frail older adults migrated towards 10nM fMLP; chemotaxis has been calculated expressed as $\mu\text{m}/\text{min}$. Figure 5.4A demonstrates the correlation of chemotaxis to fMLP with adjusted hand grip strength and figure 5.4B the correlation of chemotaxis to fMLP with Stroop interference score. Each individual also had grip strength adjusted for gender and BMI (fold change of cut-off for lowest 20% for gender and BMI in over 65) calculated and Stroop score obtained. All data sets are normally distributed.

A – N=73. Pearson's correlation coefficient. 2-tailed. $R=0.492$. $P<0.001$

B – N=51. Pearson's correlation coefficient. 2-tailed. $R=0.430$. $P=0.002$

5.6 Neutrophils exhibit an age related decline in chemotactic index to a bacterial chemoattractant but a frailty related decline to an endogenous chemoattractant

A neutrophil's ability to migrate efficiently towards a target is dependent on both migratory accuracy and speed. To assess the overall migratory function of neutrophils in frailty and old age chemotactic index was compared across the three groups. The chemotactic index of the three groups demonstrated an identical pattern to that witnessed with chemotaxis.

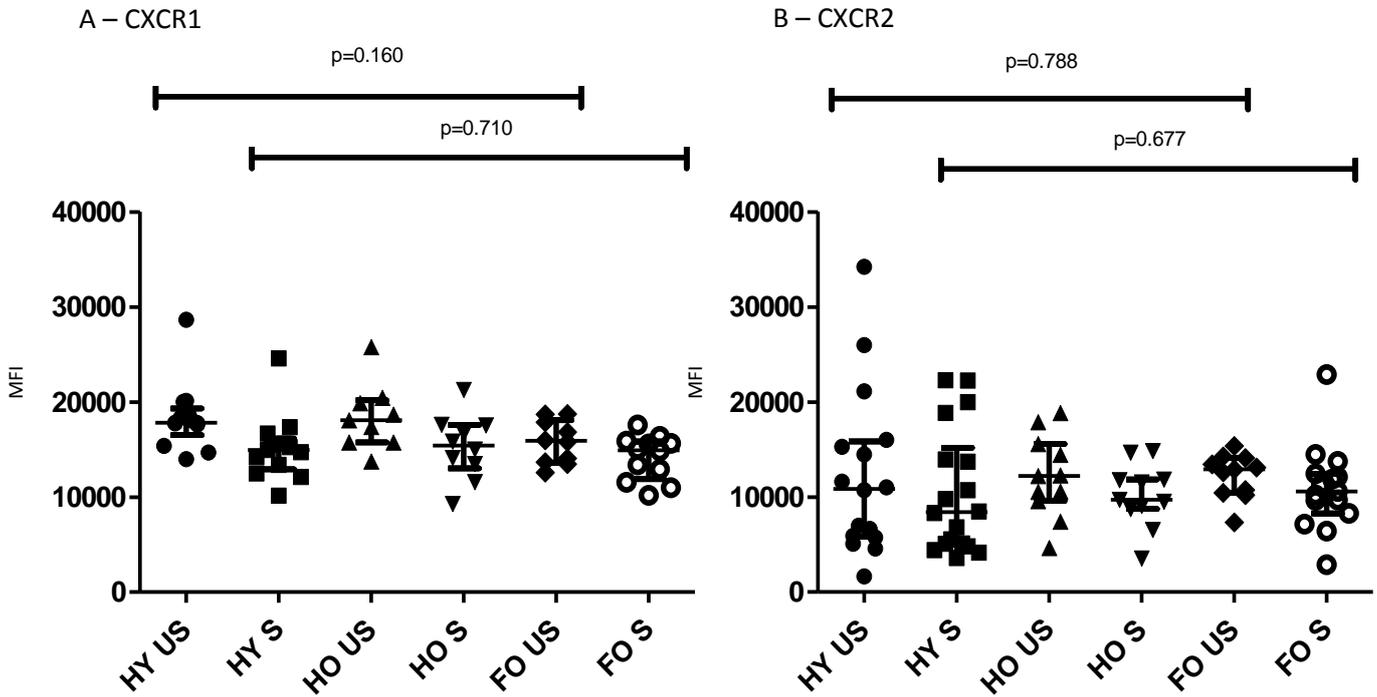
There was a significant difference between the chemotactic index to fMLP for the three groups but not IL-8. Chemotactic index towards fMLP: HY 0.38cs (0.32-0.44), HO 0.15cs (0.09-0.21) and FO 0.21cs (0.13-0.28). ANOVA $p < 0.001$. Post-hoc Tukey's: HY-HO $p < 0.001$, HY-FO $p < 0.001$, HO-FO $p = 0.481$. Chemotactic index towards IL-8: HY 0.24cs (0.17-0.31), HO 0.24cs (0.17-0.30) and FO 0.13cs (0.06-0.20). ANOVA $p = 0.057$. Post-hoc Tukey's: HY-HO $p = 0.994$, HY-FO $p = 0.063$, HO-FO. $p = 0.109$.

5.7 CXCR1 and CXCR2 expression cannot explain the differences witnessed between the migration of neutrophils from healthy older and frail older adults

The experiments described in this chapter demonstrate preserved migratory accuracy to IL-8 in healthy older adults whereas neutrophils from both healthy older and frail older adults demonstrate reduced migratory accuracy to fMLP. To investigate these differences the expression of CXCR1 and CXCR2 on the neutrophil cell surface was measured using flow cytometry and recorded as median fluorescence intensity (MFI).

There was no difference in expression of CXCR1 or CXCR2 on the neutrophil cell surface at baseline or on stimulation in frail older adults and healthy older adults when compared with healthy young adults. (Figure 5.5.) CXCR1 US MFI: HY 17512.0 +/-2804.5, HO 18092.5 +/- 4424.5, FO 15905.8 +/- 4447.0. Independent Kruskal-Wallis $p=0.160$. CXCR1 S MFI: HY 14954.0 +/- 3227.0, HO 15426.8 +/- 4556.6, FO 14930.3 +/- 3936.3 . Independent Kruskal-Wallis $p=0.710$. CXCR2 US HY 10856.5 +/- 10035.0, HO 12232.0 +/- 7181.0, FO 12931.0 +/- 3662.1. Independent Kruskal-Wallis $p=0.788$. CXCR2 S HY 8400.5 +/- 10151.6, HO 9517.0 +/- 4052.3, FO 10576.0 +/- 4151.5. Independent Kruskal-Wallis $p=0.677$.

Figure 5.5 – Expression of CXCR1 and CXCR2 on the neutrophil cell surface



The MFI of A - CXCR1 and B - CXCR2 expression on neutrophil cell surface in unstimulated cells (US) and cells stimulated with 10nM fMLP (S) in healthy young adults (HY), healthy older adults (HO) and frail older adults (FO). Data sets are median +/- IQR. Data not normally distributed.

CXCR1 – N for US = HY-13, HO-9, FO-10, N for S = HY-13, HO-10, FO-12. CXCR1 US MFI: HY 17812.0 +/- 2805.0, HO 28092.5 +/- 4424.5, FO 15905.8 +/- 4447.0, CXCR1 S MFI: HY 14954.0 +/- 3227.0, HO 15426.8 +/- 4556.6, FO 14930.3 +/- 3936.3 Independent Kruskal-Wallis test US p=0.160, S p=0.710

CXCR2 - N for US = HY-16, HO-9, FO-11, N for S = HY-18, HO-9, FO-15. CXCR2 US HY 10856.5 +/- 10035.0, HO 12232.0 +/- 7181.0, FO 12931.0 +/- 3662.1, CXCR2 S HY 8400.5 +/- 10151.6, HO 9517.0 +/- 4052.3, FO 10576.0 +/- 4151.5. Independent Kruskal-Wallis test US p=0.788, S p=0.677

5.8 Neutrophils from frail older adults have reduced expression of the adhesion markers CD11a and CD11b whereas neutrophils from healthy older adults have reduced expression of CD11b.

To consider whether differences in neutrophil expression of the β 2 integrins Mac-1 and LFA-1 (which bind to ICAM-1 during cell-cell adhesion) could explain the reduction in chemotaxis witnessed in both frail and healthy older adults the MFI of CD11a, CD11b, CD18 and activated CD11b expressed on the neutrophil cell surface was measured using flow cytometry in unstimulated, time-matched controls (denoted baseline in following text) and following stimulation with fMLP.

There was no difference in the expression of CD18 at baseline or on stimulation but there were differences in the expression of CD11a, CD11b and activated CD11b. MFI of CD18 US: HY 34431 +/- 10014, HO 30542 +/- 8825, FO 29205.5 +/- 15525. Independent Kruskal-Wallis $p=0.514$. CD18 S: HY 57389 +/- 12419, HO 57452.5 +/- 15462, FO 51621 +/- 19523. Independent Kruskal-Wallis $p=0.278$. CD18 fold change ((Stimulated – Baseline)/Baseline) baseline and stimulated: HY 0.8 +/- 0.38, HO 0.7 +/- 0.3, FO 0.7 +/- 0.5. Independent Kruskal-Wallis $p=0.787$.

Neutrophil cell surface expression of CD11a was reduced in frail older adults but not in healthy older adults at both baseline and at stimulation. MFI of CD11a US: HY 1296 +/- 318, HO 1186 +/- 332, FO 1054 +/- 174. Independent Kruskal-Wallis $p=0.004$. Bonferroni corrected pairwise comparisons HY-HO $p=1.000$, HY-FO $p=0.003$, HO-FO $p=0.028$. CD11a S: HY 1270 +/- 454, HO 1242.5 +/- 464, FO 1081 +/- 211. Independent Kruskal-Wallis $p=0.005$. Bonferroni corrected pairwise comparisons HY-HO $p=1.000$, HY-FO $p=0.006$, HO-FO $p=0.031$. There was no difference in response to stimulation throughout the three groups. CD11a fold change ((Stimulated – Baseline)/Baseline) between baseline and

stimulated: HY 0.0 +/-0.1, HO 0.0 +/- 0.1, FO 0.1 +/- 0.1. Independent Kruskal-Wallis p=0.951.

Neutrophil cell surface expression of CD11b was significantly reduced in healthy older adults at baseline and on stimulation and there was a trend to reduction in frail older adults. However, the response to stimulation, upregulation, was similar throughout the three groups. MFI of CD11b US: HY 2015.8 +/- 884, HO 1665 +/- 880, FO 1649 +/- 1629. Independent Kruskal-Wallis p=0.018. Bonferroni corrected pairwise comparisons HY-HO p=0.039, HY-FO p=0.053, HO-FO p=1.000. CD11b S: HY 3997.3 +/- 1495, HO 3195.5 +/- 1241, FO 3180.7 +/- 1673. Independent Kruskal-Wallis p=0.002. Bonferroni corrected pairwise comparisons HY-HO p=0.002, HY-FO p=0.074, HO-FO p=1.000. CD11b fold change ((Stimulated – Baseline)/Baseline) between baseline and stimulated: HY 0.8 +/- 0.5, HO 0.7 +/- 0.4, FO 0.75 +/- 1.33. Independent Kruskal-Wallis p=0.937.

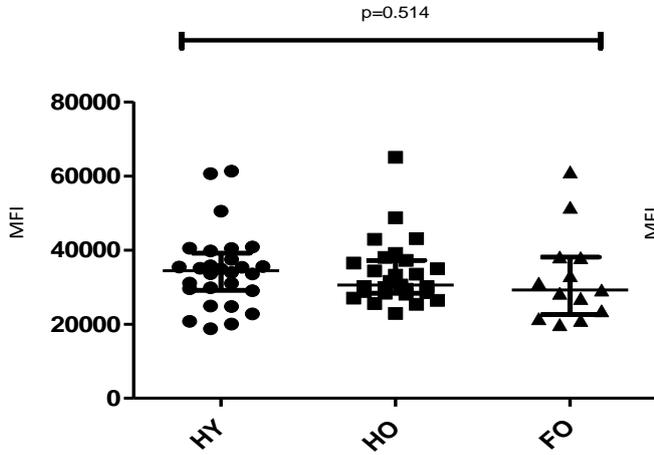
There was reduced expression of activated CD11b at baseline in healthy older adults and on stimulation in both healthy older and frail older adults. There was a significant reduction in the ability of neutrophils from healthy and frail older adults to upregulate activated CD11b with stimulation. MFI of Act11b US: HY 3149 +/- 2944, HO 2992 +/- 955, FO 2301 +/- 2260. Independent Kruskal-Wallis p=0.038. Bonferroni corrected pairwise comparisons HY-HO p=0.037, HY-FO p=0.409, HO-FO p=1.000. Act11b S: HY 11323 +/- 7118, HO 5239 +/- 5446, FO 6410 +/- 6393. Independent Kruskal-Wallis p=0.001. Bonferroni corrected pairwise comparisons HY-HO p=0.003, HY-FO p=0.021, HO-FO p=1.000. ActCD11b fold change ((Stimulated – Baseline)/Baseline) between baseline and stimulated: HY 78.4 +/- 50.5, HO 31.0 +/- 48.5, FO 36.8 +/- 45.6. Independent Kruskal-

Wallis $p=0.002$. Bonferroni corrected pairwise comparisons HY-HO $p=0.005$, HY-FO $p=0.021$, HO-FO $p=1.000$. (Figure 5.6.)

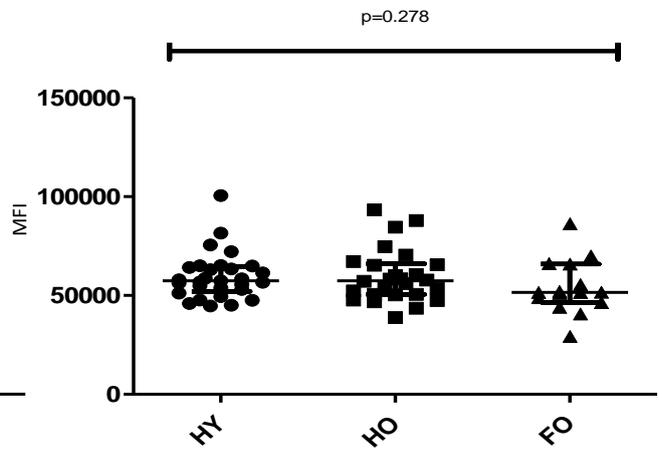
The reduction in expression of CD11b and ActCD11b correlated with reduced chemotaxis to fMLP illustrating the role of Mac-1 in neutrophil migration towards fMLP. The correlation, whilst significant, was not very strong, suggesting this was not the only process important in neutrophil migration towards fMLP. Chemotaxis to fMLP ($\mu\text{m}/\text{min}$) and expression of CD11b at baseline (MFI). $R=0.413$. $p=0.029$. Pearson's correlation coefficient. Chemotaxis to fMLP ($\mu\text{m}/\text{min}$) and expression of activated 11b at baseline (MFI). $R=0.364$. $p=0.038$. (Figure 5.7.)

Figure 5.6 – Neutrophil cell surface expression of CD18 at baseline, CD18 on stimulation, CD11a at baseline, CD11a on stimulation, CD11b at baseline and Act11b response to stimulation

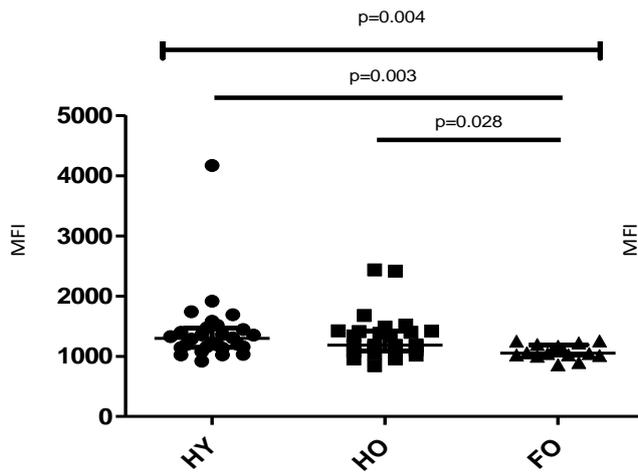
A – CD18 Baseline



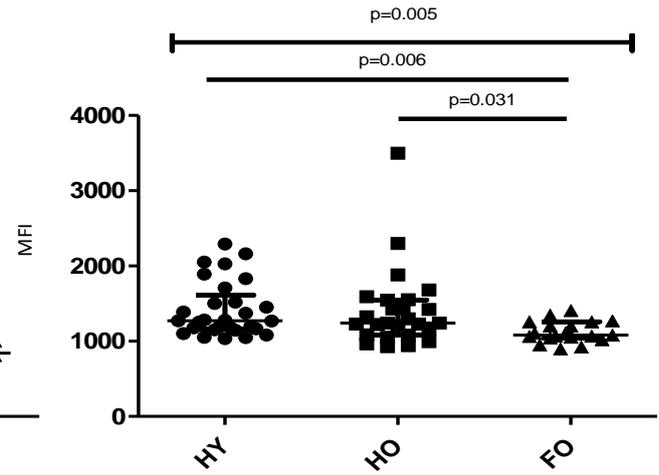
B – CD18 Stimulation



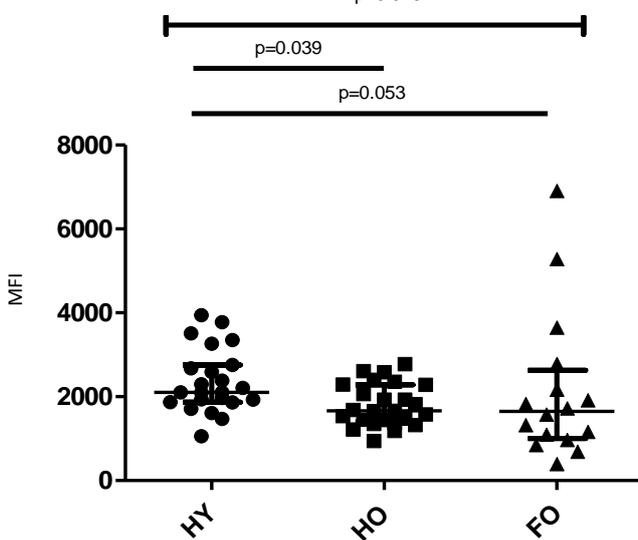
C – CD11a Baseline



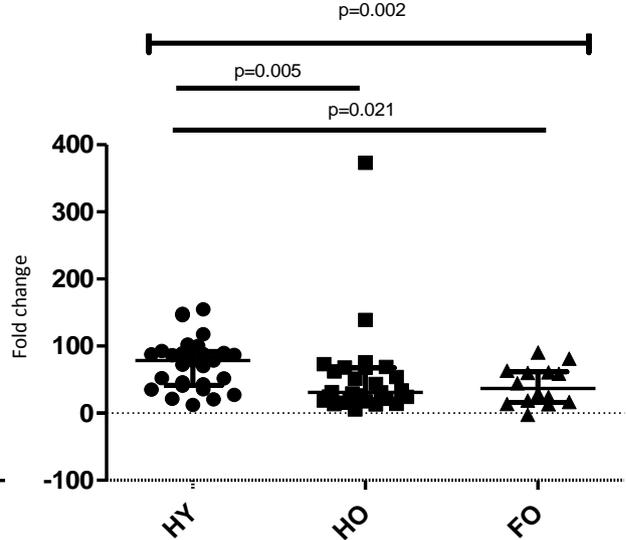
D – CD11a Stimulation



E – CD11b Baseline



F – Act11b



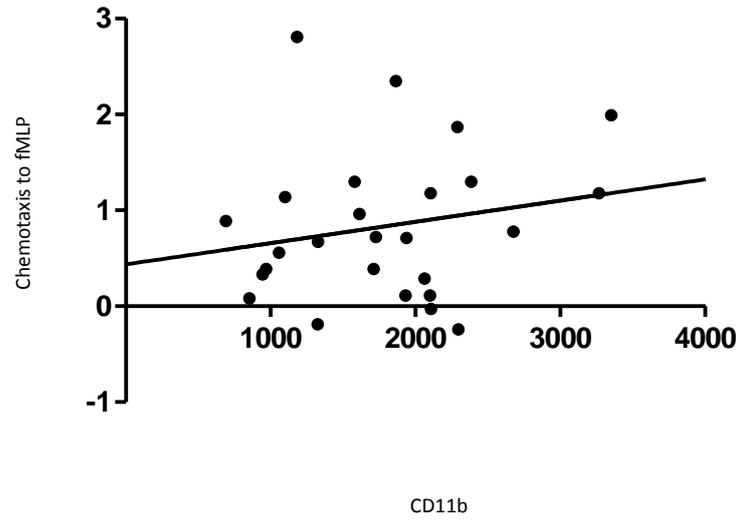
The MFI of A - CD18 baseline, B – CD18 on stimulation, C - CD11a baseline, D – CD11a on stimulation, E – CD11b at baseline and F - Act11b response to 10nM fMLP stimulation in healthy young adults (HY), healthy older adults (HO) and frail older adults (FO). Stimulation is 10nM fMLP. Data sets are median +/- IQR; data not normally distributed.

A – CD18US: HY – 28, HO – 27, FO - 13. HY 34431 +/- 10014, HO 30542 +/- 8825, FO 29205.5 +/- 15525. Independent Kruskal Wallis – p=0.514. B - CD18 S: HY – 29, HO – 26, FO – 15. HY 57389 +/- 12419, HO 57452.5 +/- 15462, FO 51621 +/- 19523. Independent Kruskal Wallis – p=0.278.

C - CD11a US: HY – 28, HO – 27, FO – 16. HY 1296 +/- 318, HO 1186 +/- 332, FO 1054 +/- 174. Independent Kruskal Wallis – p=0.004. Post-hoc pairwise comparison with Bonferroni correction: HY-HO p=1.000, HY-FO p=0.003, HO-FO p=0.028. D - CD11a S: HY – 29, HO – 26, FO – 19. HY 1270 +/- 454, HO 1242.5 +/- 464, FO 1081 +/- 211. Independent Kruskal Wallis – p=0.005. Post-hoc pairwise comparison with Bonferroni correction: HY-HO p=1.000, HY-FO p=0.006, HO-FO p=0.031.

E - CD11b US: HY – 24, HO - 25, FO – 18. HY 2015.8 +/- 884, HO 1665 +/- 880, FO 1649 +/- 1629. Independent Kruskal Wallis – p=0.018. Post-hoc pairwise comparison with Bonferroni correction: HY-HO p=0.039, HY-FO p=0.053, HO-FO p=1.000. F - Act11b fold change: HY – 28, HO – 27, FO – 14. HY 78.4 +/- 50.5, HO 31.0 +/- 48.5, FO 36.8 +/- 45.6. Independent Kruskal Wallis – p=0.002. Post-hoc pairwise comparison with Bonferroni correction: HY-HO p=0.005, HY-FO p=0.021, HO-FO p=1.000.

Figure 5.7 – Correlation of cell surface expression of CD11b with chemotaxis to fMLP



The correlation of cell surface expression of CD11b at baseline expressed in MFI and chemotaxis to fMLP ($\mu\text{m}/\text{min}$). Data sets are normally distributed. $N = 25$. $R=0.413$. $P=0.029$. Pearson's correlation coefficient.

5.9 Neutrophils from healthy and frail older adults have a poorer response to stimulation.

The expression of β 2 integrins Mac-1 and LFA-1 are in part regulated by the activation molecules and selectins CD62L, CD63 and CD66b which are expressed on the neutrophil cell surface. To explore the effect of frailty on these activation molecules, the MFI was measured on neutrophils using flow cytometry both at baseline and following stimulation with fMLP.

At baseline there was no difference in the expression of all three cell surface markers. (Table 5.2.) However, there were significant differences in how all three cell surface markers were expressed on stimulation (with upregulated CD63 and CD66b and shedding of CD62L) and the neutrophil's response to stimulation, Independent Kruskal-Wallis $P=0.001$, with neutrophils from healthy older and frail older adults less able to upregulate CD63 and CD66b and less able to shed CD62L on stimulation . (Figure 5.8.)

Table 5.2 – Median fluorescence intensity (MFI) of CD62L, CD63 and CD66b in healthy younger, older and frail older adults at baseline and on stimulation

	HY	HO	FO	I K-W	Pairwise
CD62L US	27510 +/- 11358.5	32110.0 +/- 10115.5	23626 +/- 20221.5	p=0.073	
CD62L S	8498 +/- 8296	16247.8 +/- 8809.6	15619 +/- 12829	p=0.002	HY-HO p=0.002 HY-FO p=0.051 HO-FO p=1.000
CD62L Fold	-0.7 +/- 0.3	-0.5 +/- 0.2	-0.4 +/- 0.5	p<0.001	HY-HO p=0.004 HY-FO p=0.001 HO-FO p=0.961
CD63 US	653.5 +/- 269	578 +/- 325.5	571.5 +/- 403	p=0.638	
CD63 S	998 +/- 569	718 +/- 501.2	615 +/- 608.3	p=0.033	HY-HO p=0.102 HY-FO p=0.065 HO-FO p=1.000
CD63 Fold	0.4 +/- 0.4	0.2 +/- 0.4	0.1 +/- 0.3	p=0.001	HY-HO p=0.014 HY-FO p=0.004 HO-FO p=1.000
CD66b US	9093.5 +/- 2601	9075.5 +/- 2058	9937 +/- 6444	p=0.609	
CD66b S	23338 +/- 9760	15897 +/- 4451	18385 +/- 9431	p=0.001	HY-HO p=0.001 HY-FO p=0.105 HO-FO p=0.339
CD66b Fold	1.61 +/- 1.0	0.83 +/- 1.0	0.92 +/- 1.0	p<0.001	HY-HO p=0.001 HY-FO p=0.002 HO-FO p=1.000

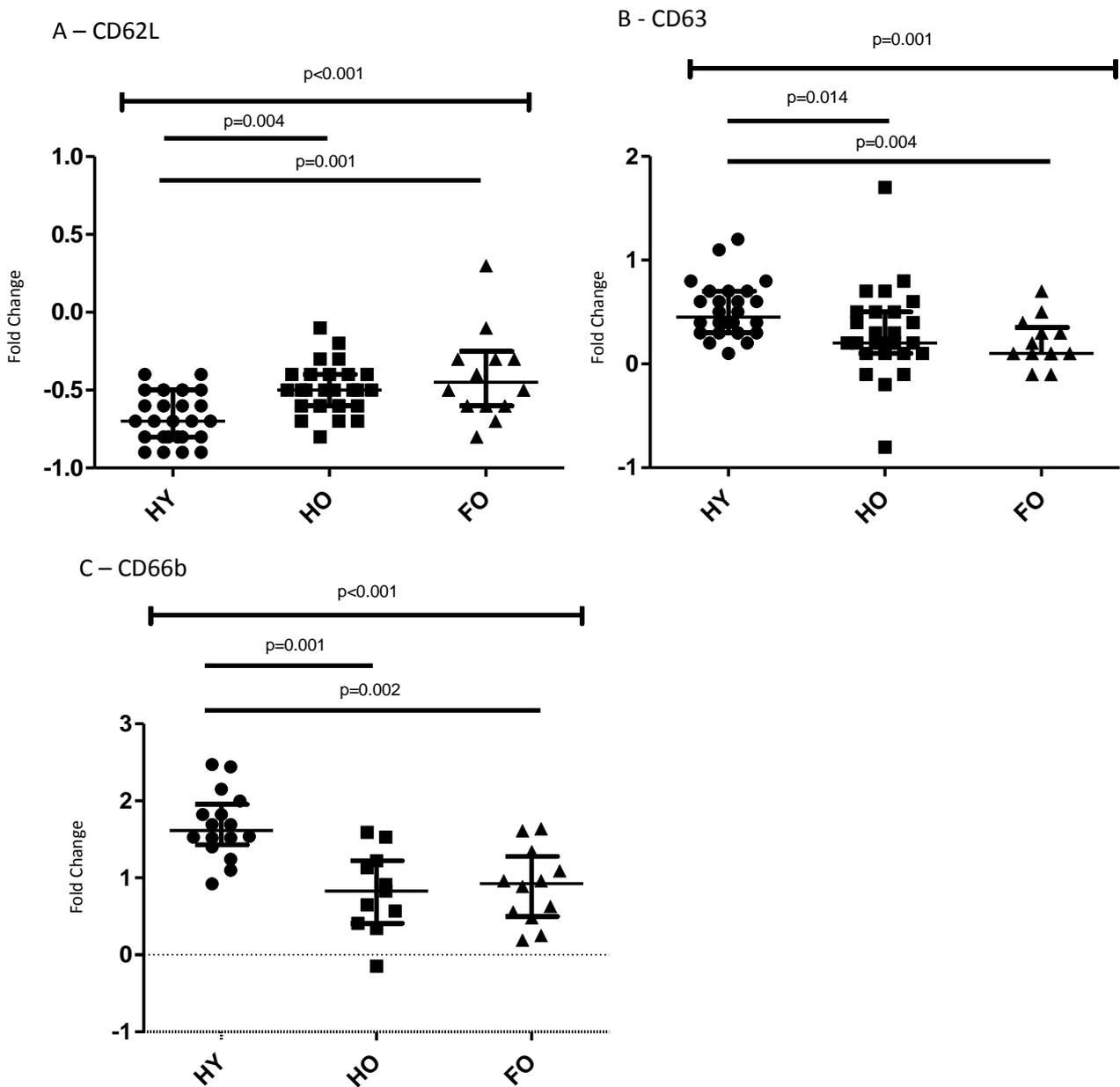
MFI at baseline and on stimulation with 10nM fMLP, and fold change with stimulation in CD62L, CD63 and CD66b in healthy younger adults (HY), healthy older adults (HO) and frail older adults (FO). Data sets are median +/- IQR; data not normally distributed. Independent Kruskal-Wallis(I K-W) test with post-hoc pairwise comparison with Bonferroni correction.

CD62L US: HY – 27, HO – 27, FO -17, CD62L S: HY – 29, HO -28, FO -16, CD62L fold change: HY – 27, HO – 27, FO – 15.

CD63 US: HY – 27, HO – 29, FO – 14, CD63 S: HY – 29, HO – 29, FO – 17, CD63 fold change: HY – 27, HO – 29, FO – 14.

CD66b US: HY – 16, HO – 11, FO – 12, CD66b S: HY – 17, HO – 11, FO – 13, CD66b fold change: HY – 16, HO – 11, FO – 12

Figure 5.8 – Change in neutrophil cell surface expression on stimulation of CD62L, CD63 and CD66b



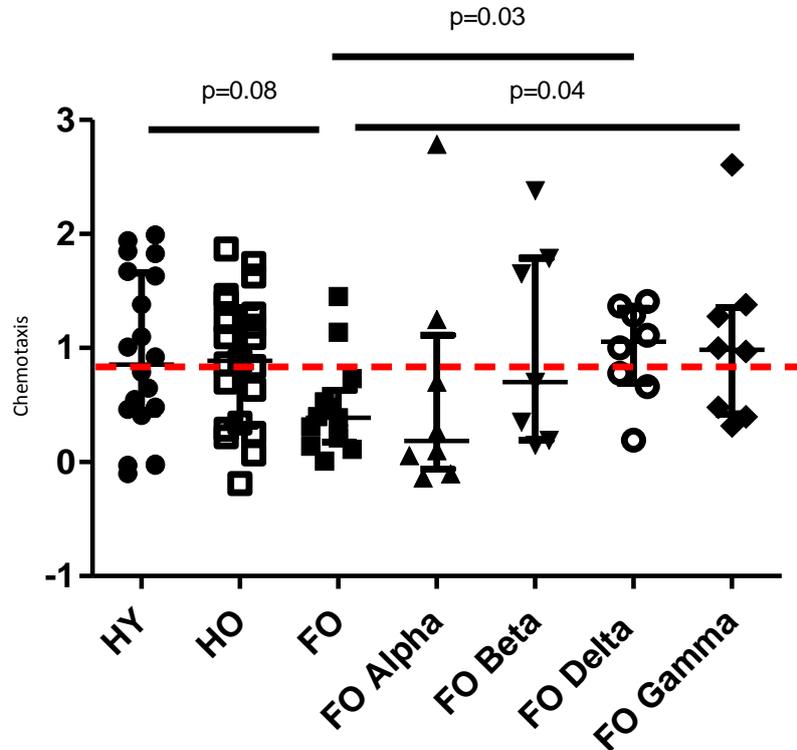
The fold change in MFI on stimulation with 10nM fMLP of A – CD62L, B – CD63 and C – CD66b. Data sets are median +/- IQR; data not normally distributed. A - CD62L fold change: HY – 27, HO – 27, FO – 15. HY - -0.7 +/- 0.3, HO - -0.5 +/- 0.2, FO - -0.4 +/- 0.5. Independent Kruskal-Wallis test $p < 0.001$. Post-hoc pairwise comparison with Bonferroni correction. HY-HO $p = 0.004$, HY-FO $p = 0.001$, HO-FO $p = 0.961$. B - CD63 fold change: HY – 27, HO – 29, FO – 14. HY – 0.4 +/- 0.4, HO – 0.2 +/- 0.4, FO – 0.1 +/- 0.3. Independent Kruskal-Wallis test $p = 0.001$. Post-hoc pairwise comparison with Bonferroni correction. HY-HO $p = 0.014$, HY-FO $p = 0.041$, HO-FO $p = 1.000$. C - CD66b fold change: HY – 16, HO – 11, FO – 12. HY – 1.61 +/- 1.0, HO – 0.83 +/- 1.0, FO – 0.92 +/- 1.0. Independent Kruskal-Wallis test $p < 0.001$. Post-hoc pairwise comparison with Bonferroni correction. HY-HO $p = 0.001$, HY-FO $p = 0.002$, HO-FO $p = 1.000$.

5.10 Poor migratory accuracy in neutrophils from frail older adults can be rescued by incubation with selective PI3kinase inhibitors

Previous work has demonstrated that decline in chemotaxis of neutrophils with ageing is due to dysregulated intracellular mechanisms and in particular the PI3kinase-Akt pathway; poor chemotaxis could be rescued in vitro in the neutrophils from healthy older adults by incubation with PI3kinase inhibitors (163). The data shown already in this chapter demonstrates that migratory accuracy to IL-8 is frailty dependent and independent of age. To consider whether frailty related decline in migratory accuracy to IL-8 was a consequence of dysregulated PI3kinase activity and could be rescued with PI3kinase inhibitors neutrophils from the three groups were incubated with PI3kinase inhibitors, α , β , δ and γ , or vehicle control and migrated towards 100nM IL-8.

Poor migratory accuracy towards IL-8 was seen again in neutrophils from frail older adults (Chemotaxis ($\mu\text{m}/\text{min}$): FO 0.57 \pm 0.64) which could be restored with PI3kinase inhibitors δ (Chemotaxis ($\mu\text{m}/\text{min}$): FO δ 1.05 \pm 0.66, Mann-Whitney Test $p=0.03$) and γ (Chemotaxis ($\mu\text{m}/\text{min}$): FO γ 0.99 \pm 0.94, Mann-Whitney Test $p=0.04$). Incubation with PI3 kinase inhibitor α and β did not alter migratory accuracy: chemotaxis ($\mu\text{m}/\text{min}$) FO α 0.19 \pm 1.17, Mann-Whitney Test $p=0.62$), FO β 0.70 \pm 1.61, Mann-Whitney Test $p=0.31$). (Figure 5.9.)

Figure 5.9 – Chemotaxis to IL-8 following incubation with PI3kinase inhibitors



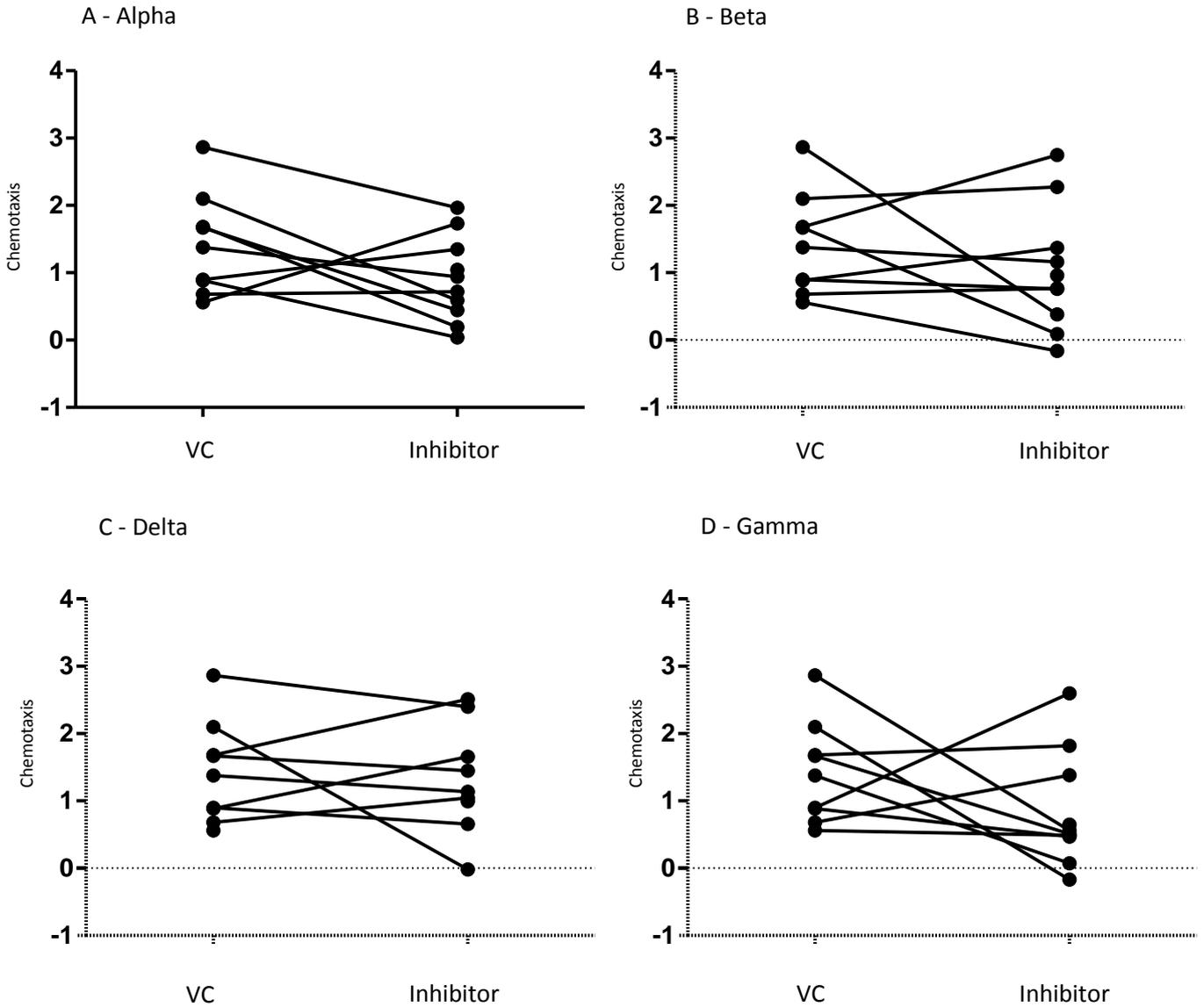
Chemotaxis to IL-8 at baseline in healthy young, healthy older and frail older adults and following incubation with PI3kinase inhibitors in frail older adults. HY – isolated neutrophils from healthy younger adults incubated with DMSO (vehicle control) migrated towards 100nM IL-8 (N=20). HO – isolated neutrophils from healthy older adults incubated with DMSO (vehicle control) migrated towards 100nM IL-8 (N=21). FO – isolated neutrophils from frail older adults incubated with DMSO (vehicle control) migrated towards 100nM IL-8 (N=13). Isolated neutrophils from frail older adults incubated with: FO Alpha – PI3K α (N=8), FO Beta - PI3K β (N=7), FO Delta – PI3K δ (N=8) and FO Gamma – PI3K γ (N=8), and migrated towards 100nM IL-8. Chemotaxis expressed as $\mu\text{m}/\text{min}$. Red line represents median chemotaxis in healthy older adults. Data sets all normally distributed except HY, FO and FO Alpha. Data sets are median \pm IQR.

Response to all four PI3kinase inhibitors was variable in healthy older adults; some individuals demonstrated an improvement, others a decline in migratory accuracy and some individuals demonstrated no response. There was no discernible pattern to the response when considering the individual inhibitor or baseline chemotaxis to the response. (Figure 5.10.)

Response to PI3kinase α in frail older adults was variable with no discernible pattern to the changes in chemotaxis. PI3kinase inhibitor δ produced the most consistent response with a similar improvement in chemotaxis in five individuals but a decrease in migratory accuracy in two individuals. One of these individuals had a baseline chemotaxis which was comparable to healthy young adults. PI3kinase inhibitor γ produced an improvement in chemotaxis in all the individuals except one but the response was less consistent between the individuals and a marginal response was present in three individuals. (Figure 5.11.)

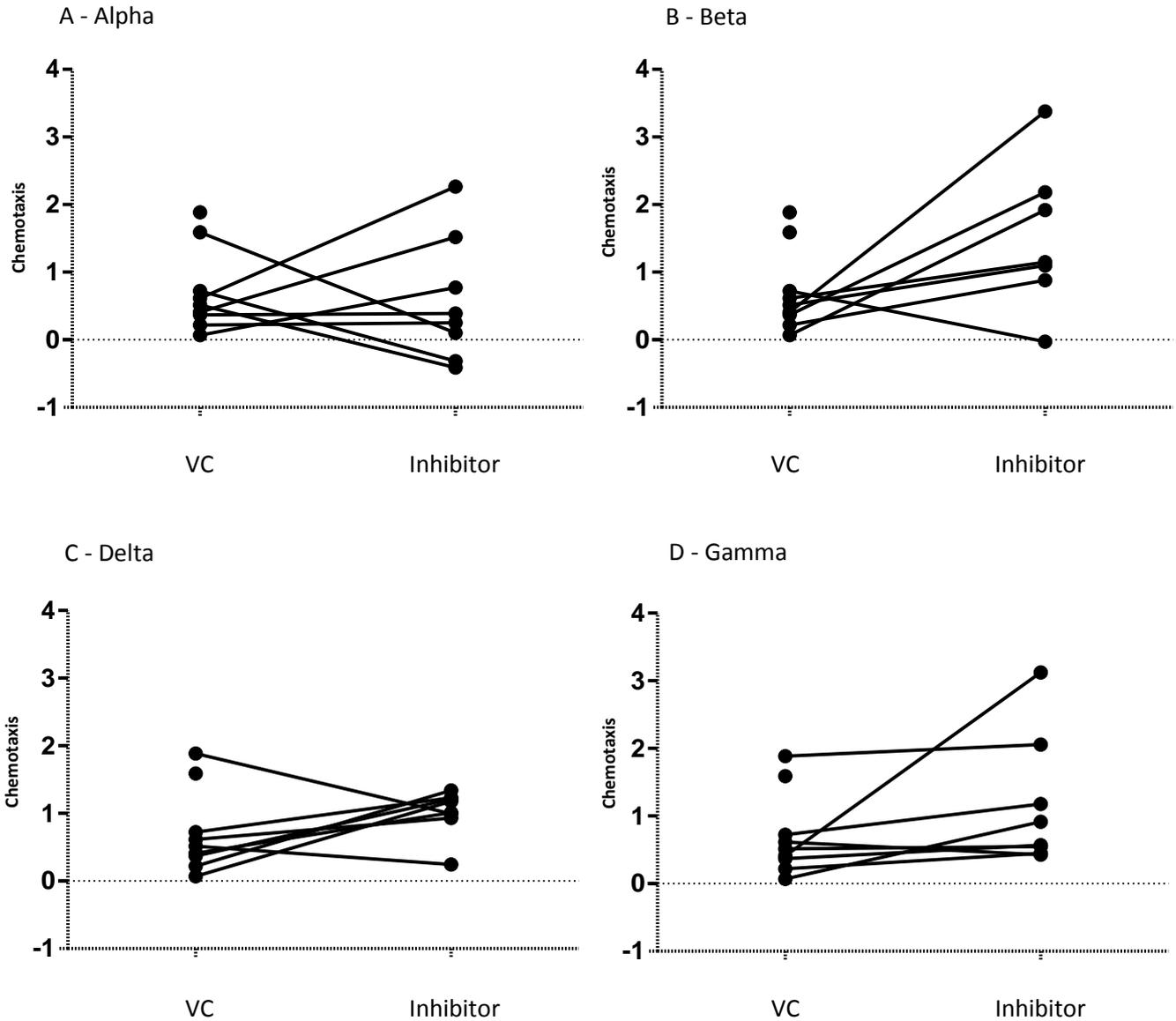
Improvement of chemotaxis in neutrophils incubated with PI3kinase inhibitors could be predicted by adjusted hand grip strength; individuals with low grip strength (adjusted for gender and BMI) had larger improvements in chemotaxis from baseline following incubation of their neutrophils with PI3kinase inhibitors. Pearson's correlation between the fold change in chemotaxis, vehicle control to PI3kinase δ and adjusted hand grip. $R = -0.577$. $P = 0.031$. (Figure 5.12.)

Figure 5.10 – Chemotaxis to IL-8 following incubation with PI3kinase inhibitors in healthy older individuals



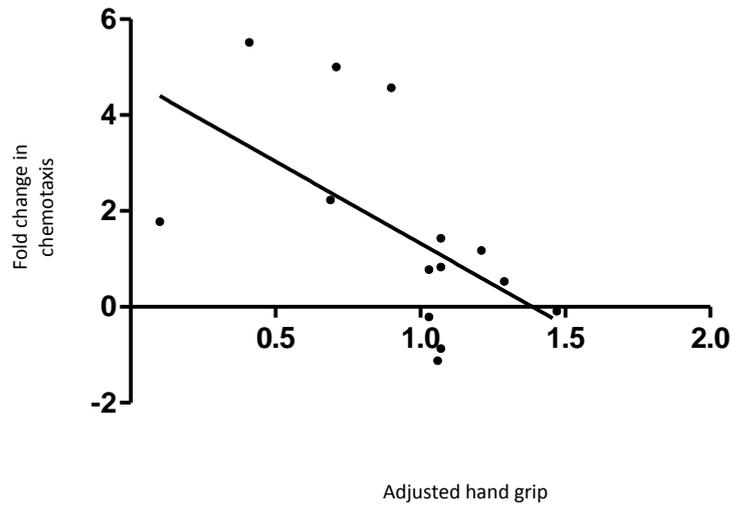
Chemotaxis to IL-8 at baseline and following incubation with PI3kinase inhibitors in healthy older individuals. Isolated neutrophils from healthy older adults incubated with DMSO (vehicle control) and migrated towards 100nM IL-8 (N=9). Isolated neutrophils from healthy older adults incubated with: A - Alpha – PI3K α (N=8), B - Beta - PI3K β (N=10), C - Delta – PI3K δ (N=9) and FO Gamma – PI3K γ (N=10), and migrated towards 100nM IL-8. Chemotaxis expressed as $\mu\text{m}/\text{min}$. Each circle represents one individual and slope represents change in chemotaxis following incubation with corresponding PI3kinase inhibitor.

Figure 5.11 – Chemotaxis to IL-8 following incubation with PI3kinase inhibitors in frail older individuals



Chemotaxis to IL-8 at baseline and following incubation with PI3kinase inhibitors in healthy older individuals. Isolated neutrophils from healthy older adults incubated with DMSO (vehicle control) and migrated towards 100nM IL-8 (N=9). Isolated neutrophils from healthy older adults incubated with: A - Alpha – PI3K α (N=8), B - Beta - PI3K β (N=7), C - Delta – PI3K δ (N=8) and FO Gamma – PI3K γ (N=8), and migrated towards 100nM IL-8. Chemotaxis expressed as $\mu\text{m}/\text{min}$. Each circle represents one individual and slope represents change in chemotaxis following incubation with corresponding PI3kinase inhibitor.

Figure 5.12 – Correlation of adjusted hand grip with fold change in chemotaxis following PI3kinase δ inhibition



Correlation of adjusted hand grip with fold change in chemotaxis following PI3kinase δ inhibition. Isolated neutrophils from healthy and frail older adults migrated towards 100nM IL-8 following incubation with vehicle control (DMSO) or PI3kinase δ inhibitor. Fold change in chemotaxis is the change in chemotaxis from vehicle control to following incubation with PI3 kinase δ inhibitor. Plotted against hand grip strength adjusted for gender and BMI (fold change of cut-off for lowest 20% for gender and BMI in over 65). Data normally distributed.

HO – 78.7, FI 0.06. FO – 84.0, FI 0.31.

A – N=14. Pearson’s correlation coefficient. 2-tailed. R= -0.577. P=0.031.

Chemokinesis towards IL-8 in neutrophils from frail older adults did not change following PI3kinase β, γ and δ inhibition compared to the vehicle control. (Chemokinesis ($\mu\text{m}/\text{min}$): FO 3.12 \pm 1.14, FO α 2.92 \pm 1.43, p=0.606, FO β 3.53 \pm 1.47, p=0.408, FO δ 3.61 \pm 0.83, p=0.200, FO γ 3.87 \pm 0.94, p=0.114. All Mann-Whitney Test.)

In contrast, incubation with PI3kinase inhibitors resulted in no effect on chemotaxis in healthy younger adults. (Chemotaxis ($\mu\text{m}/\text{min}$): HY 0.85 ± 1.24 , HY α 0.19 ± 0.49 , $p=0.133$, HY β 0.32 ± 1.27 , $p=0.393$, HY δ 0.76 ± 1.59 , $p=0.796$, HY γ 0.47 ± 0.64 , $p=0.393$.) Chemokinesis was no different following incubation with PI3kinase inhibitors. (Chemokinesis ($\mu\text{m}/\text{min}$): HY 3.16 ± 1.25 , HY α 2.71 ± 1.07 , $p=0.182$, HY β 2.75 ± 1.01 , $p=0.190$, HY δ 3.09 ± 1.47 , $p=0.631$, HY γ 3.25 ± 1.80 , $p=0.971$.) All statistical tests quoted are Mann-Whitney test.

There was a reduction in chemotaxis in neutrophils from healthy older adults incubated with PI3kinase α and β and no effect when the neutrophils were incubated with the PI3kinase δ and γ inhibitors. (Chemotaxis ($\mu\text{m}/\text{min}$): HO 0.97 ± 0.79 , HO α 0.34 ± 0.85 , $p=0.004$, HO β 0.49 ± 1.12 , $p=0.035$, HO δ 1.11 ± 0.98 , $p=0.095$, HO γ 0.36 ± 1.42 , $p=0.863$.) Chemokinesis was significantly reduced following incubation with the PI3kinase γ and there was a trend to reduction with PI3kinase β inhibitors. There was no difference in chemokinesis following incubation with PI3kinase α and δ inhibitor. (Chemotaxis ($\mu\text{m}/\text{min}$): HO 3.14 ± 0.66 , HO α 2.41 ± 1.34 , $p=0.161$, HO β 2.89 ± 1.20 , $p=0.095$, HO δ 4.03 ± 1.62 , $p=0.136$, HO γ 2.61 ± 1.10 , $p=0.035$.) All statistical tests quoted are Mann-Whitney test. (Table 5.3.)

Table 5.3 – Chemotaxis, chemokinesis and chemotactic index of the three groups for vehicle control and all four inhibitors

		HY	HO	FO
VC	Chemotaxis	0.85 +/- 1.24	0.97 +/- 0.79	0.39 +/- 0.45
	Chemokinesis	3.16 +/- 1.25	3.14 +/- 0.66	3.12 +/- 1.14
	Chemotactic Index	0.21 +/- 0.19	0.27 +/- 0.17	0.09 +/- 0.12
PI3k α	Chemotaxis	0.19 +/-0.49	0.34 +/-0.85	0.19 +/- 1.07
	Chemokinesis	2.71 +/- 1.07	2.41 +/- 1.34	2.92 +/- 1.43
	Chemotactic Index	0.09 +/- 0.1	0.14 +/-0.27	0.09 +/- 0.36
PI3k β	Chemotaxis	0.32 +/- 1.27	0.49 +/- 1.12	0.70 +/-1.61
	Chemokinesis	2.75 +/-1.01	2.89 +/- 1.20	3.53 +/-1.47
	Chemotactic Index	0.13 +/- 0.34	0.13 +/-0.35	0.14 +/-0.39
PI3k δ	Chemotaxis	0.76 +/-1.59	1.11 +/- 0.98	1.05 +/- 0.66
	Chemokinesis	3.09 +/-1.47	4.03 +/-1.62	3.61 +/- 0.83
	Chemotactic Index	0.19 +/- 0.37	0.19 +/- 0.22	0.23 +/- 0.11
PI3k γ	Chemotaxis	0.47 +/-0.64	0.36 +/-1.42	0.99 +/-0.94
	Chemokinesis	3.25 +/-1.80	2.61 +/-1.10	3.87 +/-0.94
	Chemotactic Index	0.08 +/- 0.28	0.12 +/- 0.32	0.21 +/- 0.21

VC – neutrophils incubated with DMSO (vehicle control) migrated towards 100nM IL-8 (N- HY=20, HO=20, FO=13). PI3K α – neutrophils incubated with PI3k α and migrated towards 100nM IL-8 (N- HY=9, HO=9, FO=8). PI3K β – neutrophils incubated with PI3k β and migrated towards 100nM IL-8 (N- HY=10, HO=10, FO=7). PI3K δ – neutrophils incubated with PI3k δ and migrated towards 100nM IL-8 (N- HY=10, HO=9, FO=8). PI3K γ – neutrophils incubated with PI3k γ and migrated towards IL-8 (N- HY=10, HO=10, FO=7). Chemotaxis and chemokinesis expressed as $\mu\text{m}/\text{min}$.

Data sets all normally distributed except chemotaxis HY, chemotaxis FO and chemotaxis FO Alpha. Data sets are median+/-IQR. Numbers in bold represent values that are significantly different to vehicle control values. FO PI3k δ p=0.03. FO PI3k γ p=0.04.

HY – 24.3, FI 0.01. HO – 78.7, FI 0.06. FO – 81.1, FI 0.42.

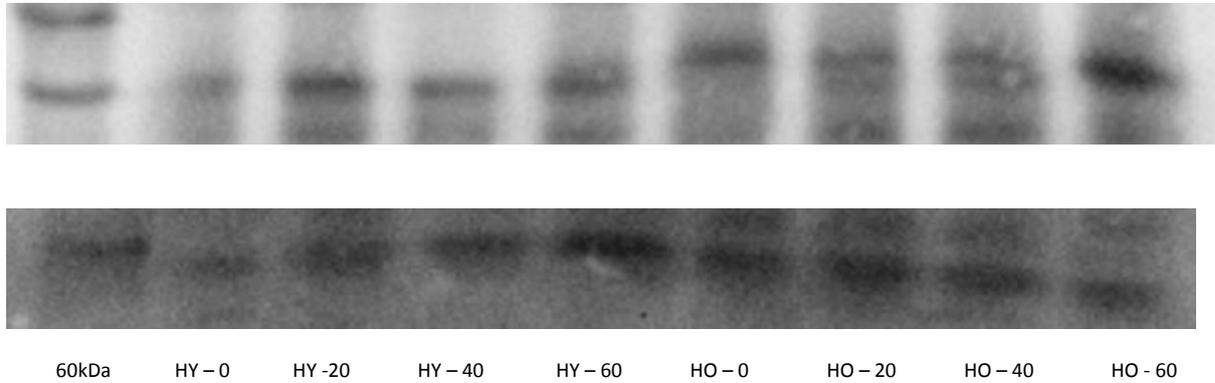
5.11 Akt signalling in neutrophils following stimulation with IL-8.

The presented data suggest that PI3kinase is involved in the control of directional migration and its dysregulation results in poor neutrophil migratory accuracy. The data have demonstrated that PI3kinase dysregulation can be corrected in frail older adults with PI3kinase inhibitors, suggesting increased expression or activity. Akt is downstream of PI3kinase and plays a central role in a number of cellular responses (see Figure 1.6 in the introduction for a description of its role within myocytes). To determine the extent of the effect of dysregulated PI3kinase on downstream intracellular activity the phosphorylation of Akt^{Thr308} was measured using β -actin as a control.

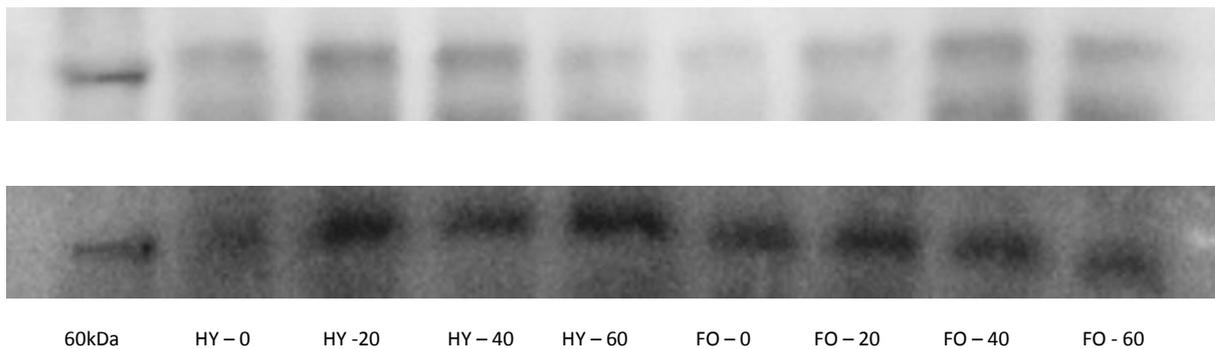
Phosphorylated Thr 308 increased in response to stimulation with IL-8 in all three groups with a maximal response at 40 seconds of stimulation. (Figure 5.13.) Healthy older and frail older adults had a trend towards the greatest increase in phosphorylation of Thr 308 but this was not significant (Area under the curve HY: 38.17, HO: 82.25 and FO: 66.71, Independent Kruskal-Wallis $p=0.066$). There was significant variability in responses between individuals, as demonstrated by the range displayed on figure 5.14.

Figure 5.13 – Phosphorylation of Akt^{Thr 308} in response to IL-8. Representative Western Blots for healthy young, healthy older and frail older adults

A

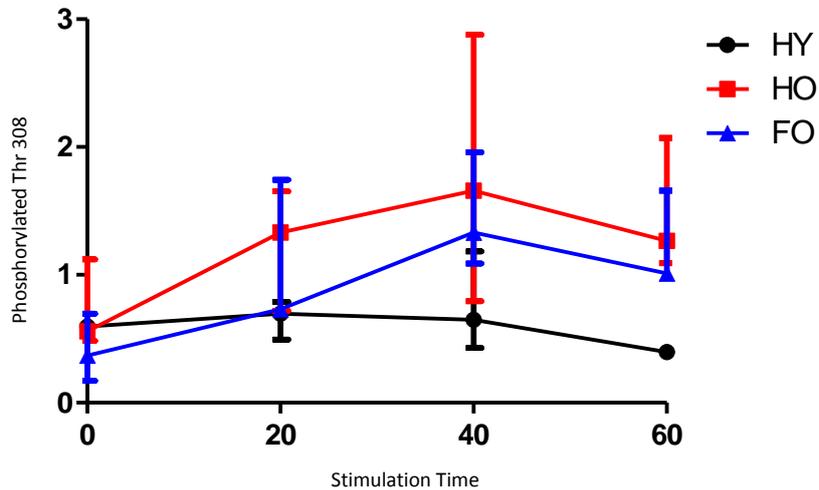


B



Phosphorylation of Thr 308 in response to increasing periods of stimulation with 100nM IL-8. Isolated neutrophils stimulated with 100nM IL-8 for increasing periods of time, 0 to 60 seconds. Figure A shows a representative western blot of a healthy young adult (HY) and healthy older adult (HO). Top western blot is phosphorylated Thr 308 and below is the beta actin. Figure B shows a representative western blot of a healthy young adult (HY) and healthy older adult (HO). Top western blot is phosphorylated Thr 308 and below is the corresponding beta actin western blot. Columns from left to right are: protein ladder (60kDa), unstimulated neutrophils, neutrophils stimulated with 100nM IL-8 for 20 seconds, neutrophils stimulated with 100nM IL-8 for 40 seconds, neutrophils stimulated with 100nM IL-8 for 60 seconds, repeated for sample 2.

Figure 5.14 - Densitometry of phosphorylated Thr 308 in neutrophils from healthy older adults, healthy younger adults and frail older adults.



Phosphorylation of Thr 308 in response to increasing periods of stimulation with 100nM IL-8. Isolated neutrophils stimulated with 100nM IL-8 for increasing periods of time, 0 to 60 seconds. Levels of phosphorylated Thr 308 were quantified using densitometry and expressed as a ratio of β -actin. X axis - time for stimulation expressed in seconds. Y axis – ratio of phosphorylated Thr 308 to β -actin. Number within each group =3. Plotted median with range. Area under the curve calculated: HY- 38.17, HO – 82.25, FO – 66.71. $p=0.066$. (Independent Kruskal-Wallis test)

5.12 Changes in neutrophil function following exposure to pooled plasma from healthy and frail older adults

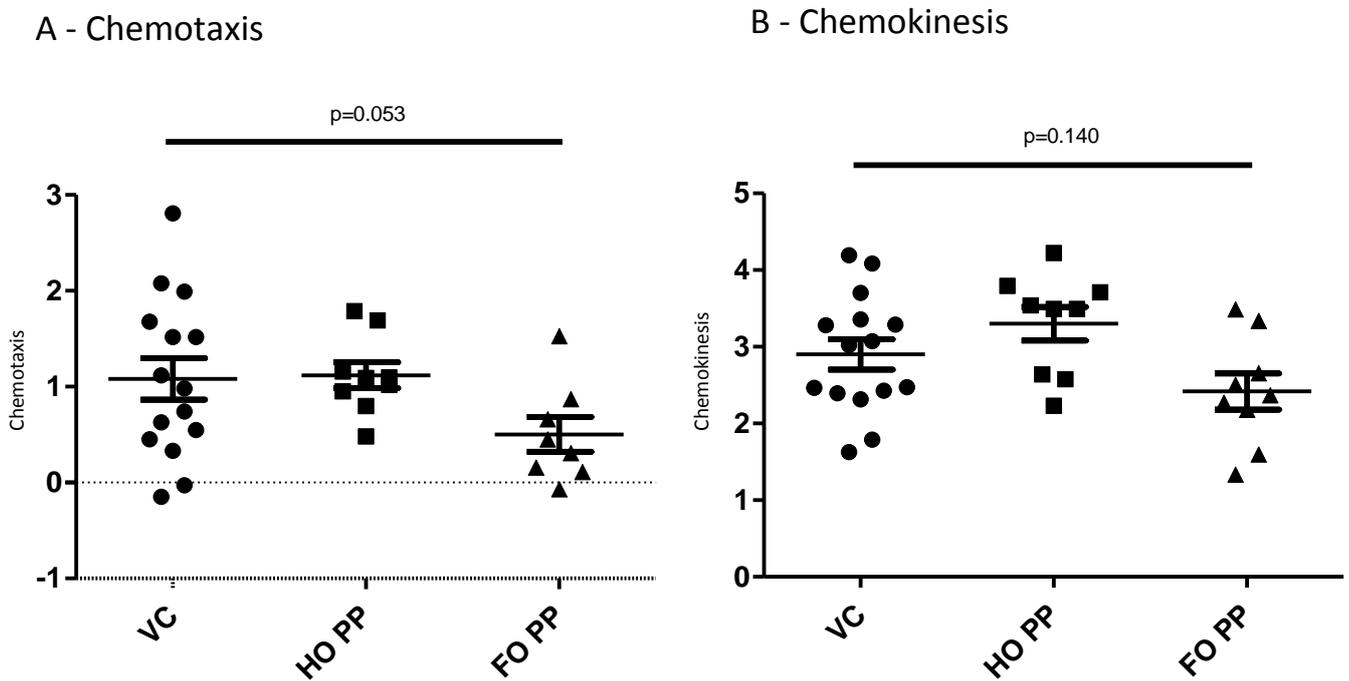
Poor migratory accuracy has been demonstrated to be the result of dysregulated intracellular mechanisms (163). However, in frailty it is not known whether this dysregulation occurs due to an acute response to an inflammatory environment or an intrinsic defect, present even in immature cells. To assess this, neutrophils from healthy younger adults were incubated with pooled plasma from healthy older and frail older adults.

There was a trend to reduction in chemotaxis of neutrophils from healthy younger adults following incubation with pooled plasma (PP) from frail older adults. In contrast, incubation with pooled plasma from healthy older adults did not change the migratory accuracy of neutrophils from healthy younger adults. Chemotaxis towards IL-8 ($\mu\text{m}/\text{min}$): VC (neutrophils from healthy younger adults) 1.08 (0.62-1.55), HO PP (neutrophils from healthy younger adults incubated with pooled plasma from healthy older adults) 1.12 (0.81-1.43) and FO PP (neutrophils from healthy younger adults incubated with pooled plasma from frail older adults) 0.45 (0.06-0.84). Independent samples T-test between VC and FO PP $p = 0.053$. (Figure 5.15)

There was no difference in chemokinesis when neutrophils from healthy younger adults were incubated with pooled plasma from healthy older adults. Similarly, there was no change in chemokinesis following neutrophil incubation with pooled plasma from frail older adults. Chemokinesis towards IL-8 ($\mu\text{m}/\text{min}$): VC 2.90 (2.47-3.33), HO PP 3.30 (2.79-

3.81) and FO 2.42 (1.88-2.96). Independent samples T-test between VC and HO PP
 $p=0.209$ and between VC and FO PP $p = 0.140$. (Figure 5.15)

Figure 5.15 – Chemotaxis and chemokinesis of neutrophils from healthy younger adults incubated with pooled plasma and migrated towards IL-8



Chemotaxis and chemokinesis of neutrophils from healthy younger adults incubated with pooled plasma and migrated towards IL-8. Isolated neutrophils from healthy younger adults incubated with either: VC – RPMI, HO PP – pooled plasma from 18 healthy older adults (Mean Frailty Index – 0.04), FO PP – pooled plasma from 18 frail older adults (Mean Frailty Index – 0.41). Neutrophils migrated towards 100 nM IL-8. Data sets are mean \pm SEM; data sets are normally distributed. Chemotaxis and chemokinesis expressed as $\mu\text{m}/\text{min}$. N= VC-15, HO PP-9, FO PP-9.

Independent samples t-test for chemotaxis. VC-HO PP $p=0.904$ VC-FO PP $p=0.053$

Independent samples t-test for chemokinesis VC-HO PP $p=0.209$. VC-FO PP $p=0.140$

5.13 Frail older adults exist in a state of chronic inflammation

To describe the systemic environment in frail older adults and explore its effect on neutrophils *in vivo*, a commercially available human cytokine multiplex and the acute phase protein hs-CRP ELISA were used to calculate cytokine and hs-CRP concentration in healthy younger and older adults, and frail older adults.

The concentrations of hs-CRP in healthy younger adults and healthy older adults were comparable but there was significantly increased concentrations of hs-CRP in the serum from frail older adults. Concentration of hs-CRP ($\mu\text{g/ml}$): HY 1.39 +/- 1.86, HO 1.34 +/- 1.65 and FO 8.87 +/- 7.97. (Figure 5.16.)

For the commercially available human cytokine kit, only IL-1ra, IL-4, IL-7, IL-8, IL-9, IL-17, Eotaxin, FGF, G-CSF, IP-10, MCP-1, MIP1a, PDGF, MIP1b, RANTES and TNF α provided results where more than 50% of values were within the measured range. Values below the detectable range were recorded as half the lowest detectable standard. Table 5.4 describes the cytokines with greater than 50% of values within the detectable range and table 5.5 describes the cytokines with fewer than 50% of cytokines with the detectable range. Only these cytokines were included for further analysis.

There was no significant difference in the concentration of TNF α throughout the three groups and the concentrations of IL-6 and IL-10 were mostly undetectable throughout the three groups.

However, there were significantly increased concentrations in both the healthy older adults and the frail older adults compared to healthy younger adults for IL-1ra, IL-8 and Eotaxin with no difference between the healthy older adult and frail older adult concentrations.

There were significantly increased concentrations of IL-4, IL-17 and IL1 β in healthy but not frail older adults. There were significantly decreased concentrations of MCP 1a in frail older adults compared to healthy older adults and significantly increased concentrations of MIP1a, resulting in a stepwise increase in MIP1a with age and frailty. (Table 5.4. Table 5.5. Table 5.6. Figure 5.16.)

Table 5.4 – Cytokines with greater than 50% of total sample within detectable range

Cytokine	Measurable Range	HY		HO		FO	
		Proportion in range (%)	Median +/- IQR	Proportion in range	Median +/- IQR	Proportion in range	Median +/- IQR
IL-1ra	10.39-2461.2	100.0	49.6 +/- 42.3	100.0	64.7 +/- 69.0	97.0	79.0 +/- 34.4
IL-4	0.61-3843.6	85.7	5.71 +/- 3.32	97.4	10.4 +/- 4.54	94.0	9.33 +/- 8.37
IL-7	7.91-1822.1	92.9	12.0 +/- 7.65	89.5	16.0 +/- 6.26	84.8	14.1 +/- 9.94
IL-8	4.82-17894.0	46.4	4.82 +/- 0.69	94.7	7.37 +/- 3.60	81.8	9.22 +/- 10.0
IL-9	7.99-8469.1	89.3	52.9 +/- 10.5	76.3	47.0 +/- 38.5	75.8	47.5 +/- 36.4
IL-17	9.12-36111.5	39.3	4.56 +/- 11.5	81.6	19.8 +/- 12.7	63.6	16.1 +/- 18.0
IP-10	3.52-13605.8	100.0	877.7 +/- 592.2	100.0	1440.2 +/- 1005.4	100.0	1521.1 +/- 909.4
Eotaxin	1.21-1366.3	100.0	102.8 +/- 68.9	100.0	167.3 +/- 83.0	97.0	161.6 +/- 105.0
FGF	12.41-3272.1	78.6	17.2 +/- 2.93	81.6	20.5 +/- 6.28	72.7	17.2 +/- 23.4
MCP-1	2.42-2412.5	100.0	25.8 +/- 12.9	100.0	30.7 +/- 24.0	93.9	19.8 +/- 23.4
MIP1a	0.53-517.8	100.0	0.97 +/- 0.62	92.1	1.41 +/- 1.35	87.9	2.83 +/- 1.90
PDGF	13.4-15304.1	100.0	82.5 +/- 13.0	100.0	82.3 +/- 74.0	100	85.9 +/- 27.0
MIP1b	1.78-1766.3	100.0	2119.8 +/- 2776.3	100.0	12442.8 +/- 13681.7	87.9	11343.0 +/- 6792.9
RANTES	8.87-9000.2	100.0	12491.4 +/- 2776.3	100.0	12441.8 +/- 13681.7	100.0	11343.0 +/- 6792.9
TNFα	17.5-17347.4	100.0	36.9 +/- 8.22	100.0	41.0 +/- 88.5	87.9	41.0 +/- 53.3

Cytokines with greater than 50% of total sample within detectable range in healthy younger adults (HY), healthy older adults (HO) and frail older adults (FO). Cytokines in horizontal rows. Vertical rows from left to right: measurable range in pg/ml (individuals with cytokine concentrations below the measurable range ascribed a value of half the lowest detectable value), proportion of individuals in the group within the measurable range (%) and median concentration of cytokine (pg/ml) within the group plus IQR. Numbers = HY: 28, HO: 38, FO: 33.

Table 5.5 – Cytokines with fewer than 50% of total sample within detectable range

Cytokine	Measur- able Range	HY		HO		FO	
		Proporti on in range (%)	Median +/- range	Proporti on in range	Median +/- range	Proporti on in range	Median +/- range
IL-1b	0.98- 1001.1	14.3	0.83 +/- 0.27	55.3	1.09 +/- 0.68	33.3	0.83 +/- 0.47
IL-2	6.16- 6286.3	0	3.08 +/- 0.00	2.6	3.08 +/- 0.00	0	3.08 +/- 0.00
IL-5	17.91- 17915.3	0	8.95 +/- 0.00	10.5	16.535 +/- 0.00	0.0	8.95 +/- 0.00
IL-6	2.81- 2825.9	0	1.41 +/- 0.00	10.5	1.41 +/- 0.00	3.0	1.41 +/- 0.00
IL-10	6.82- 6883.4	0	3.41 +/- 0.00	5.3	3.41 +/- 0.00	0	3.41 +/- 0.00
IL-12	7.39- 30354.4	0	3.68 +/- 0.00	5.3	3.68 +/- 0.00	0	3.68 +/- 0.00
IL-13	2.58- 2557.2	7.1	1.28 +/- 0.00	5.3	1.28 +/- 0.00	3.0	1.28 +/- 0.00
IL-15	43.8- 10848.3	0	21.9 +/- 0.00	2.6	21.9 +/- 0.00	0	21.9 +/- 0.00
G-CSF	41.4- 8355.7	7.1	20.7 +/- 0.00	81.6	41.4 +/- 0.00	87.9	41.4 +/- 0.00
IFN- gamma	1.01 – 3820.2	7.1	6.31 +/- 0.00	10.5	14.52 +/- 0.00	6.0	12.2 +/- 0.00
GM-CSF	2.37- 2375.9	3.6	1.17 +/- 0.00	2.6	1.17 +/- 0.00	3.0	1.17 +/- 0.00
VEGF	35.7- 40659.2	0	17.8 +/- 0.00	13.2	17.8 +/- 0.00	15.2	17.8 +/- 0.00

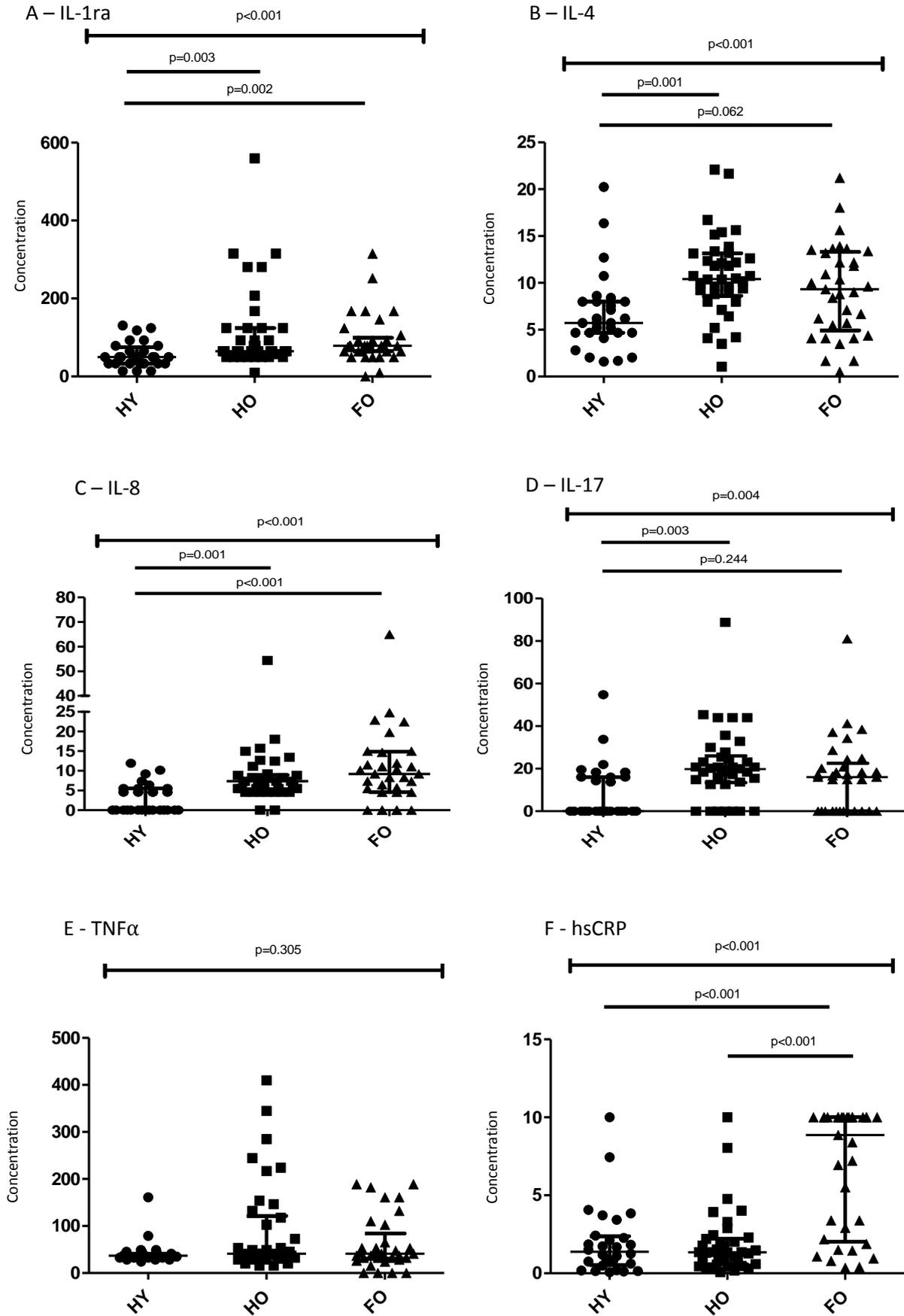
Cytokines with fewer than 50% of total sample within detectable range in healthy younger adults (HY), healthy older adults (HO) and frail older adults (FO). Cytokines in horizontal rows. Vertical rows from left to right: measurable range in pg/ml (individuals with cytokine concentrations below the measurable range ascribed a value of half the lowest detectable value), proportion of individuals in the group within the measurable range (%) and median concentration of cytokine (pg/ml) within the group plus IQR. Numbers = HY: 28, HO: 38, FO: 33.

Table 5.6 – Statistical comparisons of cytokines with greater than 50% of total sample within detectable range and IL-1 β

	Independent Kruskal-Wallis	Pairwise Comparisons
IL-1ra	p=0.001	HY-HO p=0.003, HY-FO p=0.002, HO-FO p=1.000
IL-4	p=0.001	HY-HO p=0.001, HY-FO p=0.06, HO-FO p=0.493
IL-7	p=0.116	
IL-8	p<0.001	HY-HO p=0.015, HY-FO p=0.001, HO-FO p=0.537
IL-9	p=0.266	
IL-17	p=0.001	HY-HO p=0.001, HY-FO p=0.126, HO-FO p=0.284
Eotaxin	p=0.001	HY-HO p=0.001, HY-FO p=0.023, HO-FO p=1.000
FGF	p=0.087	
G-CSF	p=0.401	
MCP-1	p=0.042	HY-HO p=0.356, HY-FO p=1.000, HO-FO p=0.041
MIP1a	p<0.001	HY-HO p=0.043, HY-FO p<0.001, HO-FO p=0.005
PDGF	p=0.949	
MIP1b	p=0.432	
RANTES	p=0.256	
TNFα	p=0.305	
IL-1β	p=0.029	HY-HO p=0.025, HY-FO p=0.705, HO-FO p=0.415

Statistical comparison of cytokines with greater than 50% of total sample within detectable range and IL-1 β . All cytokines not normally distributed. Statistical tests are Independent Kruskal-Wallis test with post-hoc Bonferroni corrected pairwise comparisons. All statistics reported in bold are significant.

Figure 5.16 - Concentration of cytokines and the acute phase protein hs-CRP in serum.



A-IL-1ra, B-IL-4, C-IL-8, D-IL-17. E-TNF α and F-hsCRP

Concentration of IL-1ra, IL-4, IL-8, IL-17, TNF α (pg/ml) and hs-CRP (μ g/ml) measured using a multiplex or commercially available ELISA. N = HY-28, HO-38, FO-33. Values below the detectable range were recorded as half the lowest detectable standard. Values above the detectable range were recorded as highest detectable value. Not normally distributed. Values are median +/- IQR. Independent Kruskal-Wallis with pairwise analysis with Bonferroni correction.

IL-1ra: p<0.001. HY-HO p=0.003, HY-FO p=0.002, HO-FO p=1.000. IL-4: p<0.001. HY-HO p=0.001, HY-FO p=0.062, HO-FO p=0.471. IL-8: p<0.001. HY-HO p=0.001, HY-FO p<0.001, HO-FO p=1.000. IL-17: p=0.004. HY-HO p=0.003, HY-FO p=0.244, HO-FO p=0.328. TNF α : p=0.305. hs-CRP: p<0.001. HY-HO p=1.00, HY-FO p<0.001, HO-FO p<0.001.

5.14 Neutrophils from frail older adults primed with TNF α demonstrate improved chemotaxis but the response is delayed.

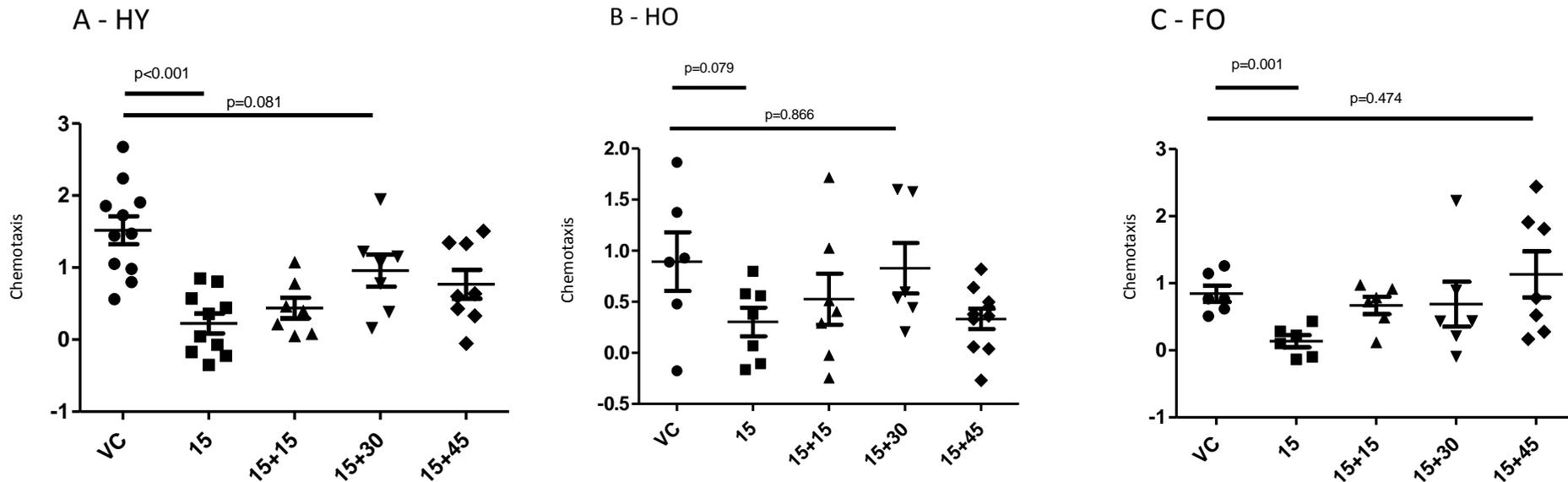
A chronic inflammatory environment has been demonstrated with increased concentrations of systemic hs-CRP, IL-1ra, IL-4, IL-8 and IL-17 in frail older adults. To determine the effect of this chronic inflammatory environment on migration of neutrophils; neutrophils were primed with of TNF α (1.15 nM) and migrated towards the bacterial end chemoattractant fMLP.

Neutrophils from healthy young and older adults, and frail older adults showed an initial reduction in chemotaxis with priming which improved with an increasing period of rest. This reduction was significant in healthy younger and frail older adults. Chemotaxis; mean (confidence intervals) (μ m/min): HY without priming 1.68 (1.40-1.95), HY following 15 minutes of priming 0.22 (0.08-0.36), independent t-test p<0.001, HO without priming 0.78 (0.49-1.08), HO following 15 minutes of priming 0.30 (0.16-0.46), independent t-test

p=0.079, FO without priming 0.92 (0.48-1.36), FO following 15 minutes of priming 0.13 (0.04-0.22), independent t-test p=0.001. (Figure 5.17.)

The maximal response to priming (the time-point chemotaxis reached the highest value following the initial priming) occurred at 15 minutes of priming and 30 minutes of rest for healthy younger and older adults but was delayed at 15 minutes of priming and 45 minutes of rest for frail older adults. At maximal response to priming, chemotaxis was: no different to chemotaxis without priming in healthy younger adults ((Chemotaxis; mean (confidence intervals) ($\mu\text{m}/\text{min}$): HY without priming 1.68 (1.40-1.95), HY with 15+30 priming 0.83 (0.36-1.31) p=0.081) healthy older adults ((Chemotaxis; mean (confidence intervals) ($\mu\text{m}/\text{min}$): HO 0.78 (0.49-1.08), HO with 15+30 priming 0.93 (0.22-1.64) p=0.866), and frail older adults ((Chemotaxis; mean (confidence intervals) ($\mu\text{m}/\text{min}$): FO 0.92 (0.48-1.36), FO with 15+45 priming 1.35 (0.35-2.34)p=0.474). Not significantly different using independent t-test between vehicle control and maximal response to priming. (Figure 5.17.)

Figure 5.17 – Chemotaxis of neutrophils primed with TNF α



Neutrophils primed with 1.15nM TNF α for 15 minutes, washed to remove stimulus and then undergoing increasing periods of rest following priming (eg. VC – vehicle control 15+15 – 15 minutes of priming with TNF α , wash to remove priming agent and 15 minutes of rest before migration) and then migrated towards 10nM fMLP. Data sets are all normally distributed and data shown is adjusted to vehicle control (chemotaxis without priming). Vehicle control data not shown. Chemotaxis is expressed as μ /min. Data shown is mean +/- SEM. P values are derived from independent t-tests.

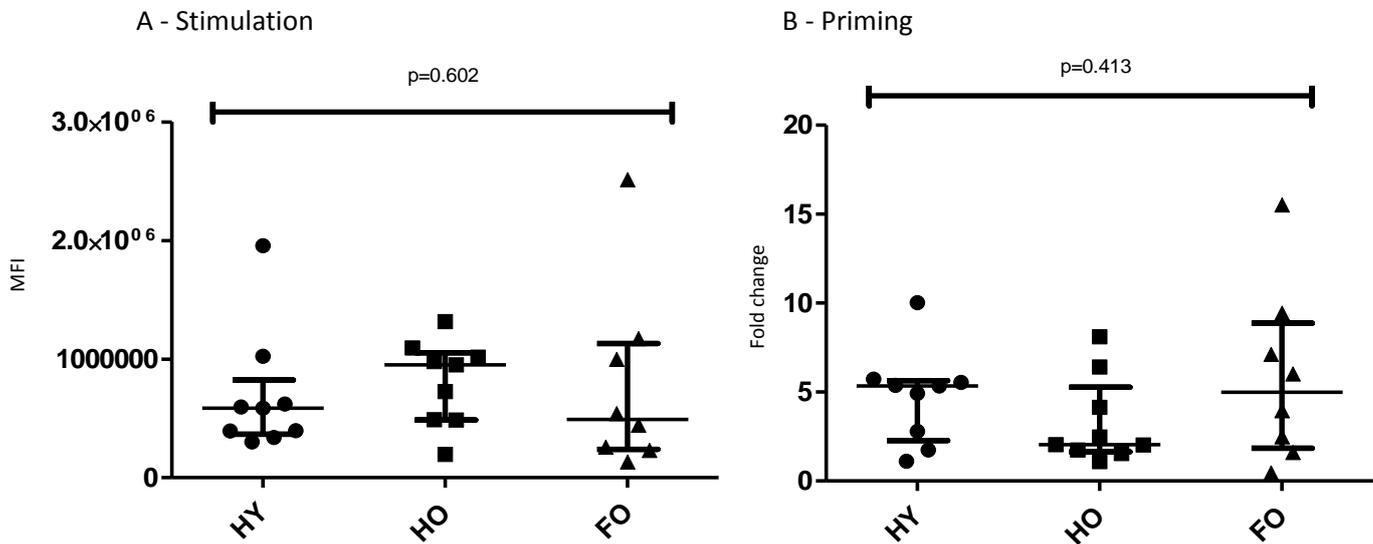
N – HY – VC = 11, 15 = 10, 15+15 = 7, 15+30 = 7, 15+45 = 8. HO – VC = 6, 15=7, 15+15 = 7, 15+30 = 6, 15+45 = 10. FO – VC = 6, 15 = 6, 15+15 = 6, 15+30 = 6, 15+45 = 7.

5.15 Neutrophils from frail older adults do not produce a reduced amount of superoxide on stimulation or with priming

To confirm that the experiments described above provided a sufficient stimulus to prime neutrophils from each participant group, the effect of the same concentration of TNF α (1.15nM) on neutrophil superoxide production was assessed compared to a vehicle control, and subsequent stimulation with fMLP. The oxidative burst of the neutrophils was measured using DHR-123 and flow cytometry. There was no difference witnessed throughout the time-course and all primed data were analysed together.

The data suggests that the concentration of 1.15nM TNF α was sufficient to prime neutrophils from healthy younger, healthy older and frail older adults. There was increased superoxide release from neutrophils from all three groups after fMLP exposure following priming with 1.15nM TNF α compared to the superoxide production in the neutrophils with no prior priming (vehicle control). Furthermore, there was no difference in oxidative burst production between the three groups either on stimulation or with priming preceding stimulation. Stimulation: HY 586157.0 +/- 457040.3, HO 952060.7 +/- 567821.4, FO 727388.8 +/- 892021.8. Primed and stimulated: HY 5.34 +/- 3.06, HO 2.04 +/- 3.63, FO 4.99 +/- 7.03. (Figure 5.18).

Figure 5.18 – Superoxide production in healthy younger, healthy older and frail older adults on stimulation with fMLP and following priming with TNF α and then stimulation with fMLP



Superoxide production in healthy younger, healthy older and frail older adults on stimulation with fMLP and following priming with TNF α and subsequent stimulation with fMLP.

A – MFI of isolated neutrophils stimulated with 10nM fMLP in healthy younger (HY), healthy older (HO) and frail older (FO) adults. B – Fold change in MFI with priming isolated neutrophils with 1.15nM TNF α prior to stimulation with 10nM fMLP. Data sets are not all normally distributed and data shown mean +/- SEM. N: HY – 9, HO – 9, FO – 8.

A - HY 586157.0 +/- 457040.3, HO 952060.7 +/- 567821.4, FO 727388.8 +/- 892021.8. Independent Kruskal-Wallis test. P=0.602.

B - HY 5.34 +/- 3.06, HO 2.04 +/- 3.63, FO 4.99 +/- 7.03. Independent Kruskal-Wallis test. P=0.413.

5.16 Functional differences in neutrophils from frail older adults cannot be explained by either differences in neutrophil subpopulations or activation and senescence status.

As described in section 5.11 frail and healthy older adults exist in a state of chronic inflammation but the effect this systemic state has on neutrophils is uncharacterised. To investigate both subpopulations and the activation status of neutrophils in frail older adults the expression of cell surface receptors were measured using flow cytometry.

There was a small but significant difference in some neutrophil subpopulations between the three groups.

1. There was no difference in the proportion of immature granulocytes (CD16 dim CD62L bright). Proportion of immature granulocytes of total number of neutrophils: healthy younger adults 3.18 +/- 2.73, healthy older adults 2.33 +/- 2.22, frail older adults 2.87 +/- 3.83, Independent Kruskal-Wallis $p=0.177$.

2. There was a significant increase in the proportion of neutrophils which had surface markers which have been described as being consistent with reverse transmigration (CD16 bright CD62L dim) in frail older adults; with frail older adults having double the proportion of neutrophils expressing the 'reverse transmigration' phenotype in comparison to healthy younger and older adults. Proportion of neutrophils expressing the 'reverse transmigration' phenotype: healthy younger adults: 0.45 +/- 0.56, healthy older adults 0.40 +/- 0.33, frail older adults 0.86 +/- 1.60, Independent Kruskal-Wallis $p = 0.004$. (Table 5.7.)

Table 5.7 – Proportion of neutrophils within the CD16 CD62L defined subpopulations

	HY	HO	FO		
CD16 bright CD62L bright Phenotypically mature neutrophils	96.17 +/- 3.56	97.25 +/- 2.26	95.79 +/- 5.55	p=0.029	HY-HO p=0.121
					HY-FO p=1.000
					HO-FO p=0.044
CD16 dim CD62L bright Immature granulocytes	3.18 +/- 2.73	2.33 +/- 2.22	2.87 +/- 3.38	p=0.177	
CD16 bright CD62L dim 'Reverse transmigration' phenotype	0.45 +/- 0.56	0.40 +/- 0.33	0.86 +/- 1.60	p=0.004	HY-HO p=1.000
					HY-FO p=0.018
					HO-FO p=0.006

Neutrophils stained with anti CD16 and anti CD62L antibodies and gated on these populations. Data shown for healthy younger adults (HY) healthy older adults (HO) and frail older adults (FO). Data sets all not normally distributed. Data shown is median +/- interquartile range of proportion of the subpopulation of total number to total number of neutrophils. CD16 bright CD62L bright = phenotypically normal, CD16 dim CD62L bright = immature granulocytes, CD16 bright CD62L dim = older phenotype. Data set numbers as follows: HY – 29, HO – 27, FO - 18. P values in bold represent statistically significant values.

CD16 bright CD62L bright: HY – 96.17 +/- 3.56, HO – 97.25 +/- 2.2.6, FO 95.79 +/- 5.55. Independent Kruskal-Wallis. P=0.029. Post-hoc pairwise analysis with Bonferroni correction HY-HO p=0.121, HY-FO p=1.000, HO-FO p=0.044.

CD16 dim CD62L bright: HY – 3.18 +/- 2.73, HO – 2.33 +/- 2.22, FO – 2.87 +/- 3.38. Independent Kruskal-Wallis. P=0.177.

CD16 bright CD62L dim: HY 0.45 +/- 0.56, HO – 0.40 +/- 0.33, FO 0.86 +/- 1.60. Independent Kruskal-Wallis. P=0.004. Post-hoc pairwise analysis with Bonferroni correction HY-HO p=1.000, HY-FO p=0.018, HO-FO p=0.006.

Utilising a method developed in chronic inflammatory conditions to describe neutrophils as activated or senescent using neutrophil surface marker expression, there was no difference in activation or senescence throughout the three groups (305-307).

As described in section 1.7.4 of the introduction, in senescence it is hypothesised that CXCR2 is downregulated and CXCR4 is upregulated. There was no significant difference in the expression of CXCR2 in frail older adults. There was a significant reduction in the expression of CXCR4 in healthy older adults when compared to healthy younger adults. (Table 5.8 and Figure 5.19.) However, analysed together these data suggest there was no difference in the proportion of senescent neutrophils throughout the three groups.

Table 5.8 – Median fluorescence intensity (MFI) of CXCR2 and CXCR4 markers of senescence in healthy younger, older and frail older adults at baseline

	HY	HO	FO	I K-W	Pairwise
CXCR2	10856.5 +/- 10035.0	12232.0 +/- 7181.0	12931.0 +/- 3662.1	p=0.788	
CXCR4	539.0 +/- 68.5	415.0 +/- 53.5	467.5 +/- 145.3	p=0.002	HY-HO p=0.001 HY-FO p=0.132 HO-FO p=0.330

Median fluorescence intensity (MFI) of CXCR2 and CXCR4 in healthy younger (HY), older (HO) and frail older (FO) adults at baseline a. Data sets are median +/- IQR; data not normally distributed. Independent Kruskal-Wallis test with post-hoc pairwise comparison with Bonferroni correction. P values in bold represent statistically significant values.

CXCR2: HY – 17, HO – 9, FO – 11.

CXCR4: HY – 17, HO – 14, FO – 11.

In activation, it has been described that CD18, CD11b, CD63 and CD66b are upregulated and CXCR1, CXCR2 and CD62L are downregulated (306-308). There was no difference in how these cell surface markers were expressed throughout the three groups suggesting neutrophils from healthy and frail older adults are not activated. (Table 5.9, figure 5.20, figure 5.21)

Table 5.9 - Median fluorescence intensity (MFI) of CD18, CD11, CD63, CD66b, CXCR1, CXCR2 and CD62L markers of activation in healthy younger, older and frail older adults at baseline

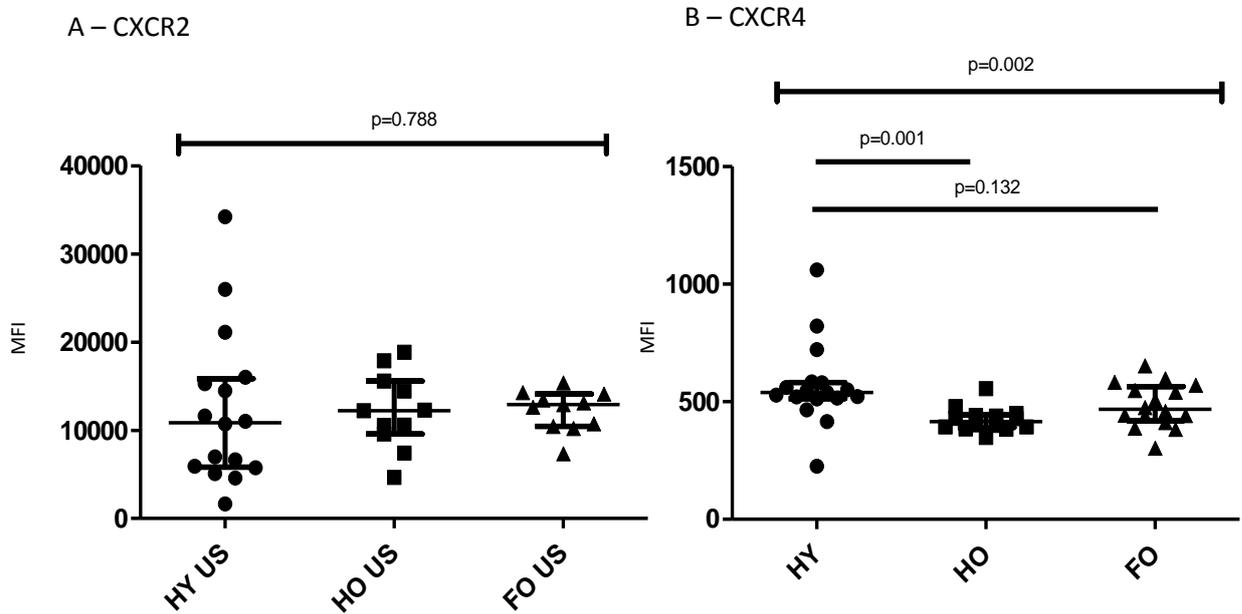
	HY	HO	FO	I K-W	Pairwise
CD18	34431 +/- 10014	30542 +/- 8825	29205.2 +/- 15525	p=0.514	
CD11b	2105.8 +/- 884	1665 +/-880	1649 +/- 1629	p=0.018	HY-HO p=0.039 HY-FO p=0.053 HO-FO p=1.000
CD63	653.5 +/- 209	578 +/- 325.5	571.5 +/- 403	p=0.636	
CD66b	9093.5 +/- 2601	9075.5 +/- 2058	9937 +/- 6444	p=0.609	
CXCR1	17812.0 +/- 2805.0	28092.5 +/- 4424.5	15905.8 +/- 4447.0	p=0.160	
CXCR2	10856.5 +/- 10035.0	12232.0 +/- 7181.0	12931.0 +/- 3662.1	p=0.751	
CD62L	27510 +/- 11358.5	32110.0 +/- 10115.5	2362 6 +/- 20221.5	p=0.073	

Median fluorescence intensity (MFI) of CD18, CD11, CD63, CD66b, CXCR1, CXCR2 and CD62L markers of activation in healthy younger, older and frail older adults at baseline. Data sets are median +/- IQR; data not normally distributed. Independent Kruskal-Wallis test with post-hoc pairwise comparison with Bonferroni correction.

Numbers included in each set of experiments are given below:

CD18: HY – 28, HO – 27, FO – 13, CD11b: HY – 24, HO – 25, FO – 18, CD63: HY – 27, HO – 29, FO – 14, CD66b: HY – 16, HO – 11, FO – 12, CXCR1: HY – 13, HO – 9, FO – 10, CXCR2: HY – 16, HO – 9, FO – 11, CD62L: HY – 27, HO – 17, FO – 17

Figure 5.19 – Neutrophil cell surface markers associated with senescence

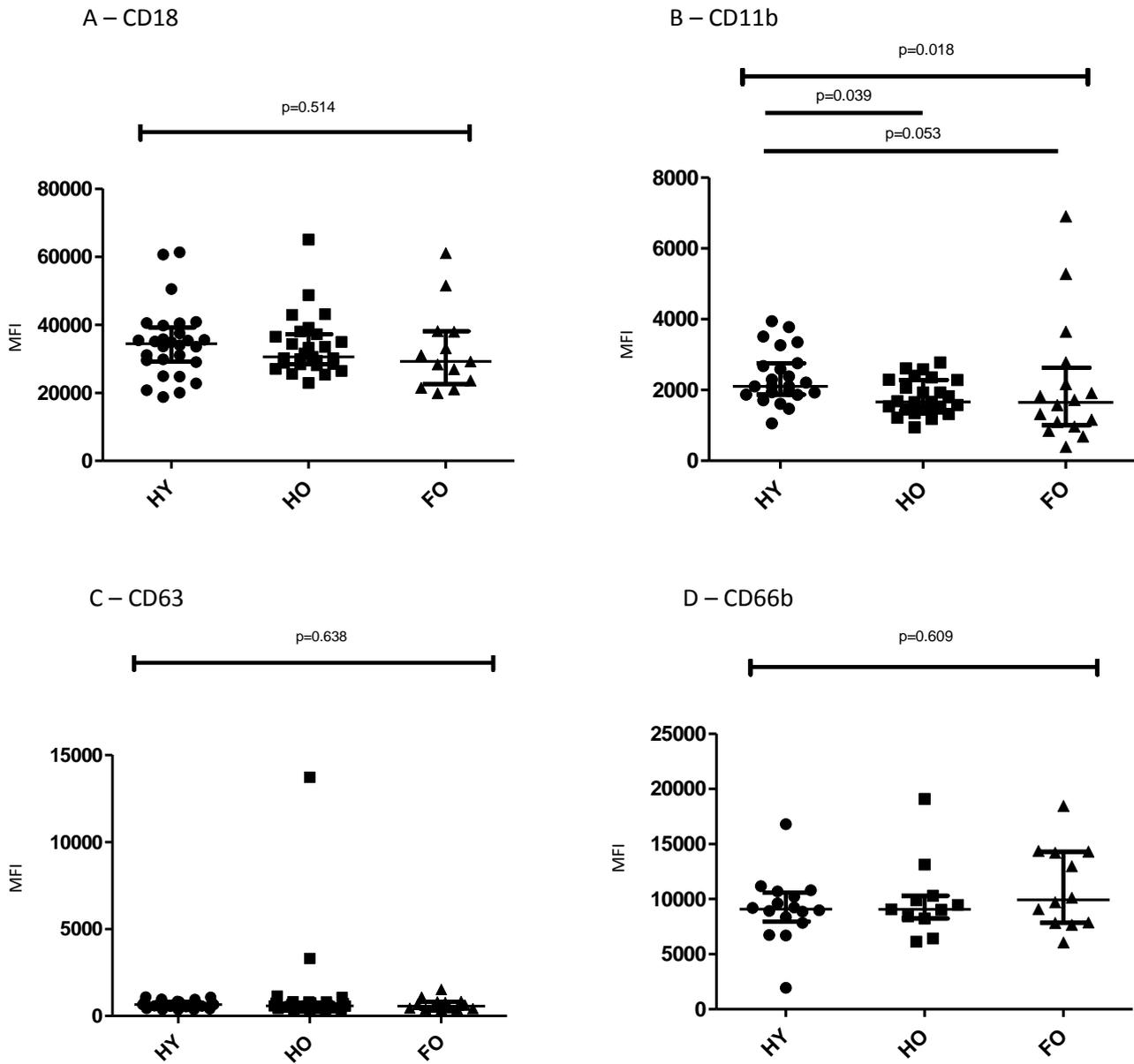


Neutrophil cell surface markers downregulated (CXCR2) and upregulated (CXCR4) in senescence. MFI of A - CXCR2 and B - CXCR4 on neutrophils at baseline from healthy younger adults (HY), healthy older adults (HO) and frail older adults (FO). Data sets are not normally distributed. Data shown are median with IQR. with.

A - CXCR2. Numbers in each group: HY-17, HO-16, FO-14. HY 10856.5 +/- 10035.0, HO 12232.0 +/- 7181.0, FO 12931.0 +/- 3662.1. Independent Kruskal-Wallis test. P=0.788.

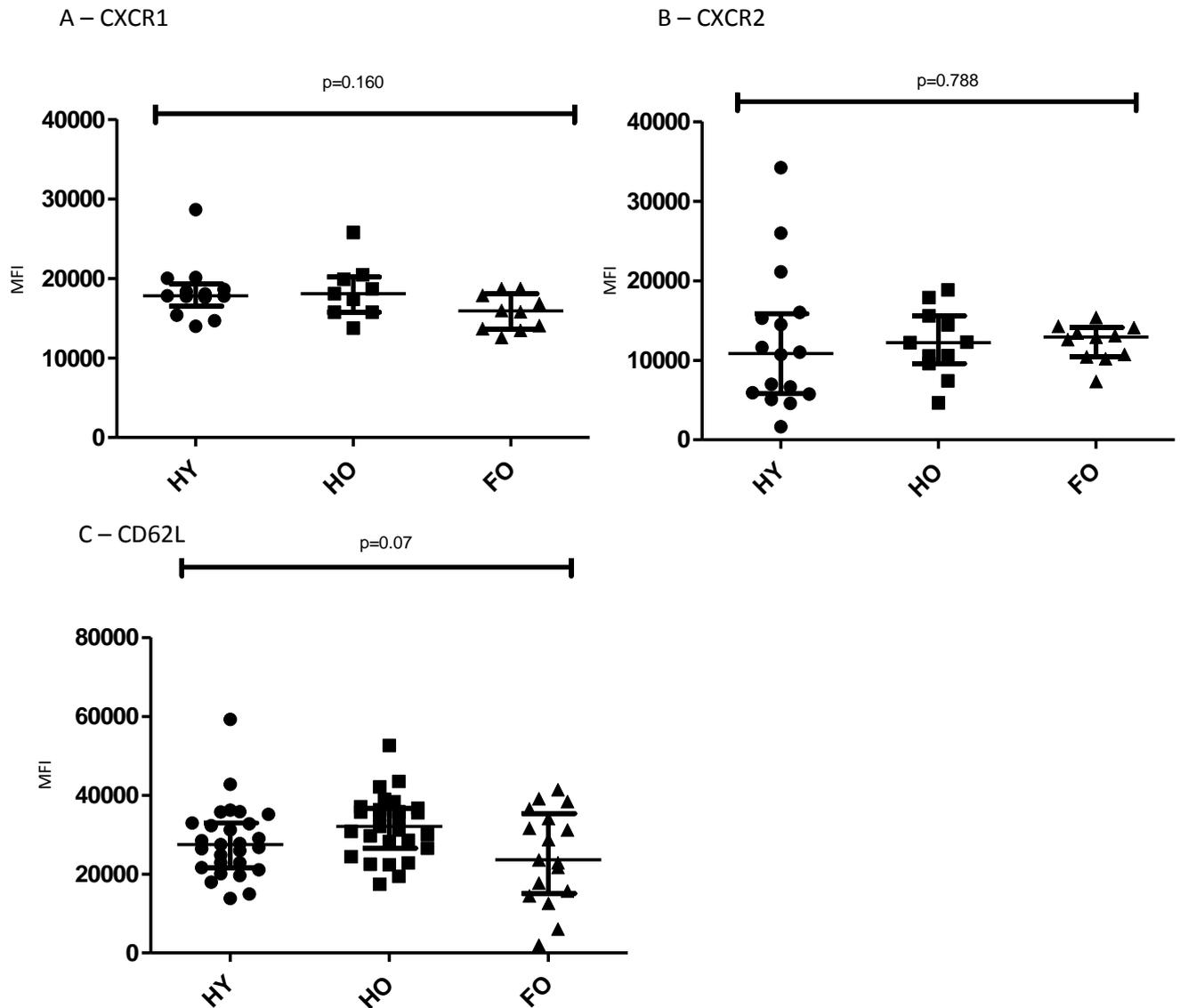
B - CXCR4. Numbers in each group HY-17, HO-14, FO-16. HY-539+/-68.5, HO-415+/-53.5, FO-467.5+/-145.3. Independent Kruskal-Wallis test. P=0.002. Post-hoc test with Bonferroni correction. HY-HO p=0.001, HY-FO p=0.132, HO-FO p=0.330.

Figure 5.20 – Neutrophil cell surface markers upregulated in activation



MFI of A – CD18, B – CD11b, C – CD63 and D - CD66b on neutrophils at baseline from healthy younger adults (HY), healthy older adults (HO) and frail older adults (FO). MFI. Data sets are not normally distributed. Data shown are median with IQR. CD18. Numbers in each group: HY-28, HO-27, FO-13. HY-34431 +/- 10014, HO -30542 +/- 8825, FO -29205.2 +/- 15525. Independent Kruskal-Wallis test. p=0.514 CD11b. Numbers in each group: HY-23, HO-25, FO-16. HY -2105.8 +/- 884, HO- 1665 +/- 880, FO -1649 +/- 1629. Independent Kruskal-Wallis test. P=0.018. Post-hoc pairwise comparison with Bonferroni correction. HY-HO p=0.039, HY-FO p=0.053, HO-FO p=1.000. CD63. Numbers in each group HY-27, HO-29, FO-14. HY - 653.5 +/- 209, HO - 578 +/- 325.5, FO - 571.5 +/- 403. Independent Kruskal-Wallis test. p=0.638. CD66b. Numbers in each group; HY-16, HO-11, FO-12. HY - 9093.5 +/- 2601, HO - 9075.5 +/- 2058, FO - 9937 +/- 6444. Independent Kruskal-Wallis test. p=0.609.

Figure 5.21 – Neutrophil cell surface markers downregulated in activation



MFI of A – CXCR1, B – CXCR2 and C-CD62L on neutrophils at baseline from healthy younger adults (HY), healthy older adults (HO) and frail older adults (FO). Data sets are not normally distributed. Data shown are median with IQR. Data sets are not normally distributed.

CXCR1; Numbers; HY-14, HO-14, FO-13. MFI: HY 17812.0 +/- 2805.0, HO 28092.5 +/- 4424.5, FO 15905.8 +/- 4447.0. Independent Kruskal-Wallis test. p=0.160

CXCR2. Numbers; HY-17, HO-16, FO-14. MFI HY 10856.5 +/- 10035.0, HO 12232.0 +/- 7181.0, FO 12931.0 +/- 3662.1. Independent Kruskal-Wallis test. p=0.751

CD62L; Numbers; HY-27, HO-27, FO-17. HY-27510 +/- 11358.5, HO-32110.0 +/- 10115.5, FO-23626 +/- 20221.5. Independent Kruskal-Wallis test. p=0.073.

CD16 expression on the neutrophil cell surface increases with stimulation. CD16 expression was significantly increased in frail older adults both at baseline and on stimulation when compared to both healthy younger and older adults. There was also a significant reduction in upregulation with stimulation in the frail older adults. (Table 5.10 and Figure 5.22.)

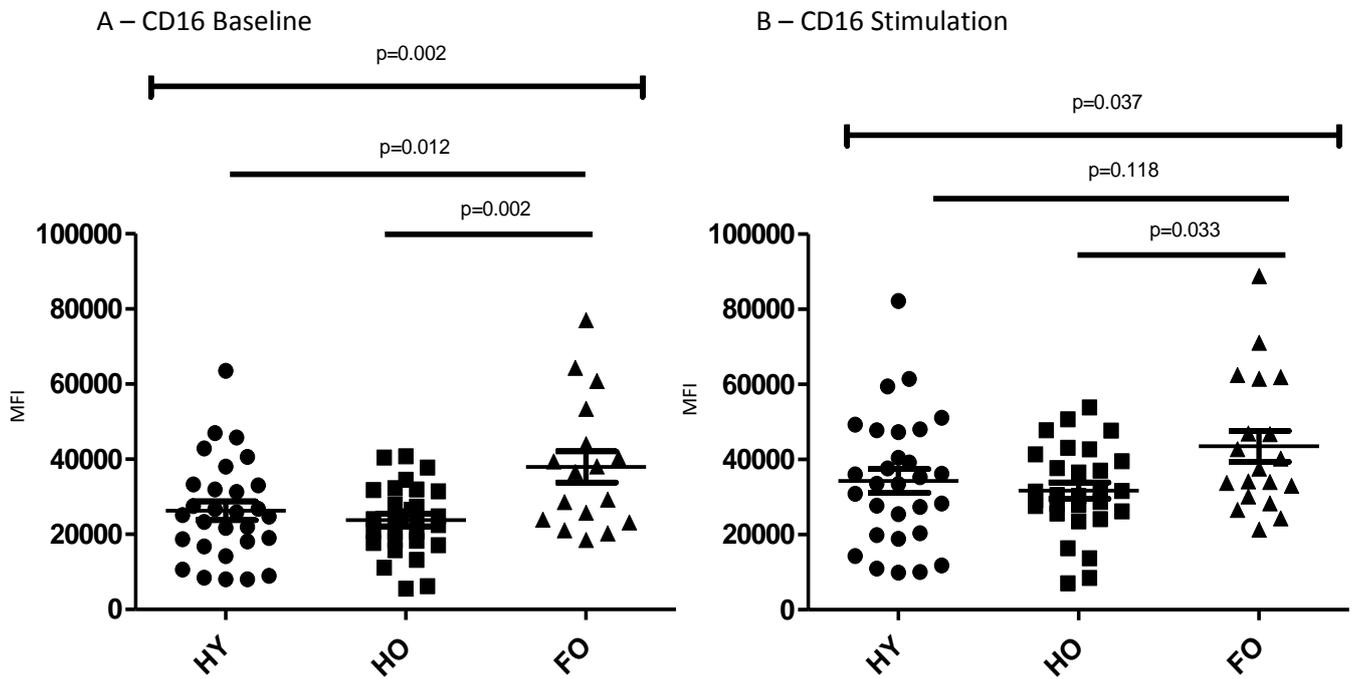
Table 5.10 - Median fluorescence intensity (MFI) of CD16 at baseline, on stimulation and the fold change in expression with stimulation in healthy younger, healthy older and frail older adults

	HY	HO	FO	ANOVA	Post-hoc Tukey
CD16 unstimulated	26284.2 (21218.6-31349.8)	23833.3 (20363.2-27303.3)	37944.8 (29102.0-46787.5)	p=0.002	HY-HO p=0.751 HY-FO p=0.012 HO-FO p=0.002
CD16 stimulated	34291.6 (27747.2-40836.1)	31726.1 (27279.1-36173.1)	43507.4 (34820.3-52194.5)	p=0.037	HY-HO p=0.806 HY-FO p=0.118 HO-FO p=0.033
Fold change in CD16 expression	0.31 (0.25-0.37)	0.36 (0.25-0.47)	0.17 (0.09-0.25)	p=0.015	HY-HO p=0.686 HY-FO p=0.074 HO-FO p=0.012

Median fluorescence intensity (MFI) of CD16 at baseline, on stimulation and the fold change in expression with stimulation in healthy younger (HY), healthy older (HO) and frail older adults (FO). Data given as mean with confidence intervals; data normally distributed. Statistical tests are ANOVA and post-hoc Tukey for pairwise comparison.

Numbers; CD16US: HY – 29, HO – 29, FO – 17, CD16S: HY - 29, HO - 29, FO – 19, CD16 fold change: HY -29, HO - 29, FO - 17

Figure 5.22 – Neutrophil cell surface expression of CD16



MFI of A – CD16 expression at baseline, B – CD 16 expression on neutrophils on stimulation from healthy younger adults (HY), healthy older adults (HO) and frail older adults (FO). Data sets are mean+/-SEM. All normally distributed. N- US: HY=29, HO=29, FO=17. S: HY=29, HO=29, FO=19.

CD16 US HY: 26284.2 (21218.6-31349.8), HO: 23833.3 (20363.2-27303.3), FO: 37944.8 (29102.0-46787.5). ANOVA p=0.002, Post-hoc Tukey: HY-HO p=0.751, HY-FO p=0.012, HO-FO p=0.002.

CD16 S HY: 34291.6 (27747.2-40836.1), HO: 31726.1 (27279.1-36173.1), FO: 43507.4 (34820.3-52194.5). ANOVA p=0.037, Post-hoc Tukey: HY-HO p=0.806, HY-FO p=0.118, HO-FO p=0.033

5.17 Neutrophil elastase activity is reduced in the plasma from frail older adults in comparison to healthy older adults

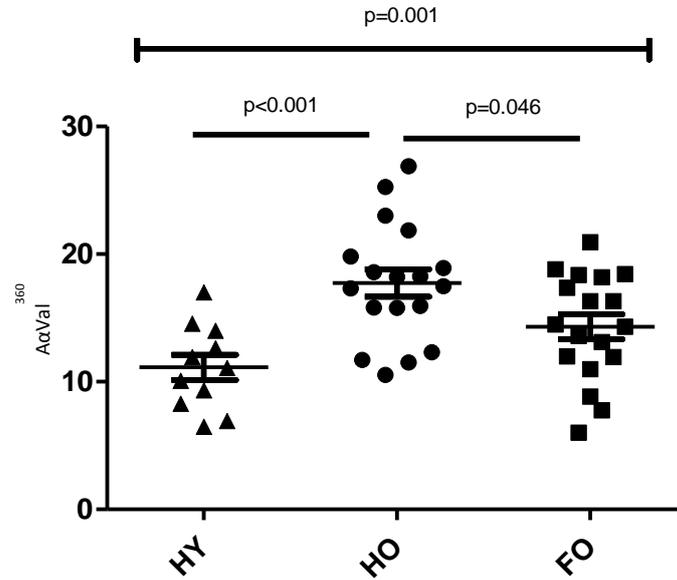
Data generated so far using the expression of neutrophil surface markers suggests that systemic neutrophils from healthy older and frail older adults are not activated.

Activated neutrophils are known to degranulate more readily (212). To confirm that neutrophils from HO or FO participants were not constitutively activated, A α Val³⁶⁰ (a validated footprint of neutrophil elastase activity) was measured (212).

Healthy older adults displayed an increase in A α Val³⁶⁰ compared to both healthy young adults and frail older adults (Figure 5.23.) A α Val³⁶⁰ HY – 11.13nM (8.91-13.35), HO – 17.74nM (15.45-20.02), FO – 14.32nM (12.24-16.39). There was no significant difference between the concentration of A α Val³⁶⁰ in healthy younger adults and frail older adults.

To determine if systemic activation of neutrophils was associated with physical parameters of frailty and sarcopenia: walk time, grip strength, BATT, ultrasound echogenicity and cognitive function were compared to A α Val³⁶⁰ concentrations. BATT significantly and negatively correlated with A α Val³⁶⁰ concentrations, R= -0.466, p=0.019. The correlation with ultrasound echogenicity and adjusted hand grip showed a trend towards significance: ultrasound echogenicity and A α Val³⁶⁰ R=0.494, p=0.086, adjusted grip strength and A α Val³⁶⁰ R= -0.329, p=0.094. There was no significant correlation between time to walk four metres and A α Val³⁶⁰ R=0.112, p=0.593, and Stroop interference and A α Val³⁶⁰ R=-0.139, p=0.582 (All Pearson's correlation coefficients).

Figure 5.23 – Neutrophil elastase activity in healthy younger, older and frail older adults



Neutrophil elastase activity in healthy younger (HY), older (HO) and frail older adults (FO). AαVal³⁶⁰, a marker of neutrophil elastase activity, calculated in serum from healthy younger adults (HY), healthy older adults (HO) and frail older adults (FO). Data sets are mean±SEM; all normally distributed. AαVal³⁶⁰ expressed as nM. N- HY=11, HO=18, FO=18. ANOVA p=0.001, Post-hoc Tukey: HY-HO p<0.001, HY-FO p=0.124, HO-FO p=0.046.

5.18 Discussion

This chapter has investigated both immunosenescence and inflammaging in frailty. It has described altered neutrophil function in healthy older and frail older adults, and explored the differences witnessed in the two groups. It is only the second study to explore function in the innate immune system in a defined population of frail older adults (208) and links a chronic inflammatory environment with neutrophil functional ability.

The most important limitation to accurate interpretation of the data quoted throughout this chapter is the fundamental differences in the healthy and frail older adults namely age and health status. Table 5.1 describes the basic demographic data of the three groups. It demonstrates that the frail older adults are significantly older than the healthy older adults and there is therefore a risk that differences attributed to frailty are actually related to ageing. However, the experiments described within this chapter do not include every individual within each cohort and therefore each experiment is a selection of individuals from the complete cohort. Where possible the data has been adjusted for age to prove that differences witnessed are due to frailty rather than ageing. The frail older adults had an increased number of chronic inflammatory conditions compared to the healthy older adults as chronic inflammatory conditions were an exclusion criteria for the healthy older adults but not the frail older adults. (See Chapter 6 for more in depth analysis.) Chronic inflammatory conditions such as COPD and cardiovascular disease are associated with immunosenescence (309). The differences described with frailty could therefore also be attributable to chronic inflammatory conditions rather than frailty itself. The method of recruitment could have been improved by age matching each of the frail older adults with a similar age healthy older adult. Another refinement to the method

would be the exclusion of chronic inflammatory conditions in the frail older adults. Both of these changes to the method of recruitment would have been possible in this study population; 40% (15) of the frail older adults did not have any chronic inflammatory conditions and the range of healthy older adults extended to 84. However, the changes may have proved difficult in practice and hindered adequate recruitment and comparing frail older adults with and without co-morbidities does not provide sufficient numbers to adequately exclude potential differences.

Sections 2.1 to 2.4 describe the reduction in chemotaxis with both ageing and frailty; reduction in chemotaxis to the end target chemoattractant fMLP in both healthy and frail older adults, and preserved chemotaxis in healthy older adults to the intermediate chemoattractant IL-8.

This is the first study investigating neutrophil migration in frail older adults but several studies have demonstrated reduction in chemotaxis with increasing age to several different chemoattractants including fMLP and IL-8 (163, 310, 311). The SENIEUR protocol (protocol outlining the inclusion and exclusion criteria for studies investigating the effect of age on the immune system) on which most gerontological studies base their inclusion and exclusion criteria does not identify or exclude frail individuals from ageing research (312). Previous studies which have demonstrated reduction in chemotaxis with increasing age may have included frail individuals within their study populations and therefore, the demonstrated decrease in neutrophil chemotaxis to IL-8 with age may actually represent a decrease with frailty, particularly as the prevalence of frailty increases with age. This supports the routine identifications of frailty in future ageing

immune studies and either the exclusion of frail individuals or preferentially their inclusion as a separate comparator group. Whilst this is the first study investigating chemotaxis in frail older adults a single study investigating chemotaxis in healthy older adults with differing levels of daily physical activity had similar results; the most active healthy older adults had preserved chemotaxis to IL-8 whereas the least active healthy older adults had reduced chemotaxis to IL-8 in comparison to healthy younger adults (313). Section 2.2 demonstrated that neutrophil function correlated with markers of both physical and cognitive health and this was independent of age. Considering the data presented in this chapter along with data already reported in the literature suggests there are two different patterns of immunosenescence occurring in the neutrophils of older adults: the chronological age dependent, chemotaxis to fMLP and biological age or frailty dependent, chemotaxis to IL-8. Appropriate neutrophil response to both intermediate (IL-8) and end target (fMLP) chemoattractants are required for organised and efficient migration to sites of infection. The reduction in migratory accuracy towards fMLP might in part explain the increased rates of infection in all older adults whereas the additional reduction in migratory accuracy to IL-8 in frail older adults can partially explain the exponentially increased rates of infection in frail older adults (314).

An alternative explanation for the apparently preserved chemotaxis to IL-8 in healthy older adults is the sample size was too small to determine the actual difference between the healthy older and frail older adults and the current study lacked power. Reviewing the data and statistical tests confirms there is a significant difference in the chemotaxis to IL-8 in neutrophils from healthy younger and frail older adults. However, there is no significant difference in the chemotaxis to IL-8 of healthy older adults to either healthy

younger or frail older adults. This means it's true value could lie anywhere between the values for chemotaxis for healthy younger adults and frail older adults. To confirm whether there is actually preserved chemotaxis to IL-8 in healthy older adults a statistical difference between the chemotaxis of healthy older adults and frail older adults is required. A sample size of 19 in each group has been calculated to be adequate to demonstrate a statistical difference between healthy and frail older adults.

As discussed in brief above differences witnessed in the frail older adults could be due to the presence of chronic inflammatory conditions rather than frailty itself. Statistical analysis of the data suggests there is no difference in the chemotaxis to IL-8 in frail older adults without chronic inflammatory conditions and those with chronic inflammatory conditions. Chemotaxis to IL-8 ($\mu\text{m}/\text{min}$): frail older adults without chronic inflammatory conditions 0.57 (0.03-1.12) and frail older adults with chronic inflammatory conditions 0.46 (0.15-0.77). Independent t-tests $p=0.658$. However, as the sample populations are small (frail older adults without chronic inflammatory conditions – 5 and frail older adults with chronic inflammatory conditions – 12) this should be interpreted with caution and further investigation is required.

Similar to other studies investigating migratory function in older adults there was no significant differences in chemokinesis (163, 313) although there was a trend towards a reduction across the three groups in migration towards fMLP. This small reduction in chemokinesis did not affect the chemotactic index, the overall migratory function. This suggests neutrophils from healthy and frail older adults can move just as effectively as neutrophils from healthy younger adults but their directional migration is impaired.

The Insall chamber assay is an imperfect technique for the quantification of neutrophil migration primarily it is not physiological but it does have some advantages over alternative assays. The direct visualisation of the neutrophils as they migrate allows quantification of both chemotaxis and chemokinesis rather than an assessment of their overall migratory function as used in the transwell approach (315). The Insall chamber assay is a relatively simple technique for neutrophil migration which does not require tissue culture and has been therefore been utilised in multiple labs across the world ensuring data is widely applicable. The assay quantifies neutrophil migration in two-dimensions not the more physiological three dimensions, the advantages and disadvantages of two and three dimensional methods are discussed later. A further criticism of the Insall chamber assay is the presence of subjective bias in the analysis of the video microscopy films. Whilst the analysis protocol was standardised: randomisation of neutrophil selection, strict inclusion and exclusion criteria for neutrophils, there is still an unacceptable degree of human interpretation. Automation of the process and inclusion of all the neutrophils not just ten randomly selected cells would improve the accuracy of the data. A potential advantage of the transwell assay is that neutrophil migration can be measured across an endothelial membrane replicating a more physiological environment (27) and migratory transwell assays incorporating flow have also been described. These are reported to be more physiological because laminar flow is important in securing the adhesion of neutrophils to the endothelial wall prior to the neutrophils initiating their migration through the endothelial walls. However, they do not allow accurate separate analysis of speed and directionality. The microfluidic device utilised by Irimia to investigate neutrophil function in burns patients is reportedly able to

accurately decouple the measurement of speed and directionality (316) but cells have to enter the chemotactic viewing well, and thus only a select population of cells which can migrate into this narrow chamber are selected for study. A further advantage of this method is it utilises whole blood rather than isolated neutrophils in the assay. Isolation of neutrophils by any method results in a degree of activation, but the percol gradient method used in this study was selected as it has been shown to minimise this effect (254). It is possible to review the activation status of the neutrophils by eye under a microscope using the morphology and behaviour of the neutrophils as indicators. This is well characterised in healthy younger adults and any neutrophils believed to be activated at this point by the process of isolation can be discarded prior to the migratory assay. However, the morphology and behaviour of neutrophils from frail older adults is poorly understood and therefore the utility of this assessment is not known in this patient group.

Both chemotaxis and chemokinesis is reliant on adhesion molecules which propagate the adhesion of neutrophils to the endothelium and their subsequent migration through the endothelial cell wall. In vitro, the albumin coating the coverslips mimics ICAM-1 which binds to both LFA-1 and Mac-1 on the neutrophil cell surface.

Sections 2.6 and 2.7 describe the differences throughout the three groups in adhesion molecules expression on the neutrophil cell surface. Throughout the three groups there was no difference in expression of CD18 but differences in the expression of both CD11a and CD11b, and differences in the response to stimulation in CD62L, CD66b and CD63 expression. CD11a expression was reduced both at baseline and on stimulation in frail

older adults but CD11b and activated CD11b expression was reduced, by a similar amount, in both healthy older and frail older adults. This is comparable to other studies although there is controversy in the literature. Butcher et al found no significant difference in the expression of either CD11a or CD11b on the cell surface of neutrophils from healthy older adults (317) and a study investigating the expression of CD11b on the surface of neutrophils from nursing home residents demonstrated elevated levels of CD11b in the frail cohort (210). However, similar to the data presented here other studies have demonstrated decreased CD11b in low activity older adult cohorts and nursing home residents (208, 313). Investigation into the differing roles of Mac-1 (CD18/CD11b) and LFA-1 (CD18/CD11a) could potentially explain how the differences in CD11a and CD11b expression in healthy and frail older adults are related to the witnessed differences in chemotaxis towards different chemoattractants. Chemotaxis to fMLP has been demonstrated to be reliant on the interaction of three adhesion molecules: Mac-1 which provides traction for movement, LFA-1 and $\alpha 4$ which both provide directional signalling for movement. However, chemotaxis to IL-8 has been demonstrated to be mediated by LFA-1 only (318, 319). Reduction in CD11a and CD11b expression in frail older adults is associated with poor chemotaxis towards both IL-8 and fMLP whereas preserved CD11a expression in healthy older adults could explain the preserved migratory function towards IL-8. The relationship between CD11b, activated 11b and chemotaxis to fMLP was demonstrated in the significant correlation of the adhesion markers with chemotaxis to the bacterial chemoattractant.

CD62L, CD63 and CD66b are all implicated in the upregulation and recruitment of Mac-1. Table 5.2 demonstrates that whilst there is no difference in the expression of these three

cell surface markers at baseline there are significant differences in how these cell surface markers are expressed on stimulation in both healthy older and frail older adults. This suggests that an ineffective response to stimulation in healthy older and frail older adults may contribute towards poor neutrophil function.

A further hypothesis that could explain the apparent preserved chemotaxis to IL-8 in healthy older adults could be differences in the amount or function of IL-8 receptors, CXCR-1 and CXCR-2, expressed on the neutrophil cell surface in healthy and frail older adults. Similar to data reported in this chapter and previous studies have not elicited a difference in the cell surface expression of CXCR-1 and CXCR-2 in either healthy older adults (compared to healthy younger adults) or healthy older adults with low activity levels compared to those with higher activity levels (163, 313, 320). There is however some evidence that CXCR-2 receptor is integral to neutrophil chemotaxis; blocking CXCR-2 receptor on neutrophils resulted in significantly decreased chemotaxis but not chemokinesis (321). Therefore, despite normal cell surface expression of both CXCR-1 and CXCR-2 in frail older adults binding of IL-8 to the receptor may not result in either appropriate or maximal intracellular signalling and result in reduction in chemotaxis to IL-8.

Flow cytometry is considered a simple and reproducible technique that it is utilised for diagnostic purposes in clinical medicine in multiple conditions (322). However, there are limitations to its use in research and in particular within this study. Whilst it is an excellent tool to describe the number of cell surface markers both as an absolute number, MFI, and as a proportion of cells expressing the cell surface marker it does not

consider the functional capabilities. Therefore, the earlier reported ineffective response to stimulation due to the failure to upregulate CD62L, CD63 and CD66b in healthy and frail older adults could be erroneous. The neutrophil from the frail older adult may not need to upregulate these cell surface markers to the extent of the healthy younger adult because the neutrophil cell surface markers in the frail older adult may be able to produce a magnified response which is comparable to the response of a greater number of cell surface markers expressed on the neutrophil from the healthy younger adult. In brief, number does not necessarily reflect function. The data presented in this chapter obtained from flow cytometry only provides a snapshot of the cell surface of a neutrophil. It does not investigate internalised cell surface markers which may be important in the consideration of response to varying conditions or provide data on neutrophil response over different time-periods. The data presented in section 12 suggests that responses to stimulation may be delayed in frail older adults and therefore reported differences in response to stimulation may be incorrect and actually represent a delay in response rather than an absolute inability to respond. The flow cytometry method utilised in investigating cell surface markers reported in this chapter also relies on the similarity of commercially available antibodies in which there may be functional differences, particularly when running experiments over a period of years.

PI3kinase is believed to be central to the control of accurate and directional migration in the neutrophil; a non-specific inhibitor of PI3 kinase, wortmantin, causes significant reduction in neutrophil migration both chemotaxis and chemokinesis (323-326). Section 2.8 describes how incubation of neutrophils from frail older adults with PI3 kinase inhibitors δ and γ rescued their chemotactic ability to IL-8 and returned it to levels

expected in neutrophils from healthy younger adults. This result is similar to data published previously which demonstrated that neutrophils from healthy older adults incubated with PI3 kinase inhibitors δ and γ had improved chemotactic ability (163). However, the data presented in this chapter did not elicit an overall improvement in neutrophil chemotaxis in healthy older adults with PI3 kinase inhibition. Furthermore, contrary to previously published data (163) there was no significant reduction in neutrophil chemotaxis to IL-8 in the healthy older adult group. As demonstrated in this chapter and elsewhere (163) PI3 kinase has a restorative but not enhance effect on chemotaxis. It can be hypothesised that previously demonstrated positive responses to PI3 kinase inhibition in healthy older adults could be related to the definition of healthy older adult which did not take into account the presence of frailty. This is supported by the negative association between the physical parameter grip strength and fold change improvement in chemotaxis following PI3 kinase inhibition (figure 5.12). Not only does this ratify the close relationship between frailty, sarcopenia and immunesenescence it offers an individualised approach to a potential therapeutic target and could be used to identify a suitable target population; PI3 kinase inhibitors prescribed on the basis of grip strength.

The data presented in this chapter demonstrated that both PI3 kinase inhibitors δ and γ significantly improved chemotaxis in neutrophils from frail older adults but did not significantly affect chemokinesis, although there was a trend towards improvement. Figure 5.11 demonstrates further work is required to investigate the effect of each inhibitor on individuals. There is currently conflicting data published on the roles of the PI3 kinase inhibitors δ and γ in neutrophil migration. Whilst some studies have suggested

they are solely related to chemotaxis (163, 327, 328) recent work in three-dimensional collagen gels, a reported mimic of peripheral tissue, reports both PI3 kinase δ and γ also have an important role in chemokinesis (329). However, it may not be possible to accurately separate chemokinesis from chemotaxis when utilising a three-dimensional collagen model; the role of PI3 kinase in aligning the pseudopodia at the leading edge is essential in propelling the neutrophil forward in any direction in a confined environment such as a collagen model. The data presented in this chapter demonstrates a definite role in chemotaxis towards IL-8 but suggests there may also be a role within chemokinesis which requires further investigation.

PI3kinase inhibitors are reported to be selective: TGX 221 is quoted as being 1000 fold more selective for p110 β than p110 δ , AS 252424 is quoted as being 30 fold more selective for PI3 kinase γ than PI3 kinase α , Cal 101 is quoted as being 40-300 fold more selective for p110 δ than p110 $\alpha/\beta/\gamma$ (330). However, not all possible interactions are reported for example TGX 221 selectivity is only quoted in comparison to p110 δ not p110 γ nor p110 α . In addition whilst the PI3kinase inhibitors are reported to be selective for the various isoforms specificity is not considered and for these reasons the limitations of the inhibitors need to be considered when interpreting these data.

Akt is an important downstream effector of PI3 kinase and its activation is dependent on the generation of PIP2 and PIP3 (the products of PI3 kinase activation). Threonine 308 is situated in the activation loop of Akt and its phosphorylation results in partial activation of Akt (331). Section 2.9 demonstrated there was no significant difference between the response to IL-8 for the three groups. However, it is difficult to interpret the data with

any certainty due to varied responses from individuals and the small sample. The visual interpretation of the data (without statistical analysis) suggests neutrophils from healthy older adults and frail older adults may have an exaggerated increase in phosphorylation of threonine 308 in response to IL-8 stimulation compared to healthy younger adults. The response to IL-8 in the healthy younger adults appears minimal. However, an alternative explanation is a delayed response in the phosphorylation of threonine 308 in the neutrophils of healthy older and frail older adults compared to healthy younger adults; the phosphorylation of threonine 308 in healthy younger adults occurring almost instantaneously on stimulation with IL-8 with a rapid rise and then decline in the phosphorylated threonine 308. This is supported by the representative Western blot (figure 5.13A) which shows an increase in density at 20 seconds in the healthy young individual and at 60 seconds in the healthy older adult. Validation work of the experiment protocol suggested that there was a rapid rise in phosphorylation of threonine 308 in response to IL-8 at twenty seconds in the neutrophils from healthy younger adults (332). It is possible that differences in experimental technique led to an absence of signal from the healthy younger adults which suggests a delayed response in healthy and frail older adults rather than an exaggerated response. This section of work needs to be repeated in a greater number of samples with a shorter period of initial stimulation, for example 10 seconds rather than 20 seconds.

Sections 2.1 to 2.9 report on the reduction in chemotaxis to IL-8 in neutrophils from frail older adults and the reduction in chemotaxis to fMLP in both neutrophils from healthy older and frail older adults. It also reports on the reduction in expression of LFA-1 in frail older adults and Mac-1 in healthy older and frail older adults and the failure of CD62L,

CD63 and CD66b in both healthy and frail older adults to respond to stimulation. It proceeds to report on the dysregulation of PI3 kinase in healthy and frail older adults and the potential for individualised therapeutic targets using PI3 kinase inhibitors. Sections 2.10 to 2.15 proceed to investigate whether poor migratory function is intrinsic to neutrophils from frail older adults due to cellular senescence or whether it can be induced by the 'frail' environment.

Studies have demonstrated that chemotaxis is increased in healthy younger adults with infection conversely in states of severe sepsis it is reduced suggesting the environment can have both an enhancing and detrimental effect on neutrophil migratory accuracy (333-335) either by directly altering cellular functions or causing a different subset or phenotype of neutrophils to be released systemically. These effects might be due to exposure to priming agents such as TNF α . Previous in vitro studies have demonstrated that incubating neutrophils from healthy younger adults with pooled plasma from healthy older adults did not induce an "old" migratory phenotype of reduced chemotaxis when migrating towards both IL-8 and fMLP (163) and nor did priming the neutrophils with 1pM TNF α prior to exposure to the chemoattractant (336). These experiments were repeated in section 2.10 and in concordance with previous results there was no reduction in chemokinesis or chemotaxis in neutrophils from healthy younger adults when incubated with pooled plasma from healthy older adults. However, there was a trend towards a reduction in chemotaxis in neutrophils from healthy younger adults when incubated with pooled plasma from frail older adults; (p=0.053). It is unclear whether these studies were under-powered but these data suggest that the 'frail' environment might induce dysregulation of cellular signalling resulting in poor neutrophil migratory

function. An important extension of this work would be to increase the number of studies and to investigate the effect of pooled plasma from healthy younger adults on the neutrophils from frail older adults to consider whether these neutrophils can be rescued with plasma from healthy younger adults. Post-hoc analysis of the data suggests a sample size of 14 in each group would be required to demonstrate a significant difference in chemotaxis following the incubation of neutrophils with pooled plasma from frail older adults. Previous work using healthy younger and older adults demonstrated that poor chemotaxis in healthy older adults was not improved following incubation with pooled plasma from healthy younger adults (163). An important consideration when using pooled plasma is the effect of one individual on the pool. It is possible a single individual may change the composition of the pool significantly. This could be negated by selection of the individuals to be included in the pool following the multiplex analysis or increasing the number of individuals within the pool both of these strategies would not have been feasible in this study.

Immunesenescence is intrinsically linked with inflammaging and it is postulated that exposure to chronic inflammation can modulate the immune system and result in immunesenescence (150, 337). Frailty is associated with an increased amount of chronic inflammation in comparison to a healthy older population; there is also some evidence that inflammation predates frailty (169-171, 206, 302). Chronological age, rather than biological age or frailty, is also considered to be associated with inflammation (150, 230), however, the effect of frailty has not been considered in these studies. Section 2.11 describes a chronic inflammatory environment in both healthy older and frail older adults with significantly increased concentrations of IL-1ra, IL-8 and Eotaxin. There are

significantly increased concentrations of IL-4, IL-17 and IL-1 β in healthy older adults but not frail older adults. The concentrations of MIP1a increases with frailty; there are significantly increased concentrations in frail older adults compared to healthy older adults and healthy younger adults. There are significantly reduced concentrations of MCP-1 in frail older adults when compared to healthy older adults but not healthy younger adults. The data presented in this chapter is contrary to previous studies which have demonstrated a pro-inflammatory environment in frail older adults; further analysis and discussion will be completed in chapter 6.

The investigation of a pro-inflammatory profile in frail older adults has been previously limited to IL-6, CRP, TNF α and IL-10. A number of studies have demonstrated a positive association between IL-6, CRP and TNF α , and frailty, sarcopenia or indicators of both conditions such as low muscle strength (169, 179-181, 185, 187, 195, 202, 302, 338). CRP was significantly raised in the frail older adults and this is discussed in greater depth, below. Contrary to previous literature TNF α concentrations were not significantly different between the three groups and only 3% of the frail older adult group had a concentration of IL-6 within detectable range and none had a detectable concentration of IL-10. Reported concentrations of IL-6 in frail older adults range between 3.5-4.4 pg/ml (171, 178, 187). The lowest detectable range of IL-6 in the commercial multiplex used in this study was 2.81pg/ml. An assay with increased sensitivity at the lower end of detectable range may have resulted in an increased number of samples in all three groups with a concentration within the detectable range ensuring apparent differences are detectable. TNF α concentrations were similar between the three groups but there

was a much larger degree of variability within both the healthy older and frail older adult suggesting a linear relationship between frailty and TNF α may be too simplistic.

Furthermore, most cytokines have a short free half-life systemically due to their cognate receptors which are present on cells or shed and present in solution (such as TNFsR1 and TNFsR2 for TNF α). The utilised assay does not report if it measures free or receptor bound TNF α and measurements taken from plasma and serum would not provide a measure of cell bound TNF α , therefore it is unclear how effectively these systemic measures reflect cytokine activity.

Similar to previous data there was an increase in CRP concentration in the frail older adults. CRP is primarily produced by hepatocytes and to a lesser extent lymphocytes and monocytes; it is unlikely that CRP synthesised by other sources have much of an impact on circulating serum concentration. Pro-inflammatory cytokines, IL-6, IL-1 β and TNF α induce CRP expression in hepatocytes (339). CRP exists as two forms both of which have significant effects on neutrophil function. Pentameric CRP circulates in the serum and is believed to have mainly anti-inflammatory effects including reduction in neutrophil chemotaxis (340). Monomeric CRP is the tissue bound form and has pro-inflammatory effects including delaying neutrophil apoptosis and upregulating Mac-1 (341, 342). The plasma half-life of CRP is approximately 19 hours and is constant under all conditions of health and disease therefore the sole determinant of circulating CRP concentration is the synthesis rate (300). The frail older adults could have either a constant stimulus present, which is unmeasured in this study, an abnormal exaggerated response to the circulating cytokines, or increased production secondary to an unmeasured physiological difference.

Whilst pentameric CRP is believed to reduce neutrophil chemotaxis the demonstrated increase in concentration of CRP in frail older adults is not high enough to explain the trend towards a reduction in chemotaxis when pooled plasma from frail older adults was added to neutrophils from healthy younger adults. The concentration of CRP measured in the frail older adult population (8.87 $\mu\text{g}/\text{ml}$) is not comparable to concentrations in adults with sepsis (48.0-150 $\mu\text{g}/\text{ml}$ (343, 344)) and therefore it is unlikely the effect of pentameric CRP is having a direct effect on neutrophil migratory ability, but instead might be a marker of other serum factors which could induce change in neutrophil function.

The most significant limitation to the data reported in this chapter on CRP is that it is unable to fully describe the complete relationship between CRP and frailty. CRP concentration was measured using a hs-CRP ELISA assay which is able to accurately measure between 0.4 and 10 $\mu\text{g}/\text{ml}$. Values greater than 10 $\mu\text{g}/\text{ml}$ were recorded as 10 $\mu\text{g}/\text{m}$ and were seen in 3.6% (1 person) of healthy young adults, 2.6% (1 person) of healthy older adult and 27.3% (10 people) of frail older adults. Although this did not affect the quoted statistics (not normally distributed therefore median quoted) it does prevent full analysis of the relationship between CRP, frailty, immunesenescence and inflammaging.

The data presented in this chapter has so far demonstrated a significant difference in migratory accuracy in neutrophils from frail older adults; in vitro this can be corrected with a PI3kinase inhibitor suggesting dysregulation of internal cell mechanisms is fundamental. It has also established that an environment of chronic inflammation exists

in frail older adults and this can induce a trend towards a detrimental migratory profile in neutrophils from healthy younger adults.

Previous studies suggest that primed neutrophils produce a response to stimulation which can be up to ten times larger than a quiescent neutrophil. This has been shown to result in improved chemotaxis along with improvements in other neutrophil functions (78, 163, 320). Previous studies have demonstrated: failure to respond to priming in neutrophils from healthy older adults (163, 214) and, a reduction in the production of NETs following priming with 0.5nM TNF α and subsequent stimulation in healthy older adults. Neutrophils from healthy younger adults primed with 1pM TNF α in experiments conducted in an identical manner to the data presented here displayed an improvement in chemotaxis (163) whereas in these data priming neutrophils from healthy younger adults with 1.15nM TNF α had a detrimental effect. Subjective review of the videos suggested this may be due to increased adhesion of the neutrophils to the albumin. The neutrophils from frail older and healthy older adults primed with 1.15nM TNF α demonstrated a non-significant improvement in chemotaxis. It can be postulated this is either an adaptive response to a chronic inflammatory environment or the result of neutrophil immunosenescence associated with increasing age and frailty. Further experiments designed to review the effect of the concentration of priming agents on neutrophils from the three different groups and the relationship with background inflammatory state are necessary to support this hypothesis. The concentration of TNF α in the frail older adults in this study was 2.36pM suggesting the 1.15nM concentration used for priming is not physiological. The concentration of previously reported TNF α in healthy older adults is 0.19pM and 0.21 pM (163, 185). However, this is the systemic

concentration rather than the concentration within the tissue or the concentration during infection or inflammatory response. A significant limitation of the data presented throughout this chapter is that all the validation work for the concentration of chemoattractants, priming and stimulating agents was completed only in healthy younger adults. The data presented in this chapter also suggests a delayed response to priming in frail older adults with maximal response at 30 minutes in healthy younger and healthy older adults and at 45 minutes in frail older adults. The effect on the migratory dynamics of neutrophils primed over an extending time-period has not been investigated before but these data suggests there may be a delay in neutrophil functional response to priming in frail older adults. This supports the hypothesis that there is a lag in intracellular signalling which was also suggested by the phosphorylation of threonine 308 data. However, this does need to be interpreted with caution as the data is mostly not significant.

The number of circulating neutrophils is raised in the frailty state (210, 215) but the mechanisms and characteristics of this relative neutrophilia are unknown. Increased release of neutrophils from the bone marrow in response to an acute inflammation state would result in increased proportion of immature neutrophils but a functional deficit in the clearing mechanism of aged neutrophils would result in a shift in the neutrophil population towards senescent neutrophils. An alternative explanation is the inflammatory environment associated with frailty results in decreased apoptosis of neutrophils and extends their lifespan. The differences demonstrated in the functionality in neutrophils from frail older adults could be explained by the relative neutrophilia and increase in the subpopulations of neutrophils in these individuals. A single study

investigating the characteristics of neutrophils from nursing home residents demonstrated both an increased proportion of immature band neutrophils in frail older adults and an increased frequency of HLA-DR expression in neutrophils from frail older adults, which is associated with tissue infiltrating neutrophils, and the authors suggest this represents an increased proportion of neutrophils undergoing reverse trans-endothelial migration (210).

Section 2.14 demonstrates an increased proportion of CD16 bright CD62L dim neutrophils, reportedly aged neutrophils, in frail older adults compared to both healthy younger and healthy older adults. Three different neutrophil subsets, CD16 bright CD62L bright, CD16 bright CD62L dim and CD16 dim CD62L bright, have been observed in a range of inflammatory conditions including sepsis, cancer and trauma (345). The CD16 bright CD62L dim neutrophils are reported to be functionally different to the phenotypically normal neutrophil (CD16 bright CD62L bright) with increased expression of CD11b, increased production of hydrogen peroxide and inhibition of T cell responses in co-culture (345). CD62L dim neutrophils have a banded hypersegmented nucleus and have been reported to be aged in comparison to the other two subsets. However, recent research has suggested they may be an entirely separate subset recruited in acute inflammation independently of the other two subsets (346, 347). Whilst the increase in this sub-population of neutrophils in frail older adults represents a doubling in the population the actual numbers are small and therefore unlikely to account for the differences in neutrophil function described earlier in the chapter.

Section 2.14 also demonstrates that neutrophils from frail older adults are neither senescent nor activated using a 'traditional' method classifying neutrophil sub-populations developed in chronic inflammatory conditions and trauma (305-308, 348). This is also demonstrated in neutrophils from healthy older adults. Possible explanations for the relative neutrophilia in frail older adults associated with the inflammatory profile include a chronic increase in the release of neutrophils from bone marrow in response to chronic inflammation which whilst resulting in increased immature granulocytes in the circulation would not be detectable by the methods utilised in this study, as the immature granulocytes mature to phenotypically normal neutrophils at the same rate and the proportions of the populations remain the same, this explanation would not explain differences in neutrophil function. An alternative explanation might be that the chronic inflammatory environment results in a constant cycle of priming and then de-priming of the circulating neutrophils resulting in longer lifespans for these neutrophils. Current evidence suggests that re-primed neutrophils are not functionally inferior to their unexposed counterparts (83) but little is known about neutrophils undergoing a perpetual cycle of priming and de-priming.

There is an increase in CD16 expression in the frail older adult group both at baseline and on stimulation compared to the healthy older adults and also a failure to appropriately upregulate CD16 expression on stimulation. CD16 expression has previously been demonstrated to be reduced in healthy older adults but has never been investigated in frail older adults (349). CD16 is acquired by neutrophils as they mature in the bone marrow and in states of neutrophilia associated with acute inflammation there is both reduced expression of CD16 on the cell surface of neutrophils (350-352) and functional

impairment including reduced chemotaxis and an impaired ability to upregulate CD16 on stimulation (353). Whilst this chapter has demonstrated that neutrophils from frail older adults have functional impairment, reduced chemotaxis and impaired ability to upregulate CD16 on stimulation, and previous literature has demonstrated a neutrophilia in frail older adults (204, 210) the increased CD16 expression at baseline in the frail older adults suggests the neutrophilia is not a 'traditional' acute inflammatory response. Taking these data together suggests a polyclonal expansion of neutrophils in frail older adults rather than isolated expansion of one sub-population.

Sections 2.15 and 2.16 report that neutrophils from healthy older adults are functionally activated despite cell surface marker phenotypes that suggest otherwise. Healthy older adults have increased amounts of neutrophil elastase activity compared to healthy younger adults; a finding that replicates results from previous studies (163). However, in the frail older adults there was significantly less neutrophil elastase activity compared to the healthy older adults and the neutrophil elastase activity of frail older adults was no different to that of healthy younger adults. Section 2.11 demonstrated a pro-inflammatory environment in both healthy and frail older adults and therefore a similar level of exposure to circulating activating factors. Possible explanations of this phenomenon are poorer functional ability in the neutrophils from frail older adults to respond to these activating factors or an increased concentration of an unmeasured activating factor in healthy older adults that is not present in frail older adults.

This chapter reports unique data investigating the functional ability of neutrophils from frail older adults. It describes neutrophil migratory inaccuracy towards the end

chemoattractant, fMLP, in healthy older and frail older adults and migratory inaccuracy towards the intermediate chemoattractant, IL-8, in just frail older adults. This detrimental 'frailty' effect on neutrophil migration can be rescued by PI3kinase inhibitors and there was a trend towards inducing this phenotype in neutrophils from healthy young adults with pooled plasma from frail older adults. Migratory inaccuracy in frail older adults is related to the expression of the adhesion molecules CD11a and CD11b and their regulatory cell surface markers CD66b, CD63 and CD62L. An inflammatory environment has been demonstrated in both the healthy and frail older adults which will require exploration and this will be analysed further in Chapter 6. This chapter has also demonstrated that migratory inaccuracy in frail older adults is not related to their oxidative burst. Further work is required investigating the effect of priming on the chemotaxis of frail older adults particularly in a variety of concentrations and agents as well as confirming a dysregulated PI3kinase-Akt pathway in neutrophils from frail older adults with intracellular protein concentration determination.

6. Frailty and Sarcopenia Concordance and Chronicity

6.1 Introduction

The relationship between frailty and sarcopenia is incompletely characterised but these conditions share many of the same clinical outcomes, associations and suggested pathophysiology and are therefore considered to be closely related. There has been an assumption in the literature that because sarcopenia is more prevalent than frailty and, is in itself a component of physical frailty that it can be considered a pre-cursor syndrome. However, a recent isolated report investigating the concordance of frailty and sarcopenia in a geriatric clinic demonstrated both that frailty was more common than sarcopenia and the concordance between frailty and sarcopenia was poor suggesting a more complex relationship than a simple, unidirectional model of progression from sarcopenia into frailty (32).

The underlying pathophysiology for both syndromes is only partially understood and believed to involve a complex dysregulation of multiple systems. Immunesenescence and inflammaging are associated with both syndromes but a causative role in the development or propagation of both syndromes is as yet undefined. Higher levels of pro-inflammatory cytokines can predict a reduction in fat free mass and loss of grip strength but a causative role for inflammaging has yet to be fully established (187, 188). The drivers behind the propagation and progression of each syndrome are unknown but the role of the dysregulated immune system is likely to be fundamental.

This chapter describes a comprehensively characterised frail older adult group and investigates frailty models, screening and diagnostic tests in this population. It investigates the prevalence of sarcopenia and the overlap between frailty and sarcopenia

using different models. It then progresses to consider possible drivers behind the progression of sarcopenia and frailty.

This chapter aims to

- Describe the three different recruited groups in detail
- Describe the differences in prevalence of frailty with the employment of different models
- Consider the effect of different parameters on frailty severity
- Review the value and accuracy of two different frailty screening tests and two different frailty diagnostic tests
- Describe the prevalence of sarcopenia in both a healthy and frail older adult population using two different methods; EWGSOP and IWGS.
- Describe the overlap between frailty and sarcopenia in the healthy and frail older adult groups

Results

6.2 Clinical Characterisation

Individuals were recruited to three different groups: healthy young, healthy older and frail older adults. 40 individuals were recruited to the healthy young and older adult groups and 37 were recruited to the frail older adult group: 2 frail older adults were lost to follow up before they could undergo complete characterisation and 4 frail older adults did not have assessment of muscle size. The clinical characteristics of the three groups are described in Table 6.1 and the details of the frailty assessments described in Table 6.2.

The frail older women were significantly older (85.5 years) than then healthy older women (71.0 years) but the frail older men (77.5 years) were not significantly older than the healthy older men (76.0 years). The difference witnessed in age between the two genders was due to: one, the frail older men being younger than frail older women and two, the healthy older men being older than their female counterparts. When both genders were analysed together there was still a significant difference between the two groups: healthy older adults 71.0 +/- 9.0, frail older adults 84.0 +/- 14.8; Independent samples Mann-Whitney U test $p < 0.001$. (Table 6.1.)

There were significantly more falls, increased co-morbidity and number of medications prescribed in the frail older adults compared to both the healthy younger and older adults. Frail older adults had significantly more cognitive impairment, as described by the Addenbrooks cognitive examination, than healthy younger and older adults: HY F - 96.0, HY M - 96.0, HO F - 93, HO M - 93, FO F - 82, FO M - 70. The Stroop Interference

assessment demonstrated a step-wise progression in cognitive impairment through ageing and frailty in both genders this was significant for all pairwise comparisons apart from healthy older adults to frail older adults: HY F - 58.0, HY - M 58.0, HO - F 44.5, HO - M 50.5, FO - F 38.5, FO M - 43.0. (Table 6.1.)

As discussed previously in chapter 3 the height of the men was constant throughout the groups but the women were shorter with both increasing age and frailty: height expressed in metres HY F - 1.63, HO F - 1.60, FO F - 1.54, HY M - 1.77, HO M - 1.77, FO M - 1.75 . There was no difference in weight for either gender but due to the differences in height in women there was a significant increase in BMI in the frail older women when compared to the healthy younger women: HY F - 22.3, HO F - 24.3, FO F - 25.5, HY M - 26.3, HO M - 23.3, FO M - 23.1. As expected, and previously described in chapter 3, there is a stepwise reduction in BATT measured in mm throughout the groups for both genders: HY F - 58.0, HO F - 39.3, FO F - 25.2, HY M - 79.5, HO M - 47.5, FO M - 26.3. There is a significant reduction in the MUAC for men with age and frailty measured in cm, HY M - 32.5, HO M - 28.0, FO M - 25.5, but no real difference in the MUAC with age of frailty for women, HY F - 26.8, HO F - 28.0, FO F - 25.5. There is an increase in ultrasound echogenicity for both healthy older males and females, HY F - 0.95, HO F - 1.50, HY M - 0.95, HO M - 1.48. There is a non-significant increase in ultrasound echogenicity in frail older women in comparison to healthy older women and no difference in ultrasound echogenicity for frail older men in comparison to healthy older men, FO F - 1.99, FO M - 1.46. (Table 6.1.)

As expected there was increased frailty severity in the frail older adults for both genders for all the frailty assessments (FI, FP, PRISMA 7, CFS, EFS, walk speed). There was no difference in frailty severity between the different genders apart from the PRISMA 7 which had a one point higher average score in males for both healthy younger and older adults. The frailty index demonstrated a significantly graduated response throughout the groups in women but not men: HY F – 0.00, HO F - 0.05, FO F - 0.41, HY M - 0.00, HO M - 0.03, FO M – 0.40. All other frailty assessments (FP, PRISMA 7, CFS, EFS, walk speed) did not differentiate between healthy younger and older adults. This was similar for the short physical performance battery which also did not differentiate between healthy younger and older adults: HY F - 12.0, HY - M 12.0, HO F - 12.0, HO M - 11.0, FO F - 2.0, FO M - 2.0. Of the assessments of physical performance only grip strength demonstrated a graduated response with age and frailty in both men and women: HY F - 30.0, HY M - 50.0, HO F - 23.0, HO M - 38.0, FO F - 12.0, FO M - 26.0. (Table 6.2.)

Table 6.1 – Characteristics of healthy young adults, healthy older adults and frail older adults

	Healthy Young Adults		Healthy Older Adults		Frail Older Adults		Female		Male	
	Female	Male	Female	Male	Female	Male		Pairwise comparisons		Pairwise comparisons
Number	50% (20)	50% (20)	67.5% (27)	32.5% (13)	54.3% (19)	45.7% (16)				
Age (years)	26.0 +/-7.0	28.0 +/- 11.0	71.0 +/- 8.0	76.0 +/- 11.0	85.5 +/- 14.8	77.5 +/- 13.0	p<0.001	HY-HO p<0.001 HY-FO p<0.001 HO-FO p=0.011	p<0.001	HY-HO p<0.001 HY-FO p<0.001 HO-FO p=1.000
Height (m)	1.63 +/- 0.07	1.77 +/- 0.12	1.60 +/- 0.08	1.77 +/- 0.12	1.54 +/- 0.04	1.75 +/- 0.10	p<0.001	HY-HO p=0.033 HY-FO p<0.001 HO-FO p=0.012	p=0.456	
Weight (kg)	61.4 +/- 13.5	81.0 +/- 18.8	62.0 +/- 11.8	73.1 +/- 22.8	59.0 +/- 18.9	69.5 +/- 26.5	p=0.656		p=0.185	
BMI (kg/m²)	22.3 +/- 4.0	26.3 +/- 4.0	24.3 +/- 4.4	23.3 +/- 4.4	25.5 +/- 5.7	23.1 +/- 6.5	p=0.042	HY-HO p=0.225 HY-FO p=0.04 HO-FO p=1.00	p=0.300	
MUAC (cm)	26.8 +/- 4.5	32.5 +/- 3.5	28.0 +/- 3.0	28.0 +/- 4.0	25.5 +/- 6.3	26.5 +/- 4.4	p=0.099		p<0.001	HY-HO p=0.012 HY-FO p=0.001 HO-FO p=1.00
Co-morbidity	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 1.0	0.0 +/- 0.5	2.0 +/- 2.0	1.0 +/- 3.0	p<0.001	HY-HO p=0.308 HY-FO p<0.001 HO-FO p<0.001	p<0.001	HY-HO p=0.653 HY-FO p<0.001 HO-FO p=0.026

	Healthy Young Adults		Healthy Older Adults		Frail Older Adults		Female		Male	
	Female	Male	Female	Male	Female	Male		Pairwise comparisons		Pairwise comparisons
Falls	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 1.0	0.0 +/- 0.5	1.0 +/- 3.0	2.0 +/- 2.8	p<0.001	HY-HO p=0.221 HY-FO p<0.001 HO-FO p=0.004	p<0.001	HY-HO p=0.712 HY-FO p<0.001 HO-FO p=0.004
Medications	0.0 +/- 1.0	0.0 +/- 0.0	1.0 +/- 3.0	1.0 +/- 3.5	7.0 +/- 4.0	6.0 +/- 4.0	p<0.001	HY-HO p=0.457 HY-FO p<0.001 HO-FO p<0.001	p<0.001	HY-HO p=0.084 HY-FO p<0.001 HO-FO p=0.008
Addenbrooks Cognitive Examination	96.0 +/- +/- 5.0	96.0 +/- 8.0	93.0 +/- 5.0	93.0 +/- +/- 6.5	82.0 +/- 28.0	70.0 +/- 20.5	p=0.003	HY-HO p=0.882 HY-FO p=0.003 HO-FO p=0.036	p<0.001	HY-HO p=0.960 HY-FO p<0.001 HO-FO p=0.010
Stroop Interference	58.0 +/- +/- 12.5	58.0 +/- 9.0	44.5 +/- 9.3	50.5 +/- +/- 10.8	38.5 +/- 9.3	43.0 +/- 0.0	p<0.001	HY-HO p<0.001 HY-FO p<0.001 HO-FO p=0.481	p<0.001	HY-HO p=0.040 HY-FO p=0.072 HO-FO p=0.949
BATT (mm)	58.0 (53.0- 63.0)	79.5 (72.6- 86.4)	39.3 (36.5-42.1)	47.5 (41.6- 53.5)	25.2 (19.0- 31.4)	26.3 (18.4- 34.2)	p<0.001	HY-HO p<0.001 HY-FO p<0.001 HO-FO p<0.001	p<0.001	HY-HO p<0.001 HY-FO p<0.001 HO-FO p<0.001
Ultrasound echogenicity	0.95 +/- +/- 0.39	0.95 +/- 0.25	1.50 +/- 0.68	1.48 +/- +/- 0.48	1.99 +/- 0.73	1.46 +/- 0.98	p<0.001	HY-HO p<0.001 HY-FO p<0.001 HO-FO p=0.554	p<0.001	HY-HO p<0.001 HY-FO p=0.001 HO-FO p=1.000
Subcutaneous tissue : BATT ratio	2.8 +/- 3.1	11.4 +/- 10.1	1.7 +/- 1.2	5.8 +/- 5.8	1.4 +/- 1.1	4.7 +/- 5.7	p=0.001	HY-HO p=0.002 HY-FO p=0.003 HO-FO p=1.000	p=0.078	

Table describing the different characteristics of the three different groups: healthy young adults, healthy older adults and frail older adults.

Co-morbidity = number of chronic conditions (stroke, myocardial infarction, congestive cardiac failure, diabetes mellitus, chronic obstructive pulmonary disease, dementia, osteoporosis, fragility fracture, cancer and atrial fibrillation). Falls = number of falls in the last year. Medications = number of prescribed regular medications. BATT = bilateral anterior thigh thickness (as described in Chapter 2 and 3). Ultrasound echogenicity = average measure of echogenicity of rectus femoris bilaterally.

N= HY – 40, HO – 40, FO – 35. Incomplete data sets for: Addenbrooks cognitive examination – HY – 40, HO – 40, FO – 29, Stroop Interference – HY – 39, HO – 38, FO – 8. BATT – HY – 40, HO – 40, FO – 31, Ultrasound echogenicity – HY – 26, HO – 33, FO – 16. All data was not normally distributed except BATT which was normally distributed for each group and gender. Data given is median +/- IQR except BATT which is mean with confidence intervals. Statistical tests given are Independent Kruskal-Wallis with Bonferroni corrected pairwise comparisons except BATT which is ANOVA with post-hoc Tukeys. All p values in bold are significant ($p < 0.05$).

There are no differences in the reported data between the genders for each group except in HY – height $p < 0.001$, weight $p < 0.001$, BMI $p = 0.013$, MUAC $p < 0.001$, BATT $p < 0.001$ in HO – stroop interference = 0.033, height $p < 0.001$, weight $p < 0.001$ and BATT $p = 0.004$ and FO – height $p < 0.001$, weight $p = 0.048$. Mann-Whitney U test for not normally distributed data and independent t-test for normally distributed (BATT).

Table 6.2 – Frailty descriptors in healthy young adults, healthy older adults and frail older adults

	Healthy Young Adults		Healthy Older Adults		Frail Older Adults		Females		Males	
	Female	Male	Female	Male	Female	Male	I K-W	Pairwise comparisons	I K-W	Pairwise comparisons
Frailty Index	0.00 +/- 0.02	0.02 +/- 0.02	0.05 +/- 0.07	0.03 +/- 0.06	0.41 +/- 0.15	0.40 +/- 0.19	p<0.001	HY-HO p=0.006 HY-FO p<0.001 HO-FO p<0.001	p<0.001	HY-HO p=0.761 HY-FO p<0.001 HO-FO p<0.001
Frailty Phenotype	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0	3.0 +/- 1.0	3.0 +/- 1.0	p<0.001	HY-HO p=1.000 HY-FO p<0.001 HO-FO p<0.001	p<0.001	HY-HO p=1.000 HY-FO p<0.001 HO-FO p<0.001
PRISMA 7	1.0 +/- 0.8	2.0 +/- 0.0	1.0 +/- 0.0	2.0 +/- 0.0	5.0 +/- 2.0	5.0 +/- 2.0	p<0.001	HY-HO p=0.510 HY-FO p<0.001 HO-FO p<0.001	p<0.001	HY-HO p=1.000 HY-FO p<0.001 HO-FO p<0.001
Clinical Frail Scale	1.0 +/- 1.0	1.0 +/- 0.0	2.0 +/- 2.0	1.0 +/- 2.0	6.0 +/- 0.0	6.0 +/- 1.0	p<0.001	HY-HO p=0.087 HY-FO p<0.001 HO-FO p<0.001	p<0.001	HY-HO p=0.483 HY-FO p<0.001 HO-FO p<0.001
Edmonton Frail Scale	0.0 +/- 1.8	0.0 +/- 1.0	2.0 +/- 2.0	1.0 +/- 2.0	8.0 +/- 3.0	9.0 +/- 4.3	p<0.001	HY-HO p=0.145 HY-FO p<0.001 HO-FO p<0.001	p<0.001	HY-HO p=1.000 HY-FO p<0.001 HO-FO p<0.001
Walk Speed (m/s)	1.40 +/- 0.00	1.68 +/- 0.00	1.22 +/- 0.00	1.41 +/- 0.00	0.33 +/- 0.00	0.32 +/- 0.00	p<0.001	HY-HO p=0.079 HY-FO p<0.001 HO-FO p<0.001	p<0.001	HY-HO p=0.166 HY-FO p<0.001 HO-FO p=0.005
Grip Strength (kg)	30.0 +/- 11.0	50 +/- 11.0	23.0 +/- 8.0	38.0 +/- 9.0	12.0 +/- 8.0	26.0 +/- 9.0	p<0.001	HY-HO p<0.001 HY-FO p=0.015 HO-FO p<0.001	p<0.001	HY-HO p=0.006 HY-FO p<0.001 HO-FO p=0.014

	Healthy Young Adults		Healthy Older Adults		Frail Older Adults		Females		Males	
	Female	Male	Female	Male	Female	Male	I K-W	Pairwise comparisons	I K-W	Pairwise comparisons
Short physical performance battery	12.0 +/- 0.0	12.0 +/- 0.0	12.0 +/- 2.0	11.0 +/- 1.5	2.0 +/- 4.0	2.0 +/- 2.8	p<0.001	HY-HO p=0.473 HY-FO p<0.001 HO-FO p<0.001	p<0.001	HY-HO p=0.109 HY-FO p<0.001 HO-FO p=0.001

Table describing the different frailty characteristics of the three different groups: healthy young adults, healthy older adults and frail older adults.

N= HY – 40, HO – 40, FO – 35. Data given is median +/- IQR. All data sets not normally distributed. Statistical tests given are Independent Kruskal-Wallis with Bonferroni corrected pairwise comparisons. Statistically significant p values are in bold (p<0.05).

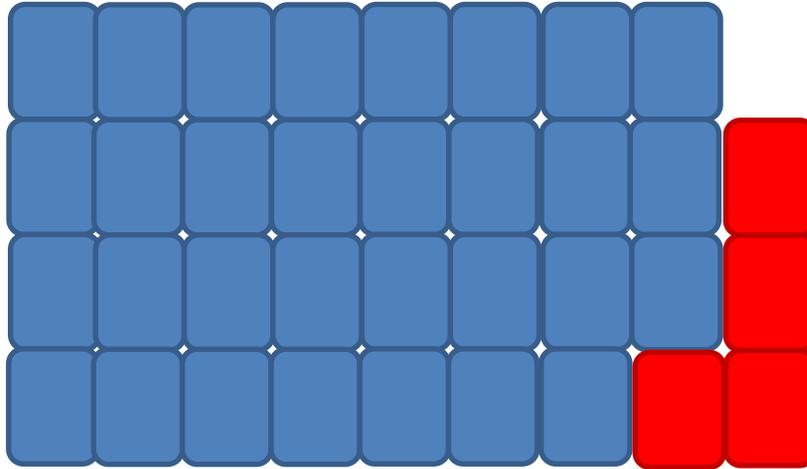
There are no differences in the reported data between the genders for each group except in grip strength for all three groups p<0.001 and PRISMA 7 for HY p<0.001 and HO p=0.002. Mann-Whitney U test.

6.3 Frailty Index identifies a higher prevalence of frailty than Frailty Phenotype

35 individuals recruited to the frail group underwent a complete assessment of frailty; all 35 were identified as frail using the frailty index. 88.6% (31 individuals) of the frailty index positive individuals were also identified as frail using the frailty phenotype. The frailty index positive but frailty phenotype negative individuals, 11.4% (4 individuals), were all classified as pre-frail using the frailty phenotype. (Figure 6.1.)

No individuals in the healthy older adult group were identified as frail using either the frailty index or frailty phenotype. 15% (6) of the healthy older adult group were positive in a single frailty phenotype criterion: 10% (4) had a grip strength indicative of frailty, these individuals were all women, 2.5% (1) reported exhaustion and 2.5% (1) had weight loss indicative of frailty.

Figure 6.1 – Prevalence of frailty identified by Frailty Index and Frailty Phenotype



Prevalence of frailty identified by Frailty Index and Frailty Phenotype. Each individual is represented by a block. Frailty Index a composite measure of 30 different characteristics using a continuous scale representing a variety of different biopsychosocial domains; a score greater than 0.2 diagnostic of frailty. Frailty Phenotype a categorical definition of frailty; greater than or equal to three diagnostic of frailty.

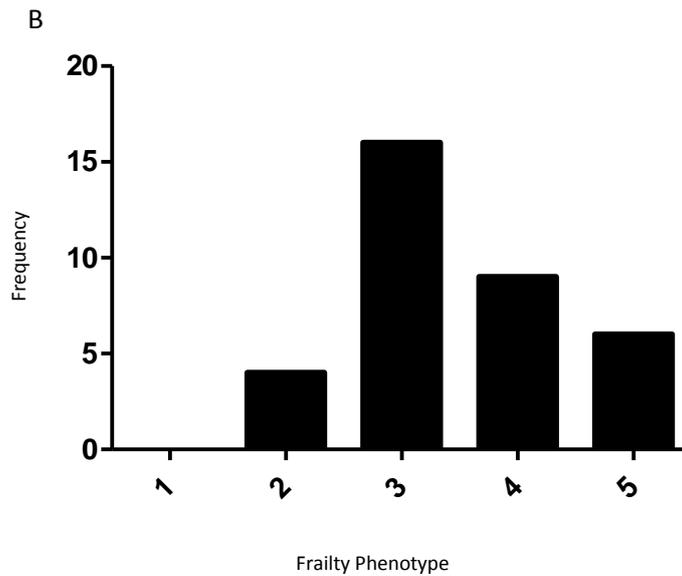
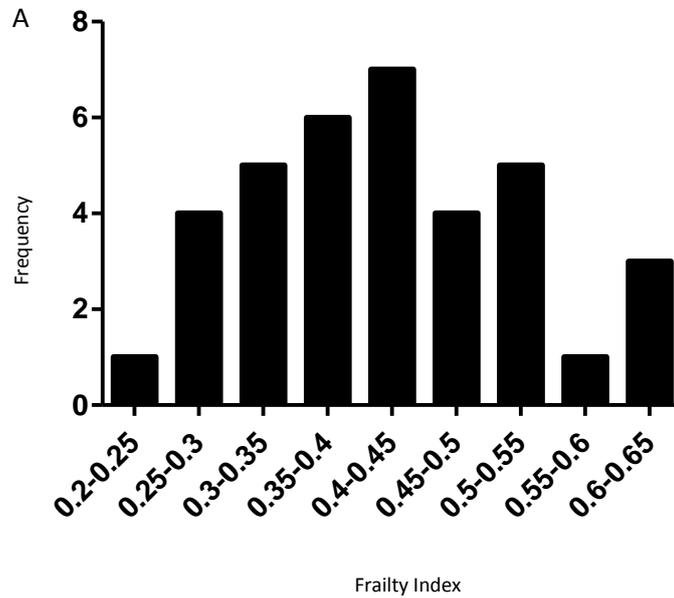
37 individuals recruited to frailty group. 35 individuals identified as frail using both frailty index and frailty phenotype (blue). 4 individuals identified as frail with Frailty Index but were not frail using Frailty Phenotype (red). 2 individuals lost to follow up.

6.4 Frailty severity measured by Frailty Phenotype and Frailty Index is comparable

The distribution of frailty severity was different for each frailty identification model; less severe frailty was more common in the frailty phenotype model but the complete spectrum of frailty was relatively equally represented with the frailty index model apart from the least frail (FI skewness = 0.494, FI kurtosis = -0.319, FP skewness = 0.285, FP kurtosis = -0.703). Frailty phenotype identification of frailty: 11.4% positive in two criteria, 45.7% positive in three criteria, 25.7% positive in four criteria and 17.1% positive in all five criteria. Frailty as described by the frailty index was more representative of a complete spectrum of frailty peaking at a frailty index of 0.4-0.45. (Figure 6.2.)

Frailty severity as described by both models is comparable. There are significant increases in the frailty index with increasing positive criteria for the frailty phenotype; this occurred in a stepwise fashion: positive in two frailty phenotype (FP) criteria frailty index (FI) 0.36, positive in three FP criteria FI 0.39, positive in four FP criteria FI 0.46 and positive in all five FP criteria FI 0.51. Correlation of severity as measured by the two frailty models was high: $R=0.885$, $p<0.001$, Spearman's correlation coefficient. (Figure 6.3.)

Figure 6.2 – Distribution of frailty severity

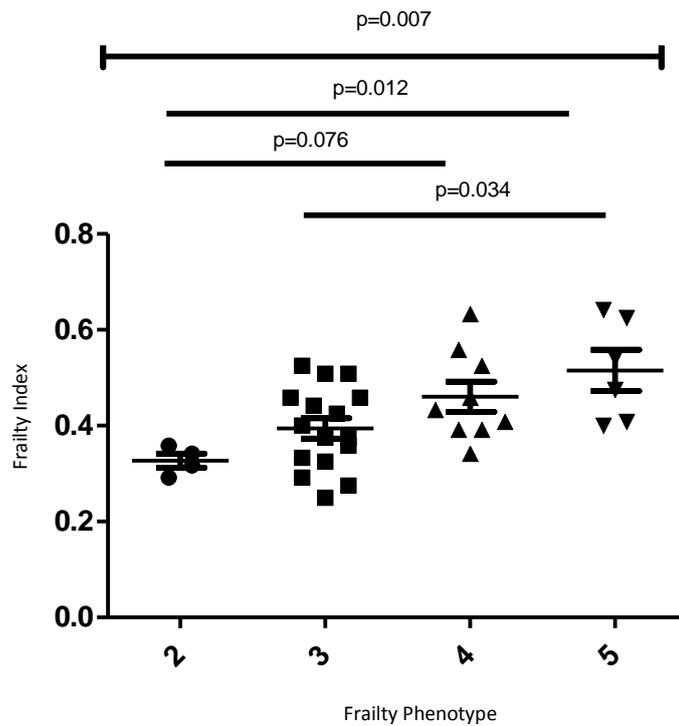


Distribution of frailty severity in frail older adults. A - Frailty Index and B - Frailty Phenotype.

A – Frailty Index a composite measure of 30 different characteristics using a continuous scale representing a variety of different biopsychosocial domains; a score greater than 0.2 diagnostic of frailty.

B - Frailty Phenotype a categorical definition of frailty; two diagnostic of pre-frailty. Greater than or equal to three diagnostic of frailty. Two is diagnostic of pre frailty.

Figure 6.3 – Comparison of frailty severity measured by the Frailty Phenotype and the Frailty Index in frail older adults



Comparison of frailty severity measured by the Frailty Phenotype on the x axis and Frailty Index on the y axis in frail older adults. Frailty phenotype a categorical definition of frailty; number of positive Fried's criteria. Frailty Index a composite measure of 30 different characteristics using a continuous scale; a score greater than 0.2 diagnostic of frailty. N=35. Data shown is mean +/- SEM. Data normally distributed.

Frailty Index for Frailty Phenotype 2 – 0.33, Frailty Phenotype 3 – 0.39, Frailty Phenotype 4 – 0.46, Frailty Phenotype 5 – 0.51.

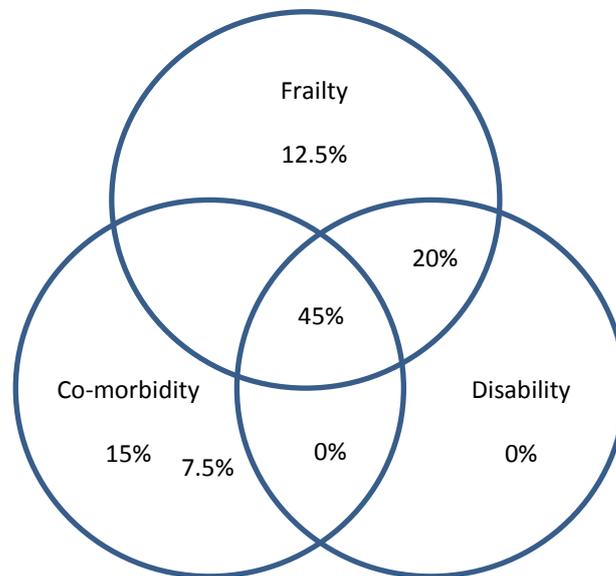
ANOVA p=0.007. Post hoc-Tukeys 2-3 p=0.528, 2-4 p=0.076, 2-5 p=0.012, 3-4 p=0.292, 3-5 p=0.034, 4-5 p=0.637.

6.5 Frailty is a state separate to disability and co-morbidity

Frailty is a separate entity to disability and co-morbidity however; there is considerable overlap between the three states of health. It is possible to exist in a state of frailty without being either disabled or co-morbid. In the study population, 12.5% were defined as just frail, 15% had co-morbidity, 7.5% had both frailty and co-morbidity, 20% were frail and disabled and 45% were frail, disabled and had co-morbidity. (Figure 6.4.)

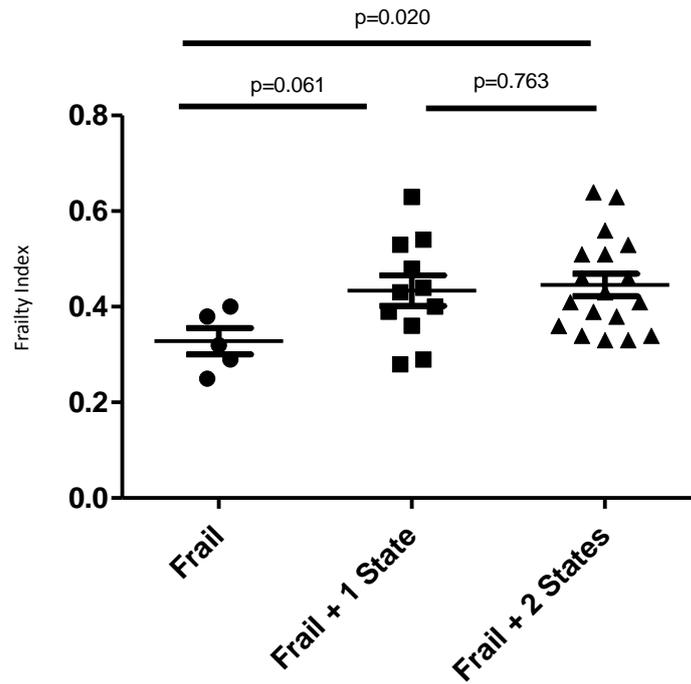
The addition of either a disabled or co-morbid state to a frailty state appeared to increase the frailty severity as measured by the frailty index: FI for frail state – 0.33, FI for frail plus one other state (either disability or co-morbidity) – 0.43 ($p=0.061$). There is a significant increase in frailty severity with the combination of all the three states: FI for frail state – 0.33, FI for frail state plus two states (disability and co-morbidity) – 0.45. This suggests that the addition of different health states to frailty may not have a cumulative effect and a saturation point may be reached. (Figure 6.5.)

Figure 6.4 – Overlap and prevalence of disability, frailty and co-morbidity in healthy and frail older adults



Overlap and prevalence of disability, frailty and co-morbidity in healthy and frail older adults. Venn diagram depicting the frequency of each health state in both healthy and frail older adults. N = 40 (35 frail older adults and 5 healthy older adults). 35 healthy older adults were neither frail, disabled nor possessed a co-morbidity and are not included in the Venn diagram. Frailty defined as positive frailty index (>0.2), disabled defined as help required with greater than one basic activity of daily living (bathing, dressing, toileting, transferring, feeding), co-morbidity defined as a past medical history of chronic disease (stroke, myocardial infarction, congestive cardiac failure, diabetes mellitus, chronic obstructive pulmonary disease, dementia, osteoporosis, fragility fracture, cancer and atrial fibrillation).

Figure 6.5 – Frailty severity with addition of different health states in frail older adults



Frailty severity with addition of different health states in frail older adults. X axis – health state: Frail = positive Frailty Index, Frail + 1 state = positive Frailty Index and either disabled or co-morbid, Frail + 2 states = positive Frailty Index and disabled and co-morbid. Y axis – Frailty Index. N = 35. Data normally distributed. Data shown is mean +/- SEM.

Frailty Index for Frail – 0.33, Frailty Index for Frail + 1 state – 0.43, Frailty Index + 2 states – 0.45.

T-test; Frail - Frail + 1 state $p=0.061$, Frail – Frail + 2 states $p=0.020$, Frail + 1 state – Frail + 2 states $p = 0.763$

6.6 The addition of sarcopenia to frailty increases frailty severity

This chapter has so far demonstrated that the Frailty Index and Frailty Phenotype models identify different populations of frailty and whilst the distribution of frailty severity is different for each model it is related; increasing frailty in one model reflected in the second model. It has also demonstrated that frailty is a separate health state to disability and co-morbidity but the addition of just one of these states to frailty has significant implications on frailty severity.

To consider whether the frailty phenotype criteria are equally weighted in their discriminative ability each criterion was analysed separately. The most frequently positive criteria were walk speed (91.4%), physical activity (82.9%) and grip strength (85.7%). Weight loss (positive in 40%) and exhaustion (positive in 48.6%) were equally likely to be positive or negative. Taken with the data already presented in this chapter which describes increasing risk of adverse outcomes (as identified by the Frailty Index) with each additional positive Frailty Phenotype criterion this suggests that weight loss and exhaustion may be discriminative for increased frailty severity. However, the average Frailty Index for an individual positive for each Frailty Phenotype criterion suggests that exhaustion and, in particular, weight loss are not discriminative of adverse outcomes: FI of exhaustion positive 0.45, FI of exhaustion negative 0.40 $p=0.084$, FI of weight loss positive 0.44, FI of weight loss negative 0.42 $p=0.539$. Physical activity and grip strength demonstrate more of a discriminative effect as dichotomous criteria: FI of physical activity positive 0.44, FI of physical activity negative 0.35 $p=0.057$, FI of grip strength positive 0.44, FI of grip strength negative 0.33 $p=0.016$. Grip strength is integral to the diagnosis of sarcopenia and low physical activity is both a direct consequence of

sarcopenia and contributor to loss of muscle mass. This suggests that the addition of sarcopenia to a diagnosis of frailty would increase frailty severity and results in worse outcomes. (Table 6.3.)

Table 6.3 – Frequency and severity of frailty for each Frailty Phenotype criterion

Frailty Phenotype Criterion				
		Negative	Positive	
Walk Speed	Number	8.6% (3)	91.4% (32)	
	Frailty Index	0.37 (0.29-0.44)	0.43 (0.39-0.47)	p=0.312
Weight Loss	Number	60.0% (21)	40.0% (14)	
	Frailty Index	0.42 (0.37-0.46)	0.44 (0.38-0.51)	p=0.539
Exhaustion	Number	51.4% (18)	48.6% (17)	
	Frailty Index	0.40 (0.36-0.44)	0.45 (0.40-0.48)	p=0.084
Physical Activity	Number	17.1% (6)	82.9% (29)	
	Frailty Index	0.35 (0.29-0.42)	0.44 (0.40-0.48)	p=0.057
Grip Strength	Number	14.3% (5)	85.7% (30)	
	Frailty Index	0.33 (0.26-0.39)	0.44 (0.40-0.48)	p=0.016

Frailty Index and frequency of each Frailty Phenotype criterion. N=35 for each criterion. Number is frequency as percentage of total with actual number in brackets. Frailty Index is mean with confidence intervals. All normally distributed. Independent t-tests. Statistically significant p values are in bold (p<0.05).

6.7 Value and accuracy of identification tools of frailty

Diagnostic screening tests are valuable tools in identifying complex clinical syndromes such as frailty but are only suitable for clinical use if the screening tool is accurate.

Figure 6.6 and Table 6.4 demonstrate that PRISMA 7 in this population is a more sensitive test than walk speed but walk speed is a more specific test when comparing both screening tests to the gold standard of frailty index. PRISMA 7 - 100% sensitivity, 95% specificity, positive predictive value 94.6%, and negative predictive value 100%. Walk

speed - 94.3% sensitivity, 100% specificity, positive predictive value 100%, and negative predictive value 95.3%. Combining the two tests together and considering it positive for frailty if both tests are positive does not improve the predictive value. PRISMA 7 and walk speed – positive predictive value 100%, negative predictive value 95.3%. Due to the methods of recruitment it is not possible to consider the effect of combining the methods and taking either result. The study design stipulated that only participants with either a positive PRISMA 7 or walk test could be included in the frail older adult group therefore it is impossible to determine whether accuracy is increased combining the tests.

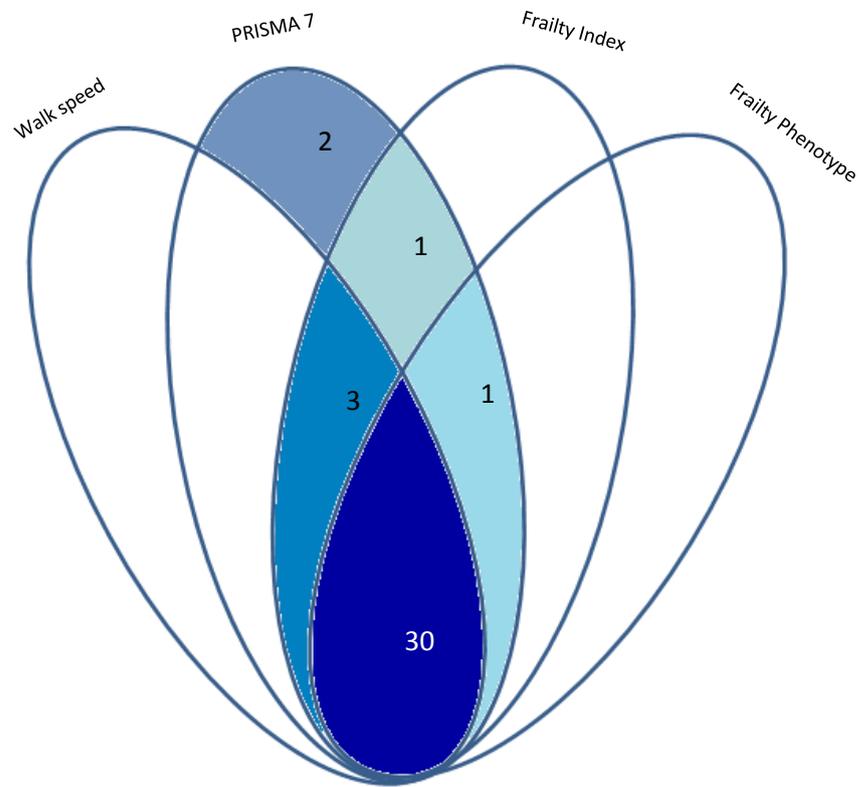
PRISMA 7 is in concept more aligned with the Frailty Index, a composite measure of multiple biopsychosocial domains, whereas walk speed is closer in concept to Frailty Phenotype, a descriptive measure of physical health. However, figure 6.6 demonstrates that both screening tests are non-specific and can identify individuals described as frail using either model. Walk speed identified three individuals who were described as frail by the Frailty Index but not the Frailty Phenotype. This suggests that the tests are applicable regardless of the frailty model being employed.

Table 6.4 – Accuracy of PRISMA 7 and walk speed individually and combined compared to Frailty Index

		Frailty Index Positive	Frailty Index Negative
PRISMA 7	Positive	35	2
	Negative	0	38
Walk Speed	Positive	33	0
	Negative	2	40
PRISMA 7 and Walk Speed	Positive	33	0
	Negative	2	40

The accuracy of screening tests compared to the frailty index. Screening test horizontally in rows and the frailty index in columns. N= 75 (40 HY and 35 FO). PRISMA 7 and Walk Speed: positive if positive for both screening tests, negative if negative in either screening test.

Figure 6.6 – Accuracy of the frailty identification methods: walk speed and PRISMA 7



Prevalence and accuracy of frailty identified by the screening tests: walk speed and PRISMA 7 in healthy and frail older adults. 37 individuals recruited to frailty group complete data available on 35. Each ellipse represents those individuals positive for either that frailty model or screening test as identified above the ellipse. Where the ellipses overlap the individual is positive for all those overlapping criteria.

30 individuals identified as frail using walk speed, PRISMA 7 frailty index and frailty phenotype (dark blue). 3 individuals identified as frail with walk speed, PRISMA 7 and frailty index but not frailty phenotype (mid-blue). 1 individual identified as frail using PRISMA 7, frailty phenotype and frailty index but not walk speed (light blue). 1 individual identified as frail using PRISMA 7 and frailty index but not walk speed and frailty phenotype (green). 2 individuals identified as frail using PRISMA 7 but not walk speed, frailty index or frailty phenotype (air force blue). 38 healthy older adults negative for both screening tests.

The Frailty Index and Frailty Phenotype are both perceived as being time consuming diagnostic models which are impractical for use in clinical practice. In an attempt to reduce the time required for clinical assessment shorter tests have been developed that include an assessment of severity; these are conceptually similar to the Frailty Index and take a biopsychosocial multi-domain approach to frailty diagnosis. Two tests were considered: the Clinical Frailty Scale a clinical diagnosis of frailty severity following comprehensive geriatric assessment and, the Edmonton Frail Scale a short questionnaire which can be administered by anyone. A potential advantage of the EFS and CFS over PRISMA 7 and walk speed is they characterise frailty into severity which can allow improved prognostication.

In this population the Edmonton frail scale and Clinical frailty scale were less accurate than PRISMA 7 and walk speed at identifying frailty compared to the gold standard of the frailty index. Edmonton frail scale: 73.5% sensitivity, 100% specificity, positive predictive value 100%, negative predictive value 81.63%. Clinical frailty scale: 91.4% sensitivity, 97.5% specificity, positive predictive value 97%, negative predictive value 92.9%. (Table 6.5.)

Whilst these tests offer the ability to score frailty severity and there is a step-wise increase in frailty severity, as measured by the frailty index, with progression through frailty group in both Edmonton frail scale and Clinical frailty scale there is a huge degree of variability in the frailty index severity. Figure 6.7 demonstrates that a number of individuals scored as moderate severity using the Clinical Frailty Scale have lower frailty index than individuals scored as vulnerable. The same is also true with the Edmonton

Frail Scale where some individuals scored as vulnerable have frailty indexes that are greater than those who are severely frail. However, the overall correlation remains strong for both diagnostic tests: FI and CFS, $R = 0.868$, $p < 0.001$; FI and EFS, $R = 0.841$, $p < 0.001$.

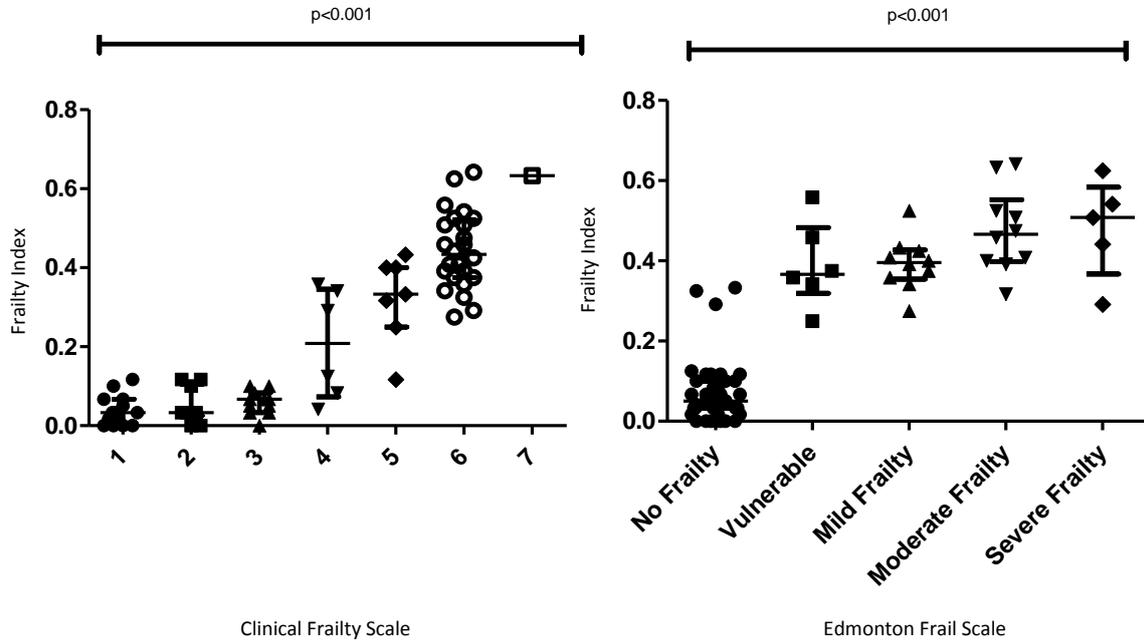
This data taken together suggests the Edmonton frail scale and Clinical frailty scale may not be as accurate as PRISMA 7 and walk speed in identifying frail individuals and the supposed advantage they confer of offering severity rating may be inaccurate compared to the frailty index.

Table 6.5 – Accuracy of Clinical Frailty Scale and Edmonton Frail Scale in comparison to Frailty Index

		Frailty Index Positive	Frailty Index negative
Clinical Frail Scale	Positive	32	1
	Negative	3	39
Edmonton Frail Scale	Positive	25	0
	Negative	9	40

The accuracy of tests compared to the frailty index. Diagnostic test horizontally in rows and the frailty index in columns. N= 75 (40 HY and 35 FO).

Figure 6.7– Comparison of frailty severity as defined by Frailty Index and Clinical Frailty Scale and Edmonton Frail Scale



A - Frailty severity as measured by clinical frailty scale and frailty index in healthy and frail older adults. Clinical frailty scale a diagnostic test of frailty severity based on comprehensive geriatric assessment. X axis – Clinical frailty scale. Y axis – Frailty Index.

B - Frailty severity as measured by Edmonton Frail Scale and frailty index in frail older adults. Edmonton frail scale a diagnostic test of frailty severity based on a biopsychosocial questionnaire. X axis – Edmonton frail scale. Y axis – Frailty Index.

Frailty Index a composite measure of 30 different characteristics representing a variety of biopsychosocial domains using a continuous scale; a score greater than 0.2 diagnostic of frailty. CFS - N=75. Data shown is mean +/- SEM. Data normally distributed. 1 - 0.03 +/- 0.07, 2 - 0.03 +/- 0.08 3 - 0.07 +/- 0.05, 4 - 0.21 +/- 0.27, 5 - 0.33 +/- 0.15, 6 - 0.43 +/- 0.15, 7 - 0.63. ANOVA - P<0.001. Unable to run post-hoc Tukey's test due to the low frequency in some CFS categories.

Edmonton Frail Scale - not frail 0-4, vulnerable 5-6, mild frailty 7-8, moderate frailty 9-10, severe frailty >11. N=75. Data shown is median +/- IQR. Data normally distributed except not frail: not frail - 0.05 +/- 0.07, vulnerable - 0.37 +/- 0.16, mild frailty - 0.40 +/- 0.07, moderate frailty - 0.47 +/- 0.15, severe frailty - 0.51 +/- 0.22. Statistical test Independent Kruskal-Wallis with Bonerroni corrected pairwise comparisons. P < 0.001, No frail to vulnerable p = 0.006, no frail to mild frailty p < 0.001, no frail to moderate frailty p < 0.001, no frail to severe frailty p = 0.001. All remaining pairwise comparisons p = 1.000.

6.9 Prevalence of sarcopenia

The prevalence of sarcopenia using the EWGSOP method of defining the diagnosis in the healthy older adult population was 2.5% (1). 52.5% (21) healthy older adults had low muscle mass and 5% (2) had low grip strength but adequate muscle mass. The remaining 40% (16) had adequate muscle mass, strength and physical performance. Considering the different genders 76.9% (10) of the healthy older men had low muscle mass and 23.1% (3) had adequate muscle mass, strength and physical performance whereas the healthy older women had a greater proportion with adequate muscle mass, strength and physical performance, 48.1% (13) and also with sarcopenia, 3.7% (1). (Figure 6.8.)

In the frail older adult population there was an increased prevalence of sarcopenia using the EWGSOP method, 90.3% (28), 80.6% (25) were categorised as severe sarcopenia and 9.7% (3) as just sarcopenia. Interestingly two individuals had both poor physical performance and low grip strength but adequate muscle mass; this diagnosis is not recognised by either the EWGSOP or IWGS method of classification of sarcopenia. When considering the different genders all the women had an element of muscle strength impairment whereas 21.4% (3) men had adequate muscle strength; 14.3% (2) of these men had poor physical performance in addition to low muscle mass and 7.1% (1) had only low muscle mass. (Figure 6.9.)

This suggests that healthy older women are more likely to have sarcopenia than healthy older men but the healthy older men are more likely to have low muscle mass than healthy older women. In frail older adults there is a similar prevalence of sarcopenia in men and women. There are however, individuals with preserved muscle mass despite

poor physical performance and low muscle strength but the majority of individuals have low muscle mass.

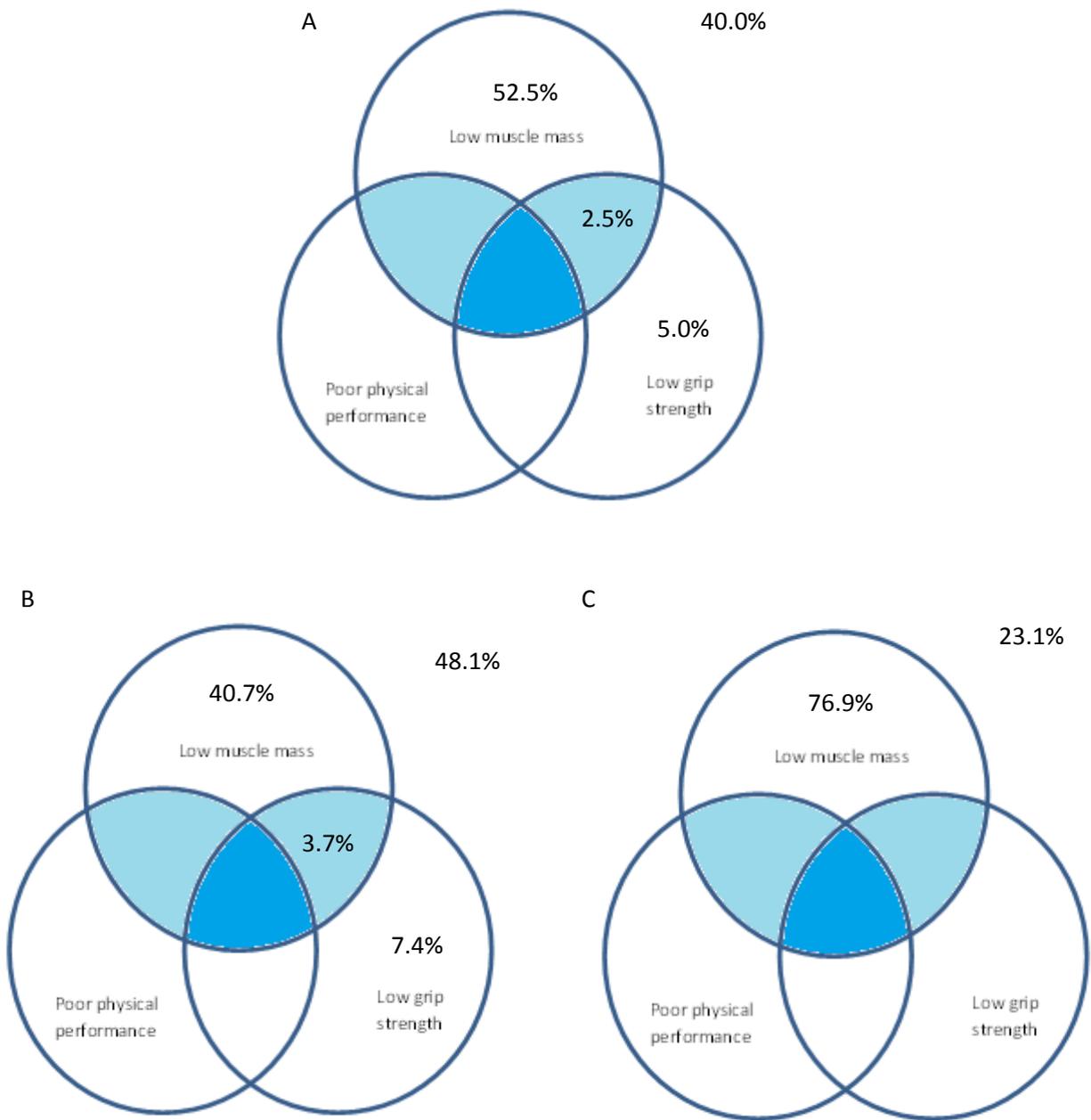
The IWGS method of diagnosing sarcopenia demonstrates an increased prevalence of sarcopenia in all of the measured populations. This is not however significant and there is a good strength of association between the two diagnostic methods. $\chi^2(1)=50.4$, $p<0.001$. Phi =0.848, $p<0.001$.

Table 6.6 – Prevalence of sarcopenia using IWGS and EWGSOP criteria

			Sarcopenia by EWGSOP	Sarcopenia by IWGS
HO	All	40	2.5% (1)	12.5% (5)
	Women	27	3.7% (1)	14.8% (4)
	Men	13	0% (0)	7.7% (1)
FO	All	31	90.3% (28)	96.8% (30)
	Women	17	88.3% (15)	100% (17)
	Men	14	92.9% (13)	92.9% (13)

Prevalence of sarcopenia using IWGS and EWGSOP criteria. EWGSOP – BATT < 2.5 standard deviations healthy younger population and either hand grip strength < 20kg for women or < 30kg for men or walk speed <0.8 m/sec. IWGS – BATT < 20% healthy younger population and walk speed < 1.0 m/sec.

Figure 6.8 – Prevalence of the components of sarcopenia in healthy older adults



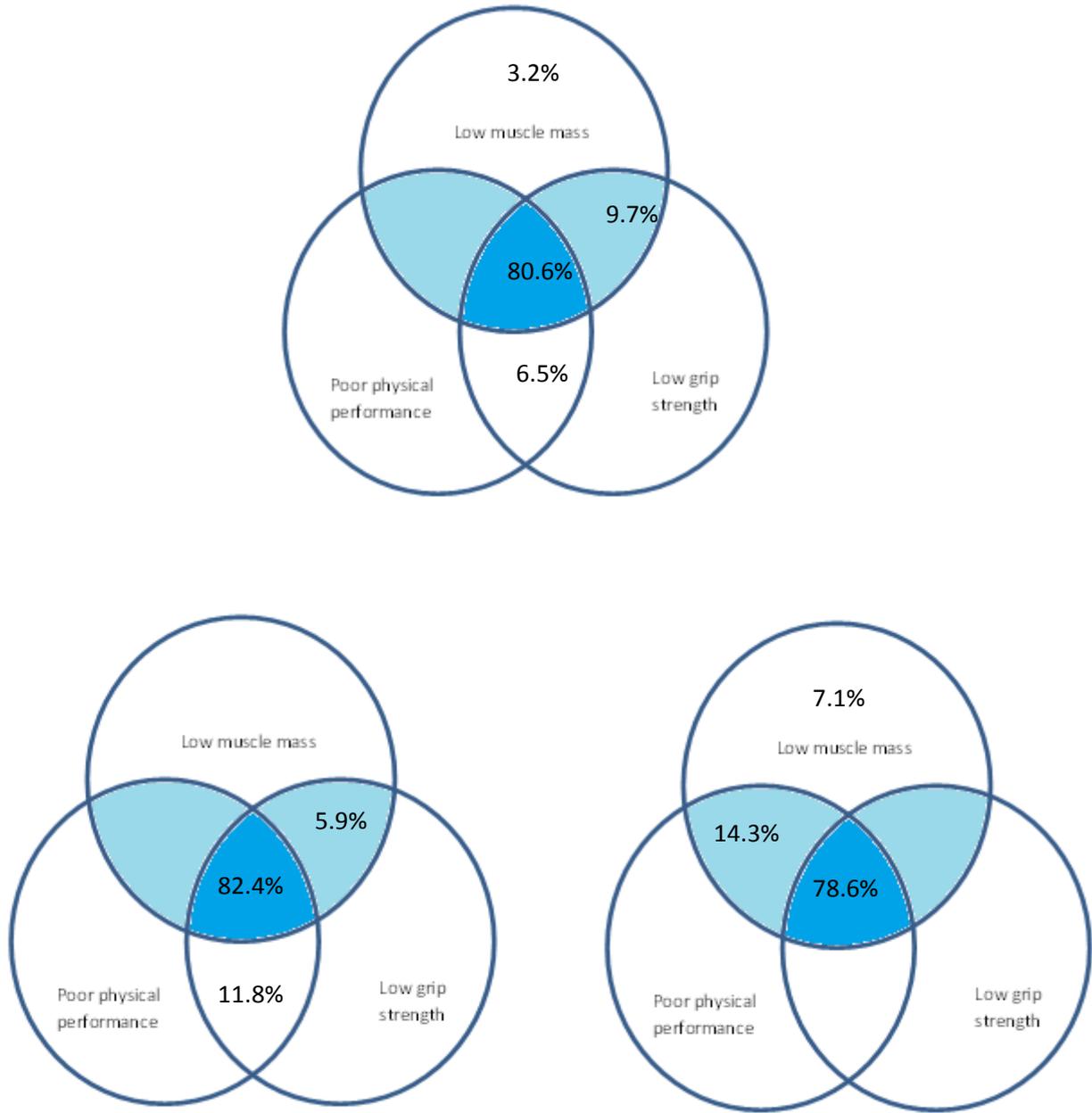
Venn diagram depicting the prevalence of sarcopenia using the EWGSOP criteria in healthy older adults in A – all healthy older adults, B – healthy older women, C – healthy older men. Low muscle mass is defined as less than two standard deviations of muscle mass in healthy younger adults – BATT < 38.55mm in women and BATT < 54.36mm in men. Low grip strength < 20kg in women and < 30kg in men. Poor physical performance < 0.8m/sec. Green area represents sarcopenia. Blue area represents severe sarcopenia.

A - N = 40. 16 adequate muscle mass, muscle strength and physical performance. 21 low muscle mass. 1 sarcopenia, 2 low grip strength with adequate muscle mass.

B - N = 27. 13 adequate muscle mass, muscle strength and physical performance. 11 low muscle mass. 1 sarcopenia. 2 low grip strength with adequate muscle mass.

C - N = 13. 3 adequate muscle mass, muscle strength and physical performance. 10 low muscle mass.

Figure 6.9 - Prevalence of the components of sarcopenia in frail older adults



Venn diagram depicting the prevalence of sarcopenia using the EWGSOP criteria in frail older adults in A – all frail older adults, B – frail older women, C – frail older men. Low muscle mass is defined as less than two standard deviations of muscle mass in healthy younger adults – BATT < 38.55mm in women and BATT < 54.36mm in men. Low grip strength < 20kg in women and < 30kg in men. Poor physical performance < 0.8m/sec. Green area represents sarcopenia. Blue area represents severe sarcopenia.

A - N = 31. 1 low muscle mass. 3 sarcopenia, 2 low grip strength and poor physical performance, 25 severe sarcopenia.

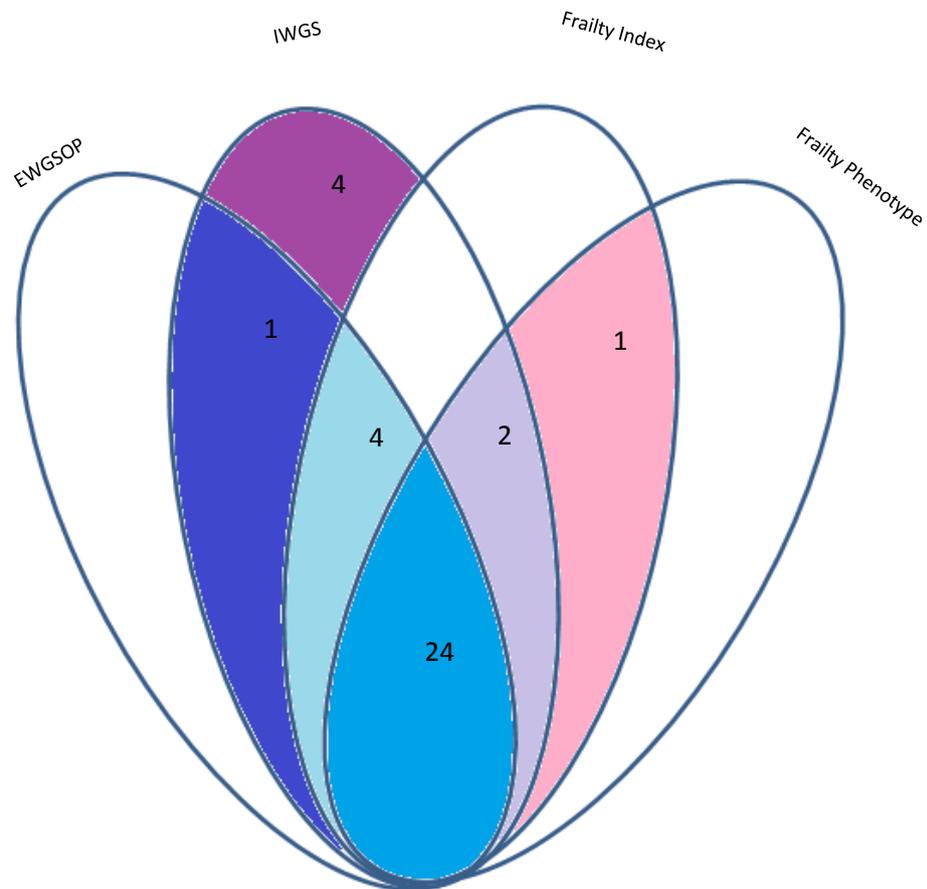
B - N = 17. 1 sarcopenia. 2 low grip strength and poor physical performance, 14 severe sarcopenia.

C - N = 14. 1 low muscle mass, 2 sarcopenia, 11 severe sarcopenia

6.10 Overlap of frailty and sarcopenia

In the frail older adult population there was a high degree of overlap between sarcopenia and frailty measured by the four different approaches, frailty index, frailty phenotype, EWGSOP and IWGS. There was a higher diagnostic rate of sarcopenia using the IWGS approach but despite this there was still a single individual with frailty who did not have sarcopenia by any definition. This suggests that in this population it was possible to have frailty without sarcopenia. However, the likelihood was low. The data from healthy older adults also suggests it is possible to have sarcopenia without frailty. (Figure 6.10.)

Figure 6.10 – Prevalence and overlap of frailty and sarcopenia in healthy and frail older adults



Prevalence and overlap of frailty and sarcopenia in healthy and frail older adults. 75 individuals in total. 33 had neither sarcopenia nor frailty and are not depicted. Each ellipse represents those individuals positive for either that frailty model or sarcopenia model as identified above the ellipse. Where the ellipses overlap the individual is positive for all those overlapping criteria.

24 individuals were positive for frailty and sarcopenia using all four models (light blue). 2 individuals were positive for frailty using the frailty phenotype and frailty index models and also sarcopenia using the IWGS criteria but not EWGSOP criteria (purple). 1 individual was positive for frailty using both the frailty phenotype and frailty index models but was not sarcopenic (pink). 4 individuals were positive for frailty using the frailty index model but not the frailty phenotype model and also positive for sarcopenia using both criteria (green). 1 individual was positive for sarcopenia using both EWGSOP and IWGS criteria but negative for frailty (dark blue). 4 individuals were positive for sarcopenia using the IWGS criteria but not the EWGSOP criteria (violet).

The relationship between frailty and sarcopenia is only partially understood and whilst there are likely to be multiple pathophysiological pathways resulting in frailty the development of low muscle mass, then sarcopenia, severe sarcopenia and finally frailty is probably a common pathophysiological pathway. The drivers propagating the syndromes are uncharacterised but a pro-inflammatory environment is considered to be fundamental and longitudinal research has shown that higher levels of pro-inflammatory cytokines can predict a reduction in fat free mass and a loss of grip strength (187, 188). A pro-inflammatory environment has also been associated with frailty leading to the hypothesis that a pro-inflammatory environment is driving the loss of muscle mass, strength and ultimately both frailty and sarcopenia.

However, Chapter 5 suggested that pro-inflammatory cytokines do not always increase in a stepwise fashion with frailty. This might suggest that frailty progression may not be driven by a pro-inflammatory environment once a state of frailty has been reached. To explore this hypothesis further the groups were expanded to include: healthy young adults, healthy older adults, healthy older adults with low muscle mass and frail older adults. Adults with sarcopenia were excluded from analysis due to their low frequency in the sample population (n=1). A commercially available human cytokine multiplex was used and cytokines with greater than 50% of values within the detectable range and IL-1 β (due to high proportion of individuals within healthy older and frail older adults with concentration of IL-1 β within detectable range) were included for further analysis. For more detailed analysis please see Chapter 5.

There are three different significant patterns within the cytokines measured. One, increased concentration of cytokine in the healthy older adults with low muscle mass compared to healthy younger adults: IL-4, IL-17, Eotaxin and FGF. Two, increased concentration of cytokine in healthy older adults, healthy older adults with low muscle mass and frail older adults compared to healthy younger adults: IL-8, IP-10 and Mip1a. IL-1ra had a higher concentrations in both healthy older adults with low muscle mass and frail older adults compared to healthy younger adults. Table 6.7 and Figure 6.11 demonstrate the large inter-quartile ranges present in the frail older adults. To consider the effect of physical parameters and frailty on the cytokines the data was analysed in a number of ways.

When the cytokine data from only the frail older adults was correlated with physical parameters and frailty there were a number of significant correlations. However, there were also a large proportion of non-significant correlations which makes the data very difficult to interpret especially in relation to the repeated statistical testing. Relevantly MUAC negatively strongly correlated with five cytokines; increased MUAC was associated with lower concentrations of cytokines. The cytokines correlating with MUAC displayed both patterns of changing cytokine concentration suggesting the differences witnessed in the cytokine concentration cannot be explained by physical parameters. (MUAC and IL-4: $R=-0.510$, $p=0.008$, MUAC and IL-8: $R=-0.393$, $p=0.047$, MUAC and Eotaxin: $R=-0.490$, $p=0.011$, MUAC and FGF: $R=-0.410$, $p=0.038$, MUAC and MIP1a: $R=-0.520$, $p=0.006$). Only IL-1ra correlated with frailty; increased frailty was associated with decreased IL-1ra. (FI and IL-1ra: $R=-0.381$, $p=0.029$, FP and IL-1ra: $R=-0.468$, $p=0.006$.)

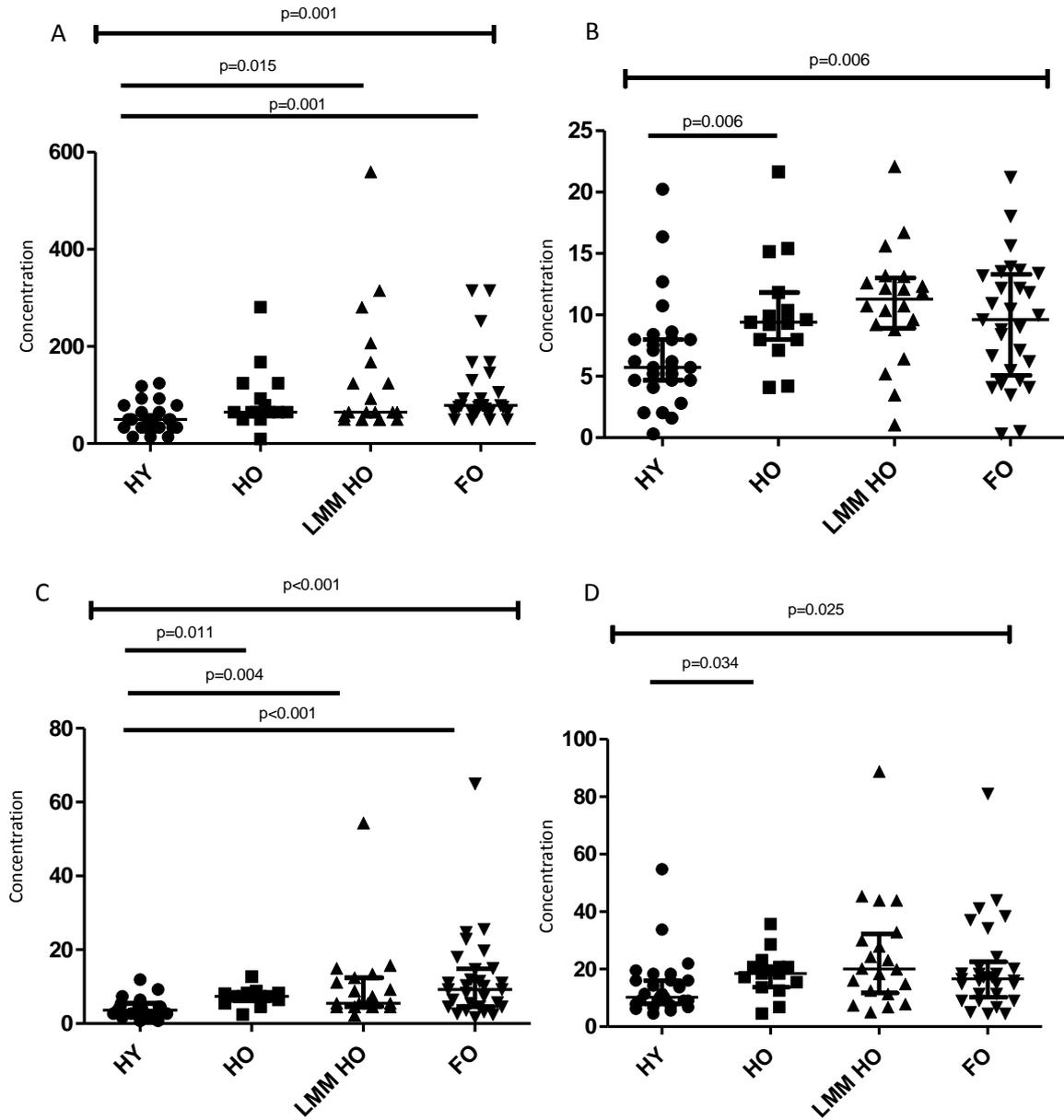
Table 6.7 – Concentration of cytokines

	HY	HO	LMM HO	FO	I K-W	Pairwise
IL-1ra	49.6 +/- 42.3	64.7 +/- 59.6	64.7 +/- 107.2	79.0 +/- 34.4	p=0.001	HY-LMM HO p=0.015 HY-FO p=0.001
IL-1β	0.83 +/- 0.27	1.09 +/- 0.53	0.96 +/- 0.8	0.83 +/- 0.57	p=0.069	
IL-4	5.7 +/- 3.3	9.4 +/- 3.8	11.3 +/- 4.1	9.3 +/- 8.4	p=0.006	HY-LMM HO p=0.006
IL-7	12.0 +/- 6.0	16.0 +/- 12.0	16.0 +/- 5.7	14.1 +/- 10.9	p=0.520	
IL-8	4.8 +/- 0.69	7.4 +/- 1.9	6.4 +/- 7.6	9.2 +/- 10.0	p<0.001	HY-HO p=0.011 HY-LMM HO p=0.004 HY-FO p<0.001
IL-9	55.3 +/- 9.8	48.5 +/- 19.5	44.0 +/- 38.8	47.5 +/- 16.1	p=0.385	
IL-17	4.56 +/- 11.5	18.4 +/- 7.0	20.0 +/- 20.5	16.1 +/- 18.0	p=0.025	HY-LMM HO p=0.034
IP-10	848.6 +/- 613.1	1426.0 +/- 896.2	1435.3 +/- 743.0	1521.1 +/- 983.8	p=0.001	HY-HO p=0.009 HY-LMM HO p=0.012 HY-FO p=0.008
MCP-10	24.9 +/- 13.1	30.1 +/- 19.4	29.5 +/- 38.2	19.8 +/- 22.3	p=0.316	
PDGF	2135.9 +/- 747.3	2349.8 +/- 4146.8	1516.9 +/- 5244.9	1909.5 +/- 2289.8	p=0.404	
MIP1α	0.97 +/- 0.62	1.41 +/- 1.57	1.65 +/- 1.51	2.83 +/- 1.9	p<0.001	HY-HO p=0.06 HO-FO p=0.037 LMM HO-FO p=0.006
MIP1β	82.9 +/- 12.7	83.8 +/- 85.7	82.3 +/- 93.7	85.9 +/- 41.3	p=0.793	
Eotaxin	102.8 +/- 68.9	158.8 +/- 75.8	181.0 +/- 81.6	161.6 +/- 105.0	p=0.009	HY-LMM HO p=0.014
FGF	17.2 +/- 3.0	20.5 +/- 6.3	23.4 +/- 32.4	17.2 +/- 23.4	p=0.010	HY-LMM HO p<0.001
Rantes	12520.3 +/- 2504.7	13223.1 +/- 12963.0	11541.9 +/- 18632.6	11674.6 +/- 7234.7	p=0.812	
TNFα	36.9 +/- 8.0	49.1 +/- 66.0	39.7 +/- 123.4	36.9 +/- 65.9	p=0.369	

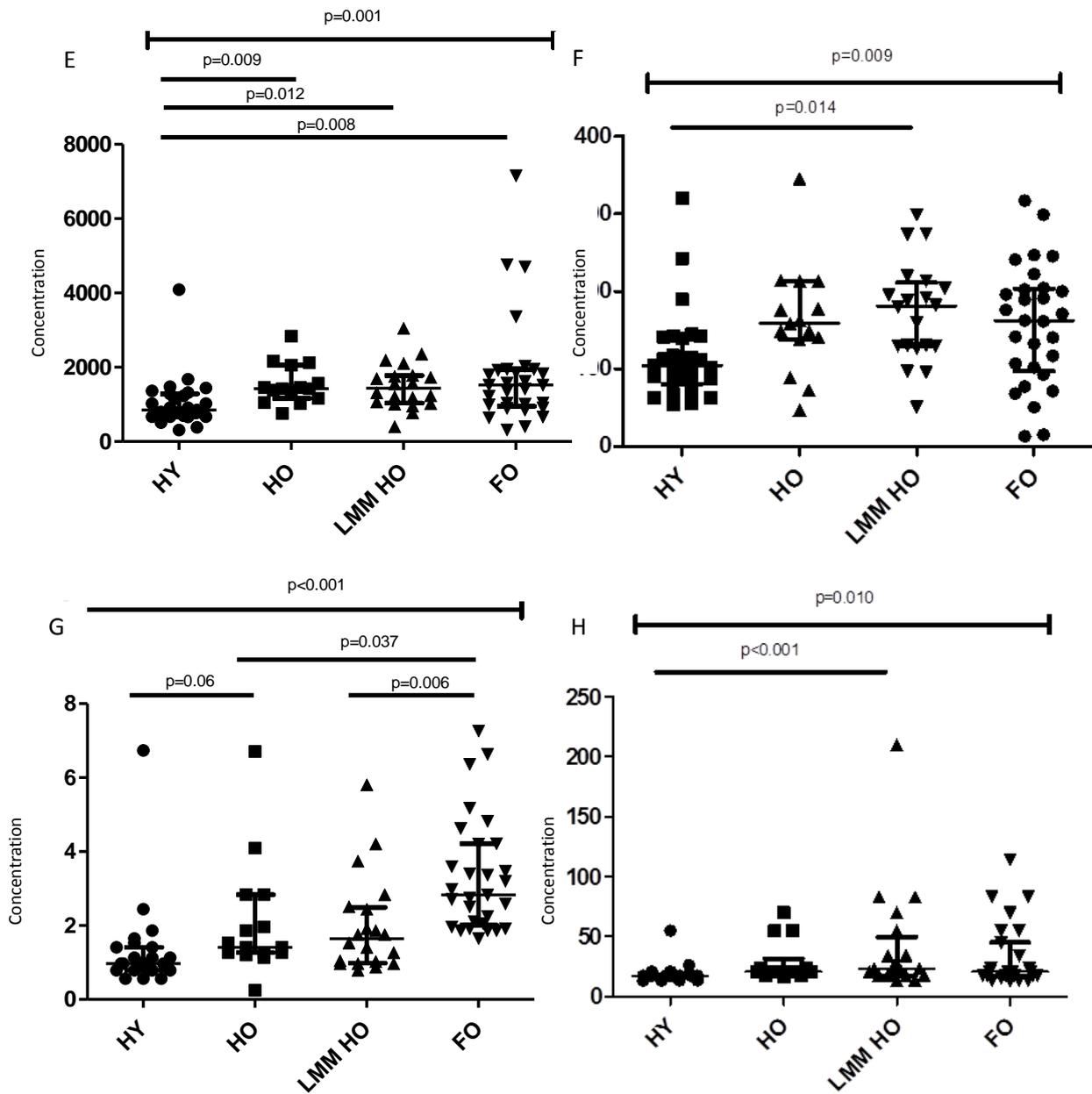
The concentration of cytokines (pg/ml) in healthy young adults (HY), healthy older adults (HO), healthy older adults with low muscle mass (LMM HO) and frail older adults (FO). N = HY -27, HO – 15, LMM HO – 20, FO – 29.

Data is not normally distributed. Median +/- IQR are shown. Statistical tests are Independent Kruskal-Wallis with Bonferroni corrected pairwise comparisons.

Figure 6.11 – Cytokine concentration



Cytokine concentration (pg/ml) of A – IL-1ra, B – IL- 4, C – IL-8, D – IL-17. Data is not normally distributed. Median +/- IQR are shown. N = HY -27, HO – 15, LMM HO – 20, FO – 29. Statistical tests are Independent Kruskal-Wallis with Bonferroni corrected pairwise comparisons. Only cytokines with statistical difference between the groups are shown.



Cytokine concentration (pg/ml) of E – IP-10, F – MIP1 α , G – Eotaxin, H – FGF. Data is not normally distributed. Median +/- IQR are shown. N = HY -27, HO – 15, LMM HO – 20, FO – 29. Statistical tests are Independent Kruskal-Wallis with Bonferroni corrected pairwise comparisons. Only cytokines with statistical difference between the groups are shown.

A further analysis was conducted to consider the effect of inflammatory burden. The 16 cytokines described in table 6.7 were analysed as a composite measure, an Inflammatory Index. (For each cytokine the individuals with a concentration in the highest 20% of the total sample were awarded a score of 1. The scores for all 16 cytokines were summed and expressed as a proportion of the total number of cytokines.) This is a similar method to the previously proposed biomarker based Frailty Index but far cruder as the relationship between frailty and cytokines is incompletely characterised (183).

The healthy older adults with low muscle mass and the frail older adults had a significantly higher Inflammatory Index than the healthy younger adults. (Table 6.8.)

There was also a weak positive correlation between the Frailty Index and the Inflammatory Index: $R=0.250$, $p=0.013$ (Spearman's Rank Correlation Coefficient).

However, age was also associated with the Inflammatory index: $R= 0.326$, $p=0.001$, and after adjusting for age frailty was no longer associated with the Inflammatory index: $R=-0.082$, $p=0.427$ (both Spearman's Rank Correlation Coefficient).

Table 6.8 – Inflammatory Index

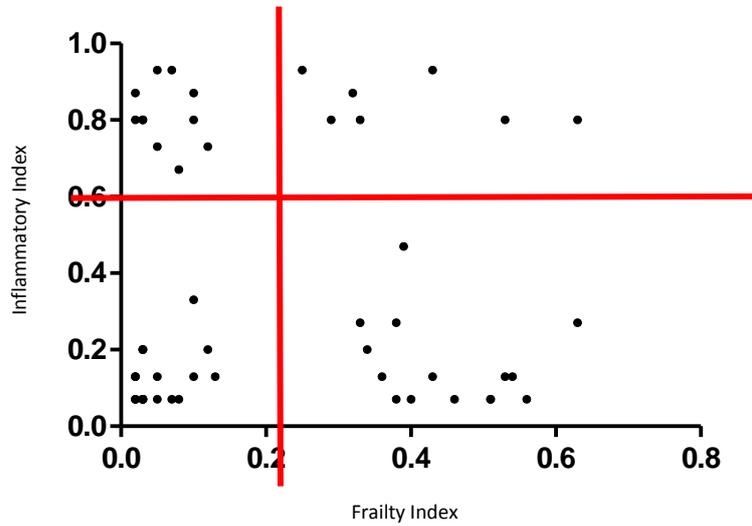
	HY	HO	LMM HO	FO	I K-W	Pairwise
Inflammatory Index	0.000 +/- 0.07	0.067 +/- 0.73	0.133 +/- 0.78	0.133 +/- 0.53	p=0.005	HY-HO p=0.124 HY-LMM HO p=0.024 HY-FO p=0.009 HO-LMM HO p=1.00 HO-FO p=1.000 LMM HO-FO p=1.000

The inflammatory index of the four groups. HY – healthy young adults, HO – healthy older adults, LMM HO – healthy older adults with low muscle mass, FO – frail older adults. Inflammatory Index composite measure of 16 cytokines with greater than 50% of whole sample within detectable range and IL-1 β . N = 27, N=15, N=20, N=29. Data is not normally distributed. Median +/- IQR are shown. Statistical test is Independent Kruskal-Wallis with Bonferroni corrected pairwise comparisons.

On plotting the data it was apparent there were two distinct populations within the two groups defined by the Inflammatory Index. (Figure 6.12.) It was not possible to draw significant conclusions about the differences between the groups due to the small sample size but there appeared to be no difference in the individuals with higher concentrations of Inflammatory Index as described by physical and cognitive parameters. (Table 6.9.)

This analysis suggests the interplay between frailty, inflammaging and physical parameters may be more complex than originally considered.

Figure 6.12 – Association between Inflammatory Index and Frailty Index



The association between the Inflammatory Index and the Frailty Index in healthy older and frail older adults. Frailty Index on the x axis and Inflammatory Index on the Y axis. N = 70 in total, HO - 38 , FO - 32. Red lines represent the separation of the two populations.

Table 6.9 – Physical and cognitive parameters of the separate populations defined by Inflammation Index

	Healthy Older Adults II<0.6	Healthy Older Adults II>0.6	MW	Frail Older Adults II<0.6	Frail Older Adults II>0.6	MW
Age	73 +/- 10	70 +/- 6.5	p=0.223	80 +/- 15	84 +/- 14	p=0.273
Frailty Index	0.03 +/- 0.07	0.05 +/- 0.07	p=0.726	0.43 +/- 0.14	0.33 +/- 0.23	p=0.263
Hand Grip	1.31 +/- 0.44	1.21 +/- 0.25	p=0.949	0.78 +/- 0.23	0.67 +/- 0.62	p=0.643
Walk Speed	1.29 +/- 0.0	1.23 +/- 0.0	p=0.679	0.33 +/- 0.0	0.30 +/- 0.0	p=0.725
ACE	93 +/- 3.0	98 +/- 8	p=0.069	79 +/- 31	70 +/- 31	p=0.672
BMI	23.3 +/- 3.85	25.4 +/- 6.36	p=0.384	25.3 +/- 7.65	23.1 +/- 3.4	p=0.643
BATT	42.1 +/- 8.9	41.4 +/- 17.3	p=0.545	28.7 +/- 12.3	28.4 +/- 23.7	p=0.598
Echogenicity	1.49 +/- 0.73	1.59 +/- 0.47	p=0.890	1.62 +/- 0.9	2.10 +/- 0.88	p=0.492

Physical and cognitive parameters of the separate populations define by Inflammation Index. Populations defined by Inflammation Index >0.6. N = HO II<0.6 – 31. HO II>0.6 – 10, FO II<0.6 – 25, FO II>7 . Data not normally distributed. Median +/- IQR given. Mann-Whitney statistical tests performed.

6.12 Discussion

This chapter has extensively described and characterised three different groups: healthy young, healthy older and frail older adult groups. It has demonstrated that individuals recruited to the frail older adult group are frail by multiple different definitions and considered the value of these definitions. It has described the differences in prevalence of frailty and sarcopenia and the concordance between the two conditions in this

population and then progressed to consider the chronicity of the conditions and potential underlying mechanisms.

This chapter has accurately described and characterised both a healthy and frail older adult group. Parameters associated with frailty representing a variety of biopsychosocial domains are significantly increased or decreased as expected in the frail older adult group (7, 354). The frail older adults are weaker and slower, with less muscle mass; they also have increased frequency of co-morbidities, falls and number of prescribed medications. There is increased prevalence of cognitive impairment in the frail older adult group as demonstrated by both the Addenbrooke's cognitive examination and Stroop Interference.

The clinical assessment described in Chapter 2 was devised to accurately identify and comprehensively describe frailty in the study population; it follows the form of a comprehensive geriatric assessment (CGA) and includes data from: cognitive assessments, nutritional assessments, reported and assessed physical function, assessments of independence, medical history and a physical examination (355). The strengths and limitations of the clinical assessment are discussed below.

Cognition is fundamental to well-being and health. Frailty was originally described as physical frailty and mental frailty. As our understanding of frailty has improved we have returned to the concept of mental frailty and its integral role within frailty as a whole. It is therefore imperative to include an assessment of cognition when diagnosing frailty. The Addenbrooke's Cognitive Examination III (ACE – III) was originally developed to detect mild cognitive impairment and is now frequently used in clinical practice and research

settings (240). The Stroop Colour and Word test conversely is very quick to administer and therefore reduces the frustration often felt by participants undergoing assessment who are self-aware of their cognitive impairment (242). It has been demonstrated that the linguistic decline in dementia can be accurately measured by the Stroop Colour and Word Test (356). It is also less reliant on education attainment and existing intelligence than cognitive tests such as the ACE-III and adjustment for age is integral to the test (357). The provision of informed consent as a requisite to inclusion within the study ensures that only mild cognitive impairment will be present within the study population. These two cognitive tests were therefore both considered ideal to identify mild cognitive impairment within the study population. During the study period it was believed that the inclusion of two cognitive assessments was placing unnecessary burden on the frail older adults and therefore both assessments were not completed in all individuals. The ACE-III was chosen preferentially over the Stroop Colour and Word Test because of familiarity and the validated criteria for diagnosis of cognitive impairment. However, following analysis of the complete data sets it is apparent that Stroop Inference may in some circumstances be a superior test to ACE-III. An important limitation that was not considered prior to the studies commencement was the reliance of both these assessments on good vision. In a population with a high prevalence of visual impairment an alternative form of cognitive assessment such as the semantic verbal fluency test may also be necessary to provide a consistent standard (358).

The maintenance of muscle mass is dependent on the intricate balance between muscle synthesis and breakdown. Nutrition, in particular the availability of protein, is fundamental to muscle synthesis (359-361). Sarcopenia has been demonstrated to be

associated with low protein intake and nutritional supplementation has shown some success in the reversal of sarcopenia (362). Recent research has also demonstrated an association between malnutrition and frailty (363-365). Therefore in the assessment of frailty and sarcopenia it is essential to include an evaluation of nutritional status. The Mini Nutritional Assessment – short form (MNA-SF) was chosen because it has been validated in an appropriate population and it is quick to complete (366). It has been used extensively in research to identify those at risk of malnutrition and is more sensitive than BMI at identifying these patients (243).

The original design of the study protocol assessed physical function with the Berg Balance test. However, in practice this was quickly found to be lengthy and time consuming and this raised concerns that the Berg Balance would be potentially tiring for frailer subjects. A successful amendment for the substitution of the Berg Balance test for the SPPB was submitted to the ethics committee (2nd March 2015) (244). The Berg Balance and SPPB are both tests of physical function with equal scientific merit and in practice the SPPB was quick and easy to administer (367, 368).

The accurate screening of frailty was fundamental to the success of the study and therefore the tools used to identify frailty were carefully considered. PRISMA-7 and gait speed were chosen on the basis of the results of a recent systematic review evaluating the diagnostic test accuracy of simple instruments used in the diagnosis of frailty (14). Whilst this is a systematic review of a relatively large number of participants, 3261, all of these participants are from a total of just three studies and therefore the findings lack external validity. All of the screening tests were also measured solely against the frailty

phenotype which reports lower rates of frailty than the frailty index. It is therefore not clear whether the reference population is accurate. The review demonstrated that both the sensitivity and specificity of the PRISMA 7 were high but wide confidence intervals suggest this may not be reproducible. The gait speed, using a 0.8m/s cut off, also had a very high sensitivity but lower specificity as did a timed get up and go test. The gait speed and PRISMA 7 were chosen as the method of screening for frailty because of their reported high sensitivity and specificity. The gait speed is a good indicator of physical function and was utilised in other assessments present in the study: diagnosis of sarcopenia, short physical performance battery and diagnosis of frailty by Fried Phenotype. For simplification and reduction of duplicity within the study gait speed was chosen rather than a timed get up and go. PRISMA 7, in contrast to gait speed reflects multiple domains, mobility, social support and health and is therefore more akin to the Frailty Index rather than the Frailty Phenotype.

The Fried Phenotype is utilised by the majority of research studies due to the relative ease of measurements. The phenotype relies entirely on physical parameters with no consideration of the psychological, social or behavioural (354). The model was constructed from criteria already collected for the Cardiovascular Health Study which was designed to determine the importance of conventional cardiovascular disease risk factors in older adults. It therefore relies on criteria not specifically chosen for their relatability to frailty. Whilst this is the most widely utilised frailty model in the literature there is huge variation in how the different components are assessed and measured. This particularly applies to the component of physical inactivity (369). To standardise the

research and make it widely applicable modifications to the Frailty Phenotype were limited to only those considered absolutely necessary.

Physical inactivity was described by as: performing no weight bearing activity, spending more than four hours sitting in a twenty-four hour period and taking less than one short walk a month. This criterion has been used in the Frailty Intervention Trial (38). This was selected due to the inherent difficulties of utilising the Minnesota Leisure Time Physical Activity Questionnaire in practice. There are alternative assessments of low activity suggested in the frailty literature but they take longer to complete. The participants underwent extensive clinical assessments and it was important to limit the number and time of assessments to reduce burden.

Slowness was measured over a 4 metre walk course rather than the 15 foot walk course used in the original Fried paper. 4 metres and 15 foot are a similar distance and 4 metres is the accepted distance for both screening of frailty and a diagnosis of sarcopenia (11, 14). To reduce the burden to participants gait speed has been calculated over a 4 metre walk course and extrapolated for the Fried Phenotype.

The Frailty Index has been adopted less frequently as a model of diagnosis of frailty due to the perceived relative complexity of application (7). It is however increasingly the more accepted concept of frailty.

Data was collected for 63 different variables and from these 30 variables were identified to be included in the Frailty Index. The seminal Rockwood paper 'A standard procedure for creating a frailty index' lists 40 variables that can be included in a frailty index (247). Of these 40 variables the study collected adequate information on 25 and these were

automatically selected. The remaining five variables were identified as variables that adhered to the Rockwood criteria. Variables were selected so as to adequately represent all the domains. The new five variables were: polypharmacy, continence, anorexia, falls and Clinical Frailty Scale.

Polypharmacy is a recognised issue in geriatric medicine. It has been associated with multiple adverse health outcomes including falls, cognitive impairment and urinary incontinence (370). It increases in prevalence with age (371). Polypharmacy is considered as more than 4 medications and excessive polypharmacy as more than 10 medications. Polypharmacy and excessive polypharmacy were both incorporated into the scoring criteria.

Continence is another important condition in the geriatric population associated with poor outcomes including length of stay in hospital and mortality (372). The criteria was scored trichotomously as it was discovered in practice that whilst the majority of participants denied partial or total incontinence of bladder or bowel more participants agreed they had 'a problem with losing control of urine when they don't want to' suggesting a hierarchy to answers.

Anorexia, or poor food intake, was included as it is one of two variables within the MNA-SF that had not been included in some form already within the frailty index. Anorexia has been suggested as an early risk factor for frailty (373).

Falls, one of the original frailty syndromes, was included as a new variable. The incidence of falls increases with age and are associated with adverse health outcomes such as fracture, loss of independence and death (374). As just one fall can have a huge impact

on health, independence and psychological well-being this has been scored as a dichotomous event.

The clinical frailty scale (CFS) was included as a physician's assessment of the individual following a comprehensive geriatric assessment (15).

A number of the variables described in the original Rockwood paper had their scoring criteria modified.

The cognitive assessment utilised in the Rockwood paper was a mini-mental state examination (MMSE). The ACE III was selected to describe our participant's cognition because of its increased sensitivity in identifying mild and cortical dementias (375). The ACE III does not categorise respondents into mild, moderate and severe groups, like the MMSE. The ACE III has two suggested cut off points with different degrees of sensitivity and specificity. The lower cut off point, 82, was chosen because whilst the sensitivity is lower the specificity is higher. In the study's population which is likely to have a low prevalence of cognitive impairment due to the nature of informed consent it is more important to have a higher specificity to reduce the number of false positives.

In the Rockwood paper walk speed was measured twice at usual and rapid pace. For ease and reduction of duplication we have used a timed walk speed over a 4 metre course. The cut off point for sarcopenia was used for a half score and the cut off point for the frailty phenotype 'slowness' has been used for a full score.

In the Rockwood paper weight loss was scored using a dichotomous criteria of either weight loss of more than 4.5kg or not. In this study the measure of weight loss, originally

part of the Fried frailty phenotype 'shrinking', has been combined with the weight loss criteria utilised in the Edmonton Frail Scale 'does clothing feel looser' to create a trichotomous criteria for weight loss. In practice some participants had not lost as much as 4.5kg or had failed to accurately measure any weight loss but felt their clothing was looser suggesting some weight loss. This identifies participants who may be at an earlier point in frailty risk.

The Clinical Frailty Scale (CFS) (15) and the Edmonton Frail Scale (EFS) (18) were both included as the CFS is more likely to be used by geriatricians and the EFS by other practitioners. This makes the results more widely applicable. On balance the clinical assessments described in Chapter 2 describe an accurate and comprehensive global assessment of frailty suitable for employment in frailty research.

The frail older adult group is an accurate representation of frailty as a syndrome. There are no individuals with a frailty index higher than 0.7 (0.64 highest FI). A frailty index of greater than 0.7 is considered incompatible with life and therefore this is an important validation of the clinical data (376, 377). The frailty phenotype has a gamma distribution, the distribution of data expected in frailty (247); with increasing frailty there is both increasing mortality and increasing isolation skewing a normal distribution towards increased frequency of less severe frailty. The distribution of the frailty index is more even than the frailty phenotype; it is less skewed and has a lower kurtosis. These differences in distribution could be due to the relatively low numbers of frail older adults or bias within the recruitment. Frail older adults were recruited following a positive screening test, either walk speed or PRISMA 7; however the decision to screen was based

on more subjective information open to bias for example information from the individual's usual care doctors. There is a tendency to consider frailty solely as a physical phenotype and therefore less frail adults by the frailty index who do not display all the hallmarks of physical frailty may have been unintentionally excluded from the study population.

The frail older adult group was older than the healthy older adult group. This difference in age is more apparent in women with a larger difference in age between the healthy and frail older groups; the healthy older women are younger and the frail older women are older than their male counterparts. In the frail older adult group the women were significantly older than men. This is likely secondary to both longer life expectancies in women; women in the UK live on average three years longer than men and differences in co-morbidity patterns (378). Analysis of these different groups therefore has to consider the effect of age as well as frailty on the sample. In Chapter 5 this has been achieved in parts by modelling the data controlling for age. A potential improvement to the study design, which would reduce the effect of age, would be to age match the frail individuals. In practice this may be difficult as the frail older adults were the most difficult group to recruit.

There was no difference in gender frequency for either the healthy younger or frail older adults. Individuals were actively recruited to the healthy younger and healthy older adult groups to reflect an equal gender split; this was not completely successful in the healthy older adult group because of the unequal gender representation in the 1000 Elders cohort (source population for healthy older adult recruitment). Frail older adults were

recruited regardless of their gender and the equal proportions of gender are reflective more of the epidemiology of frailty in the screening populations, consent of individuals and luck rather than active design. The equal gender split is surprising because females are repeatedly reported to have increased prevalence of frailty (379, 380). However, anecdotally male individuals were more likely to consent to involvement in the study, whereas women wanted to discuss their involvement with family members first or felt that it would be too burdensome and this may explain the equal gender split. The unequal gender split in the healthy older adults does mean that the male morphology and physical parameter data needs to be interpreted with caution.

There are differences in body morphology with increasing frailty for each gender which suggests that pathophysiological routes into sarcopenia and frailty may be different for men and women. As expected men and women both have a decreasing BATT with ageing and frailty. This reduction in BATT is reflected with a decrease in MUAC in men but not women. There is a significant increase in the BMI of women with frailty but not men. In men there is a trend to reduction in BMI with frailty but this is non-significant. The ultrasound echogenicity increases in healthy older men and women but there are non-significant changes with increasing frailty likely secondary to the small numbers of the frail population undergoing ultrasound echogenicity. Data presented in Chapter 3 demonstrates the correlation between increasing ultrasound echogenicity and decreasing muscle strength. There was a significant increase in the proportion of adipose tissue compared to muscle with ageing but not frailty for women. There was no significant difference in the proportion of adipose tissue compared to muscle in men but there was

a trend towards an increase in the thickness of subcutaneous tissue compared to muscle thickness.

The differences in body morphology reported in this chapter may have been affected by socio-economic differences rather than frailty or ageing. Increased obesity, reduced physical activity and poor health is associated with lower socio-economic groups (381). Individuals recruited to the healthy younger group were from the highest socio-economic group. The levels of educational attainment were significantly different for all three groups: HY 21.0 +/- 0.0, HO 18.0 +/- 6.0, FO 15.0 +/- 2.0, Independent Kruskal-Wallis $p < 0.001$, Bonferroni corrected pairwise comparisons all $p < 0.001$. The reported differences of increasing adiposity and lower muscle mass in both men and women with frailty could reflect lower socio-economic status rather than differences due to frailty. Whilst the data reported in this chapter may be biased by socio-economic status it does still reflect previous work which reports decreasing muscle mass and increasing adiposity with increasing age and frailty (271, 382). Another consideration when analysing the data is the effect of generational differences on diet, physical activity and attitudes towards body image and health. The healthy older and frail older adults were born between 1916 and 1952 a period of time defined by rationing, limited access to personal motorised vehicles and less sedentary employment. The healthy younger adults were born between 1980 and 1996 a period of time defined by increasing obesity, the rise of convenience foods and stark changes in attitude to body image. For these reasons cross-sectional analysis of data on frailty and body morphology can be difficult to interpret.

After excluding the healthy young adults from analysis the differences between the data are less apparent. Women are significantly shorter; this is discussed in Chapter 3 but in summary is secondary to osteoporosis and nutritional imbalances during puberty.

Interestingly whilst there is no real difference in BMI between healthy older and frail older adults for either gender there is an increase in the inter-quartile range in both the frail older men and women suggesting an increased polarisation and an increased number of individuals within the obese and underweight categories. Despite weight loss being a frailty defining criteria (383) and low BMI being associated with a higher risk of frailty (384) there is increasing awareness of the high prevalence of obesity in frailty and the association with an increased risk of mortality (385). As expected there is a significant reduction in BATT. Alongside a reduction in muscle mass there is an increase in adipose tissue as suggested by the decrease in BATT to subcutaneous tissue ratio and changes in the ultrasound echogenicity. The ultrasound echogenicity data does need to be interpreted with caution as there are small numbers in the frail older adult group.

This data taken with currently available literature suggests there is a change in the distribution of adipose tissue and muscle with increasing frailty. Frailty is associated with both being underweight and obese (385). The highest mortality occurs in frail individuals who are underweight, normal weight or obese; being overweight ameliorates the risk of frailty entirely in frail individuals (386, 387). There is also an association of frailty with central obesity: older adults with a large waist circumference had an increased risk of being frail at follow-up (388) and frail men were more likely to have a large waist circumference (389). The data presented here suggests the volume of intra-muscular adipose tissue increases with frailty and may be affected by gender. However, the

numbers are too small to draw conclusive results. Frailty is associated with intra-muscular adipose tissue (19, 20) and gender is known to have an effect on the distribution of adipose tissue in younger adults with increased central and visceral adiposity in men. This difference is related to sex hormones and post menopause there is an increase in visceral adiposity in women (390). Adipose tissue itself confers a pro-inflammatory state through the release of adipokines and recent research has demonstrated that frail individuals had increased intra-muscular adipose tissue and elevated IL-6 mRNA and IL-6 protein content (391). Changes in the distribution of adipose tissue can result in a localised inflammatory state which can be hypothesised contributes to propagating sarcopenia and frailty. To date evidence has demonstrated associations between states of inflammation and frailty but has yet to prove a causal link. Longitudinal mechanistic analysis is required to investigate this further.

The data presented in this chapter also identifies four parameters, Stroop interference, grip strength, BATT and frailty index, which are discriminative between healthy younger and healthy older adults. The Stroop data does need to be interpreted with caution as it was only performed on eight frail older adults due to time constraints and perceived burden on the frail older participants. However, this does suggest a number of parameters both physical and cognitive which could be used as sensitive indicators or descriptors in longitudinal research. The alternative parameters were too insensitive to discriminate between healthy younger and older adults and would not be good descriptors of change in longitudinal research investigating transitions between health and frailty states. The substitution of Stroop for the less sensitive and more time

consuming assessment of cognitive impairment the Addenbrook's cognitive examination may add value to frailty studies particularly in longitudinal research.

There was a higher prevalence of frailty diagnosed by the Frailty Index than the Frailty Phenotype; 100% FI positive in frail older adult group and 88.6% FP positive in frail older adult group. This was expected as the models are known to describe slightly different groups (8). The concordance and correlation between the two models is higher than previously reported (8, 32). This is likely secondary to the manner of recruitment and the introduction of bias towards screening more physically frail individuals.

Due to nature of the study design, primary objective to recruit to three different groups, two healthy and one frail, there are two very distinct populations a healthy population and a frail population. For this reason it is not a particularly good test of diagnostic test accuracy in an unselected population. However, it does demonstrate that in healthy older adults with no chronic inflammatory conditions and in physically frail older adults both walk speed and PRISMA 7 are accurate tests to identify frailty with high sensitivity and specificity. The calculated sensitivities of 100% for PRISMA-7 and 94.3% for walk speed and specificities of 95% for PRISMA 7 and 100% for walk speed are similar to reported results but do show an improved accuracy (14). A further significant limitation to the study design is that the frailty assessments were not conducted in isolation and this could introduce bias as the investigator was not blinded to the results of the other frailty assessments. An improvement in study design would be the involvement of a second investigator who could conduct the PRISMA 7 and walk speed assessments independently from the principle investigator who would complete the remainder of the

assessment and analysis. An alternative approach would be to ask individuals to complete the PRISMA 7 questionnaire themselves prior to assessment but this would have been impractical in this cohort of frail older adults due to the high prevalence of visual impairment.

The tests which categorised severity showed poorer test accuracy than the simpler tests: Edmonton frail scale: 73.5% sensitivity, 100% specificity, positive predictive value 100%, negative predictive value 81.63%, Clinical frail scale: 91.4% sensitivity, 97.5% specificity, positive predictive value 97%, negative predictive value 92.9%. The diagnostic methods of frailty identification categorise the severity of frailty similar to the Frailty Index. When compared to the Frailty Index the correlation for both diagnostic tests is good, 0.868 for Clinical frail scale and 0.841 for Edmonton frail scale but the variability within each group is large. The accurate description of frailty severity is crucial in intervention studies where the heterogeneity of the studied population often precludes significant results and meaningful data. A recent systematic review of interventions in frailty demonstrated that multiple different definitions of frailty are currently in use with the Fried phenotype being the most common but definitions not considered a gold standard are also in use (10). This supports the exclusive use of gold standard diagnostic tests such as the Frailty Index and Frailty Phenotype in frailty studies.

The prevalence of sarcopenia in the healthy older adults was lower than expected; estimates of sarcopenia vary wildly from 3-30% depending on both definitions and methodology. A recent study investigating sarcopenia in a male community dwelling UK population reported a prevalence of 4.6% (299). This is higher than the data on

sarcopenia prevalence reported in this chapter: 3.7% for women and 0% for men. As discussed previously, the design of the study prioritises the immunesenescence work and therefore the healthy older adult group is not typical of a community dwelling older adult population: all adults with frailty, cancer and chronic inflammatory conditions were excluded. Additionally the sample pool from which the healthy older adults were recruited contains a self-selected group of highly motivated, well-educated individuals interested in health research.

There has been limited research into the concordance of frailty and sarcopenia. Previous research has demonstrated that in certain populations, community dwelling attendees at an outpatient geriatric clinic (392) and community dwelling older adults in Japan (33) frailty is more prevalent than sarcopenia. This is counter-intuitive to our current understanding because prevalence data suggests sarcopenia is more prevalent than frailty. Interestingly in the outpatient geriatric clinic concordance between sarcopenia and frailty was poor: 16.7% of attendees had both sarcopenia and frailty and only 36.4% of frail older attendees by Fried criteria and 20% by Rockwood had sarcopenia (392). The data presented in this chapter demonstrates a far higher concordance between the two conditions; depending on the definitions of sarcopenia used 96.8% (IWGS) or 90.3% (EWGSOP) of the frail older adults also had sarcopenia. The difference in both concordance and prevalence of the two conditions in comparison to previous reports could be solely due to study design. 54.3% of the frail older adults were recruited during an inpatient hospital stay where they may have lost muscle mass secondary to immobilisation or cachexia increasing the prevalence of sarcopenia and the aforementioned unconscious bias prior to screening could have increased the prevalence

of physical characteristics of frailty including sarcopenia. However, this data has demonstrated that in a population recruited for frailty the prevalence of sarcopenia is high and when analysing data from this population the high concordance should be considered as a potential limitation to the study design. The data also confirms that frailty is a separate condition to sarcopenia not just an extension of sarcopenia.

The data presented in this chapter confirms that frailty described in the frail older adult group is a separate entity to both co-morbidity and disability and whilst there is a large degree of overlap the differences described elsewhere in this thesis are not secondary to either just co-morbidity or disability. This is similar to data presented elsewhere which suggests frailty is a separate condition which confers additional risk of adverse outcomes independent of disability and co-morbidity (5, 37). The addition of either a disabled or co-morbid state to the frailty state increases the frailty severity. The addition of either one or two states does not seem to make a difference to the frailty severity which suggests either: one, one of the states is not associated with increased frailty severity or two, a saturation point is reached where the addition of another state has no impact on frailty severity. Due to the low numbers it was impossible to consider co-morbidity and disability separately but further investigation of the relationship between frailty, co-morbidity and disability is essential to consider practical measures to prevent progression of frailty.

The data presented suggests that the addition of sarcopenia to a diagnosis of frailty increases frailty severity. Analysing all the Fried phenotype criteria separately suggested that positive grip strength, a component of sarcopenia, and physical activity, a

contributor and consequence of sarcopenia, resulted in more severe frailty. This implies that the addition of sarcopenia to frailty worsens prognosis. This analysis is reliant on frailty severity as described by Frailty Index being associated with mortality and poor outcomes but a recent paper by Rockwood has succinctly demonstrated this in multiple age groups and both humans and mice (393). Further analysis of the effect of sarcopenia, co-morbidity and disability on frailty severity and therefore long-term outcomes is essential to better understand the effects of interplay and chronicity on all these conditions. Longitudinal analysis would be the most appropriate form of investigation.

The drivers propagating frailty and sarcopenia are incompletely uncharacterised but a pro-inflammatory environment is considered to be fundamental and longitudinal research has demonstrated that higher levels of pro-inflammatory cytokines can predict a reduction in fat free mass and a loss of grip strength (188, 394). A pro-inflammatory environment has also been associated with frailty (179-181) leading to the hypothesis that a pro-inflammatory environment is driving the loss of muscle mass, strength and ultimately both frailty and sarcopenia. However, the data in this chapter suggests the relationship is more complex than a simple association of systemic inflammation with sarcopenia and frailty.

Although the data is inconclusive due to the small numbers there appears to be two patterns of changing inflammation with increasing frailty: one, the step-wise increase with frailty and two, the increase in inflammation with just low muscle mass but not frailty. This is supported by significant increased concentrations of cytokines in just healthy older adults with low muscle mass in some cytokines, IL-4, IL-17, Eotaxin and FGF,

and significant increased cytokines in both healthy older adults with low muscle mass and frail older adults, IL-8, IP-10, MIP1a and IL-1ra.

The second suggested pattern mirrors the differences reported in neutrophil elastase activity demonstrated in Chapter 5 and suggests that the production of cytokines is also vulnerable to immunesenescence. It is unclear why certain cytokines appear to be more vulnerable to immunesenescence than others and whether in extreme frailty all cytokine expression is reduced. Analysis of the relationship between physical and cognitive parameters of frailty and sarcopenia with inflammation did not reveal any definitive correlations. MUAC significantly correlated with multiple cytokines but has to be interpreted with caution because of the incompletely described relationship of MUAC with frailty and sarcopenia in both genders. MUAC is a crude measure of health incapable of distinguishing between muscle mass and adipose tissue; the proportion of adipose tissue to muscle is essential to consider due to the relationship of both muscle and adipose tissue with inflammation.

The analysis of the burden of inflammation using the Inflammatory Index also does not reveal any significant relationships between frailty and sarcopenia with inflammatory burden apart from the increase with age. The graphical representation of Frailty Index and Inflammatory Index suggests there are two separate populations within both healthy older adults and frail older adult. Whilst the high Inflammatory Indexes in both groups could just represent individuals with infection or ongoing inflammatory process such as osteoarthritis this separate population does not exist in the healthy younger adults suggesting it may be more complicated.

The Inflammatory Index is a crude composite measure based on the Frailty Index (395, 396). It does not consider the implications of pro or anti-inflammatory effects of each cytokine, the pathological concentration of each cytokine or accurately reflect a Frailty Index with at least 30 variables. However, it could be a useful tool for investigating the inflammatory burden in frailty and sarcopenia particularly in longitudinal studies.

This chapter has accurately described a frail older group recruited for cross-sectional analysis of immune parameters. It has also demonstrated that for both the complex analysis of the interplay and relationship between sarcopenia and frailty and also the inclusion of non-phenotypically frail individuals in the frail sample whole older adult populations need to be screened to remove bias towards the phenotypically frail. Whilst the concordance between sarcopenia and frailty reported in this chapter are much higher than expected it does confirm they are separate conditions able to exist in isolation. This chapter has also demonstrated that co-morbidity and disability are separate conditions to frailty and suggests the addition of either co-morbidity, disability or sarcopenia to frailty worsens outcomes. Changes in body morphology with frailty have been described and linked with existing data to suggest some of the drivers propagating both sarcopenia and frailty. The role of inflammation in both frailty and sarcopenia has been previously considered fundamental to driving both syndromes but the data presented here suggests a more complex interplay between the different states of immunosenescence, inflammaging, frailty and sarcopenia which needs to be investigated further, particularly considering a non-linear relationship between frailty and inflammation. The Inflammatory Index has been described for the first time and used to investigate the complex relationship of inflammaging with frailty. The data presented in this chapter

supports a proposal for more investigation into the relationships and interplay between frailty, sarcopenia, immunesenescence and inflammaging. However, all the data presented needs to be interpreted with caution because it is inadequately powered.

7. General Discussion

7.1 Summary and key findings

This thesis has linked several key concepts in frailty, sarcopenia and immunesenescence and attempted to explain some of the shared pathophysiology. As outlined in Chapter 1, frailty, and to a lesser extent sarcopenia, research faces a number of challenges. Frailty is not a new concept but it has only relatively recently been medicalised and adopted as a geriatric syndrome [1, 2]. The initial lack of an internationally agreed clinical definition and two fundamentally different models of frailty with apparent equal scientific merit has narrowed frailty research over the previous twenty years. Research has predominantly focused on description, epidemiology and outcomes of the syndrome and the investigation of pathophysiology and the relationship between frailty and sarcopenia has been neglected. Consequently despite sarcopenia featuring as a central component of the frailty cycle, originally proposed by Fried in 1998, the first research reviewing the concordance of the two conditions was only published in 2016 (32, 397). Additionally, apart from the consideration of the role of immunesenescence and inflammaging there has also been little advancement in the understanding of the causative and propagating factors of the conditions.

One of the fundamental challenges facing frailty and sarcopenia research is the presence of multiple tools describing similar and overlapping conditions. A hierarchical linear progression of the conditions with sarcopenia considered both a pre-cursor and a pre-requisite of frailty is frequently proposed (11, 354). However, research has demonstrated that the concordance of frailty and sarcopenia in a community based older adult populations was poor and the prevalence of frailty was higher than sarcopenia (8, 32, 33). The relationship between the different models of frailty is also incompletely described and understood. Cross-sectional studies investigating both physical frailty, as described by Fried's Frailty Phenotype, and frailty as an accumulation of deficits, as described by Rockwood's Frailty Index demonstrate the models identify related but separate, distinct populations of frailty. It has been suggested the models should be considered complementary rather than alternative methods of diagnosis (5, 7, 8). Frailty is usually considered to be a separate entity to both disability and co-morbidity and although all three conditions are related the relationship is incompletely characterised (37). The relationship between all these conditions is poorly understood particularly with respect to the common pathophysiology, including causative and propagating factors, and the temporal effect. Longitudinal research comprehensively investigating the accumulation of these conditions in individuals over a period of time is essential to accurately describe the temporal and pathophysiological relationship. Rockwood and colleagues have described an elegant computational approach to modelling health trajectories which identifies the connections between non-specific nodes as the determining factor in mortality and frailty (398). This highlights the importance of the connections between health conditions rather than conditions per se. It is possible this method could be

modified to be used in a more focused manner to investigate the relationships between frailty, sarcopenia, disability and co-morbidity further.

A second significant barrier to the advancement of understanding in frailty research is the separation of research of the clinical condition from fundamental science research.

Ageing research investigating the effect of age on biological systems such as the immune and endocrine system has failed to consider the effect of frailty and co-morbidity whereas, frailty research has tended to concentrate on describing the condition and correlating it with outcomes. There has been a lack of true translational research typified by rigorous clinical characterisation of an individual prior to the fundamental science research. Amplifying this problem is both the perceived difficulties of conducting research in frail older adults and research interests and expertise in academic geriatricians that does not align with fundamental science. Consequently the role of inflammageing and immunesenescence in the development and propagation of frailty and sarcopenia is poorly understood.

Chapter 3 briefly précised the importance of the correct and timely diagnosis of sarcopenia in both clinical practice and research. There are barriers to the identification of low muscle mass in an elderly and frail population and this work supports the use of ultrasound as a potential diagnostic tool in sarcopenia, which is also able to assess 'muscle quality', utilising ultrasound echogenicity as a surrogate marker of intramuscular adipose tissue.

Current gold standard diagnostic methods for the identification of low muscle mass are neither easily accessible nor suitable for assessing frail older adults particularly those

based within the community setting. MRI, CT and DXA are expensive and multiple physical and cognitive barriers exist preventing a frail population from accessing these imaging techniques. In addition, analysis of the images is not immediate and therefore diagnosis and management can be delayed; this is particularly important in a time-critical condition in a population who are frequently lost to follow up. BIA, although a bedside test, has not been validated in a frail older adult population and the high prevalence of contra-indicated conditions, such as dependent oedema and heart failure, suggest it cannot be used widely in this population.

Additionally, ultrasound can measure changes in muscle architecture and composition such as muscle echogenicity, pennation angle and fibre length. Recent work in several large cohort studies has suggested that muscle strength is a more accurate predictor of poor functional outcomes than muscle mass (399-401). Muscle quality, defined as muscle function (strength or power) per unit of muscle size (mass or cross-sectional area), is affected by: the morphological characteristics of the muscle, aerobic capacity, fibrous tissue, motor units and intramuscular adipose tissue. The quantification of intramuscular adipose tissue by ultrasound echogenicity has been validated with muscle biopsy and high ultrasound echogenicity has been associated with poor outcomes and low muscle strength in older adults (257, 402, 403).

The current work presents a simple ultrasound scanning protocol which includes measures of both muscle quantity (muscle thickness) and muscle quality (ultrasound echogenicity). The work contained in this thesis includes a robust assessment of the reliability and reproducibility of ultrasound using the suggested protocol and finds this to

be good when measuring muscle thickness but inadequate in measuring echogenicity when using different machines and sonographers. This initial work suggests ultrasound echogenicity may be a useful tool in single centre research studies where it is possible to standardise machines and sonographers but is not currently appropriate for clinical use or in large multi centred studies.

The chapter then proposes the BATT and the corresponding criteria for low muscle mass. The criteria for low muscle mass have been calculated from a group of healthy younger adults as suggested by EWGSOP (11). The chapter then proceeds to test the BATT in both a healthy older and frail older adult population and demonstrates a relationship between increasing frailty and decreasing muscle thickness. It also shows a correlation between frailty and the markers of physical function with ultrasound echogenicity.

The key findings in Chapter 3 are the proposed BATT and the association of ultrasound echogenicity with frailty; these require further investigation but have potential to change clinical practice. The comparison of BATT to a gold standard method such as MRI or CT in the target population is necessary before BATT can be utilised in clinical practice as a method of identification of low muscle mass. A recent systematic review of thirteen studies conducted in older adults, both healthy and with chronic conditions, concluded that muscle measurements obtained using ultrasound were reliable when compared to a gold standard (404). This suggests the suggested protocol using ultrasound to measure muscle thickness would be an accurate method of muscle measurement in an older adult population. It should however be confirmed in a frail older adult population. Whilst unadjusted muscle thickness has been frequently used as a surrogate marker for muscle

mass (249, 274, 275) comparing the BATT values with whole body or appendicular lean muscle mass would validate BATT, an unadjusted aggregate measure of muscle thickness, against muscle volume. Once the method had been appropriately validated further studies expanding the BATT criteria to include values for different ethnicities are important. The current BATT criteria presented in Chapter 3 has amalgamated data from healthy young adults of different ethnicities; to improve the reliability of the tool the study numbers should both be increased and re-analysed to produce criteria for each ethnicity. The BATT is the first proposed criteria for low muscle mass in sarcopenia using ultrasound measurement of muscle thickness. Repeating this study in different global populations would improve the validity of the method, increase the size of the reference population and therefore, improve the accuracy of the BATT criteria for low muscle mass. Criteria for reduced muscle mass based on a healthy young adult population are arbitrary and do not necessarily relate to functional outcome. Linking data on muscle thickness with muscle strength and physical performance may establish more accurate criteria for the identification of low muscle mass in older adults. However, an advantage of the current relatively high values for the BATT criteria is that it acts as a screening step. This allows earlier identification of pathology and subsequently earlier intervention which is likely to be associated with the increased success (10).

The relationship between sarcopenia and ultrasound echogenicity is less well defined and the data presented in Chapter 3 highlights the inconsistency in measurements of echogenicity in practice. Research should therefore first be concentrated on standardising ultrasound echogenicity. This will require liaison with industry to create an ultrasound scanner able to regulate the pressure and angle of the probe as well as in-built

algorithms that account for overlying subcutaneous tissue and muscle depth. Only once this is available could this be considered an appropriate tool for use in clinical practice. Combining a standardised measure of ultrasound echogenicity with automatic depth measurements in a portable ultrasound scanner would improve access to sarcopenia diagnosis and ultimately lead to increased diagnosis.

Chapter 4 builds on the theme of 'muscle quality' to consider the measurement of muscle quality via tissue analysis of: the morphological characteristics of the muscle, aerobic capacity, intramuscular adipose tissue, fibrous tissue and motor units. This analysis requires muscle tissue from frail older adults who are not acutely unwell (for example not taken intra-operatively in patients with a fractured neck of femur) and Chapter 4 reports on the difficulties in obtaining samples in a frail older adult population recruited primarily for investigation of immunesenescence and inflammaging. The feasibility of muscle biopsy in a frail older adult population is considered in two respects: the contraindications to a percutaneous muscle biopsy using a needle biopsy technique and the prevalence of a muscle depth shallow enough to prevent a successful biopsy in older adults. The chapter then discusses strategies to improve the success of muscle biopsy as a technique in both healthy older and frail older adults.

The key finding in Chapter 4 is that muscle biopsy using a needle biopsy technique was not feasible in many of the adults recruited to this frail older adult cohort. This is particularly relevant as it is contrary to previously published research and highlights the importance of proper study design (287, 289, 290). Chapter 4 discussed many of the strategies that could improve the success of muscle biopsies in research including utilising

an ultrasound prior to biopsy and the different biopsy techniques. It also discussed the importance of study design as previously successful biopsy studies have screened large population pools from which a small numbers of individuals were selected for inclusion within the study (287, 289, 290). However, this group of frail individuals is unlikely to represent the frail population as a whole; they are likely to be less frail, with fewer co-morbidities and disabilities. Whilst the muscle biopsy data from frail individuals with no contraindications to muscle biopsy will be applicable for the investigation of certain hypotheses it is essential that the data is interpreted with caution and the results are not extrapolated to the frail population as a whole.

There are a number of alternatives to elective muscle biopsy including techniques involving the removal of muscle tissue and techniques that circumvent the need for muscle tissue. Muscle for many research studies is obtained intra-operatively and there are two main options, one during elective surgery and two, during emergency surgery. Elective surgery is preferential because it negates the effect of acute illness on the muscle sample. However, elective surgery in older adults is often performed for musculoskeletal indications. Alternative indications for surgery in older adults include semi-elective surgery for abdominal aorta aneurysmal repairs but these are only offered to fitter frail older adults. The most frail older adults only undergo emergency operations for acute life-threatening conditions. Samples obtained during these operations would be appropriate for the analysis of muscle architecture but not for accurate analysis of intracellular proteins or intra-muscular inflammatory status. An alternative to muscle biopsy that does not require muscle tissue is metabolomics. Metabolomics, the systematic identification and quantification of the small molecule metabolic products, can be utilised

to analyse muscle synthesis and degradation amongst other biological processes (405). It does not require muscle tissue but uses plasma or urine samples. This is a relatively recent technique which warrants further investigation particularly for populations where muscle biopsy is contra-indicated. However, a recent study found only modest correlations between muscle and plasma metabolite levels suggesting further work on is required before this can be accurately used as a substitute for muscle tissue (290).

Chapter 5 describes neutrophil function in well-defined populations of healthy and frail older adults. It is only the second investigation of innate immune function in frail older adults and the first to provide clear associations between parameters of frailty, including physical and cognitive function, and immunosenescence. The chapter describes inaccurate neutrophil migration in frail older adults to two chemoattractants and investigates the fundamental processes that may be contributing to neutrophil immunosenescence including cell surface markers and the PI3kinase/Akt pathway. It demonstrates that poor neutrophil accuracy in frail older adults can be rescued with the PI3kinase inhibitors which are most highly expressed in neutrophils (delta and gamma) and there was a trend towards this phenotype being induced in neutrophils from healthy young adults following incubation with pooled plasma from frail older adults. It also describes a state of chronic inflammation in frail older adults and investigates the effect constant exposure of neutrophils to priming and stimulating agents may have on neutrophil function. It proceeds to investigate neutrophil subpopulations and neutrophil states (for example primed and activated neutrophils) to consider the link between the demonstrated poor neutrophil function with the relative neutrophilia found in frail older adults.

The key finding from Chapter 5 is that inaccurate neutrophil migration in frail older adults is both age independent and associated with physical and cognitive markers of frailty. Inaccurate neutrophil migration is correctable with Class 1 A delta and Class 1 B gamma selective PI3kinase inhibitors and this suggests a potential therapeutic target for future research in both frailty and immunesenescence within frailty.

Therapeutic PI3kinase inhibitors have already been developed for use in a number of malignancies and auto-immune and inflammatory driven conditions (406). Idelalisib, a selective PI3kinase δ inhibitor, has been approved by the FDA for treatment of haematological malignancies and a number of other pan and selective PI3kinase inhibitors are in clinical development (407). However, the side effect profile of these drugs may preclude their use in frail older adults. Serious side effects reported for Idelalisib include neutropenia, liver damage and colitis (408, 409). Even the mild side effects (nausea, diarrhoea and anorexia) reported in the phase one trials and tolerated well by middle-aged, previously healthy adults could have significant consequences in a frail older adult population (410). The dose required in oncological management is far higher than would likely be required for the correction of immunesenescence in frail older adults but the side effect profile and expense may still prevent PI3kinase inhibitors from clinical use.

A further consideration is the effect of PI3kinase inhibitors on intra-cellular feedback responses and whether long-term exposure will result in 'resistance' to the inhibitors. This has been described in cancer and is reported to be as a consequence of insufficient pathway inhibition, pathway reactivation or activation of complementary pro-survival

pathways (411). The intra-cellular pathways and mechanisms resulting in pathology are very different in cancer and immunesenescence but the effect of chronic PI3kinase inhibition would have to be investigated before it could be considered as a viable therapeutic option in frail older adults. The use of PI3kinase inhibitors as an adjuvant therapy during infection or periods of acute frailty, such as following a fractured neck of femur, may negate this potential effect but again would need to be investigated further.

The repurposing of current treatments is popular for a number of reasons including the reduced cost of drug development and the subsequent cost of a prescription.

Repurposed treatments are particularly attractive in frail older adults as the side effect profile has already been well characterised and characterised in a frail population rather than a study population. Observational studies of statins have demonstrated an association with both a reduction in infections and mortality in a number of populations(412-414). Statins reduce the production of all products down-stream of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) including isoprenoid. Isoprenoid is utilised by cells for post-translational modification and activation of small GTP-ases such as Ras and Rho (235). These molecules are essential in a number of immune cell functions such as adhesion and migration and importantly are also down-stream of the PI3kinase-Akt pathway (235). In a small clinical trial, statins were associated with improved neutrophil migration in a trial of healthy older adults (415) and adjuvant statins were associated with survival benefit in hospitalised patients with community acquired pneumonia including a cohort of older adults (236). Statins target a downstream effector of PI3kinase and offer a therapeutic option for immunesenescence in frailty which is

cheaper, safer and more readily available than PI3kinase inhibitors. Whilst there are a number of issues that would need to be resolved prior to the consideration of statins as a therapeutic option for immunesenescence in frail older adults, particularly related to their effect on muscle at high doses, statins appear to be a better therapeutic option.

The data presented in Chapter 5 demonstrated an association between physical and cognitive parameters of frailty and neutrophil function. However, this study was limited to commenting on association and was unable to provide evidence of a causative link between immunesenescence and frailty. There are a number of potential hypotheses: one, immunesenescence causes and drives frailty, two, frailty causes and drives immunesenescence, three, frailty is associated with immunesenescence but the conditions are not causally linked, four, and the most likely, frailty and immunesenescence are co-causative, each condition driving the other in a spiral of propagation. A potential study method for considering causation would be the reversal of frailty and the investigation of the subsequent effect on immunesenescence. To achieve this comprehensive clinical and immune characterisation should be conducted on a group of frail individuals before and after a frailty intervention programme. If frailty reversal is associated with an improvement in immunesenescence then a causative link can be established. However, this study design is unable to comment on initial causation and temporal associations.

To investigate this further and comprehensively consider causation between immunesenescence and frailty multiple additional studies would be necessary. The first study would investigate the effect on immunesenescence of the reversal frailty with a

multi-factorial intervention. Additionally a second study investigating the effect on frailty of the in vivo reversal of immunesenescence with either a statin or PI3kinase inhibitor would be necessary. If both the studies demonstrated a positive effect then the in vivo reversal of immunesenescence with either a statin or PI3kinase inhibitor and the reversal of frailty with a multi-factorial intervention could be investigated in conjunction to consider whether there is a synergistic effect of the two interventions together.

Chapter 5 provides unique evidence of associations between parameters of frailty, including physical and cognitive function, and immunesenescence.

Chapter 6 describes the three groups, healthy young, healthy older and frail older adults, in detail and demonstrates the experiments described within Chapters 3, 4 and 5 were conducted on appropriate populations. The chapter does also highlight some differences between the groups, particularly the socio-economic class and age; this may have had some effect on the reported results. The chapter then proceeds to examine the nature of the frailty identified in the frail older adult group and suggests this group may not be representative of all frail older adults. The role of inflammation in frailty and sarcopenia is then considered with respect to the propagation of these conditions. The experiments were underpowered for conclusive results but suggest the relationship between inflammation and frailty is more complex than a positive linear association. The key finding from Chapter 6 is that it is feasible to recruit a well characterised frail older adult population for mechanistic immunesenescence work and relate parameters of physical health with immune health. Chapter 6 also suggests multiple avenues for further investigation and these will be discussed later.

7.2 Strengths and limitations

The strengths and limitations of the work presented in this thesis are discussed in depth within each relevant chapter. Validated and reputable laboratory techniques were employed throughout to ensure the results generated were accurate, representative and translatable. This is discussed in more detail in Chapter 5. The clinical methods were equally rigorously selected as discussed in Chapter 6.

There are a number of additional limitations to the laboratory techniques and data that have not been adequately discussed in Chapter 5. The study relied on isolated neutrophils extracted from whole blood for migratory and oxidative burst studies and neutrophils present in whole blood for flow cytometry studies. These intravascular cells may not be representative of extravascular neutrophils and it is possible that impaired chemotaxis in frail older adults is not present in neutrophils transmigrating through tissue. Throughout the experiments non-physiological methods were used: two-dimensional model of migration without endothelial cells or laminar flow and non-physiological concentrations of priming and stimulating agents. Attempts were made to make conditions as physiological as possible, for example, the addition of albumin coating to the coverslips prior to neutrophil migration.

The most significant limitation of the clinical methods utilised in this study is the use of ultrasound as the sole diagnostic tool for the identification of low muscle mass in sarcopenia. Whilst this tool has been suggested before as a potential method for diagnosis of sarcopenia it has not been validated in this population and diagnostic criteria

for the identification of low muscle mass are not available. Chapter 3 provides some evidence for the utilisation of ultrasound as a diagnostic tool but due to cost limitations was unable to compare ultrasound to a gold standard technique.

Throughout the results chapters one strength and one limitation of the work persist and therefore deserve further analysis and discussion.

The rigorous characterisation of the three different groups is the greatest strength of the work presented in this thesis. The results presented in these chapters link immunesenescence data with clinical characteristics. The employment of a translational study design is particularly important in this study because older and, in particular, frail older adults are not heterogenous groups. The meticulous characterisation of each individual allowed the relationships between the parameters of frailty, such as physical and cognitive performance, with immunesenescence and inflammaging to be considered. Whilst not unique to this study it is the first time extensive investigations of neutrophil function have been associated with parameters of frailty. Previous research reviewing neutrophil function in older adults has either neglected to consider the effect of frailty or assumed its presence without proper characterisation (290, 313). The data presented in Chapter 5 succinctly illustrates the mirroring between the decline in physical and cognitive function, and neutrophil function.

Whilst the meticulous characterisation of all the individuals in this study is unique in the field of immunesenescence the depth and breadth of characterisation is also unusual in more clinical studies of frailty. The multi-faceted description of each individual permitted extensive comparisons and associations to be made within the clinical data. The

availability of this data facilitated the rigorous analysis of muscle biopsy failure in frail older adults, disparity in frailty severity measured by different methods and the association of frailty severity with different parameters of Fried phenotype and other unique conclusions.

The greatest limitation of the study can also be considered a strength of the study. The majority of the work described in this thesis had never been completed before in this population and therefore calculating accurate sample sizes prior to study recruitment was impossible. The data is therefore unique but also has to be considered pilot data and as a consequence a number of the experiments were underpowered. This makes it difficult to interpret the data conclusively and furthermore, most of the underpowered data followed a non-parametric distribution which makes calculating the desired sample size problematic. The discussion in Chapter 5 reports on a number of post-hoc calculated sample sizes in data that was not originally statistically significant. This suggests the sample sizes needed to be increased but not by a substantial number. On occasions in the thesis data is presented where an alternative study design would be preferential. However, due to the paucity of research in frailty and sarcopenia particularly related to the association of these syndromes with immunesenescence and inflammaging it was important to report all these results as the data may contribute to current understanding and suggest future directions for research.

A limitation with all the data presented in this study is that it is entirely cross-sectional. This is adequate to consider the main hypotheses of this thesis but when considering the more complex relationships between frailty, sarcopenia and immunesenescence cross-

sectional data it is an unsuitable form of investigation. Perhaps the greatest limitation of this study was its inability to confirm whether witnessed dysregulation of PI3kinase was present in the muscle of frail and sarcopenic older adults. The failure of muscle biopsy in frail older adults prevented the investigation of intra-myocyte PI3kinase dysregulation and prohibited the coupling of frailty and sarcopenia with a single intra-cellular pathway. .

7.3 Future directions

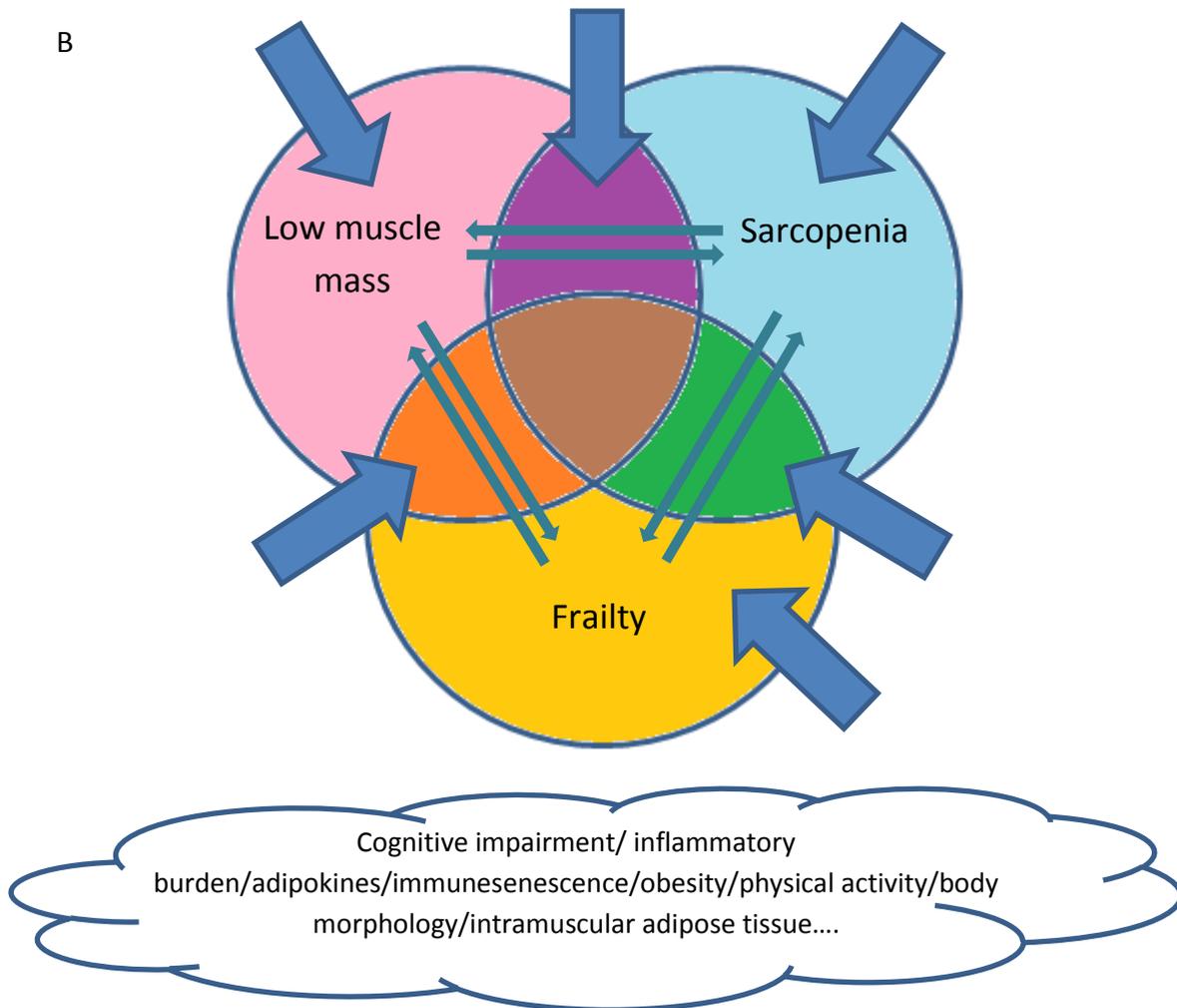
The work presented in this thesis has described immunosenescence and inflammaging in a cross-sectional population of older adults with both frailty and sarcopenia. The results generated have inevitably led to further questions particularly regarding the relationship between frailty and sarcopenia and the drivers and propagators of both syndromes. Whilst Chapter 6 affirmed that sarcopenia and frailty can exist independently it also suggested that although they are intrinsically linked a simple progressive linear relationship is unlikely to fully describe the relationship. Figure 7.1 depicts both the simplistic linear relationship and a more complex hypothesised relationship between the conditions that does not necessitate sarcopenia as a precursor to frailty. Whilst many of the drivers of the conditions have been considered their role in the progression and pathophysiology of the syndromes is incompletely characterised and further research in to how these different conditions and health states all link together is essential.

Figure 7.1 – Relationship between sarcopenia and frailty

A



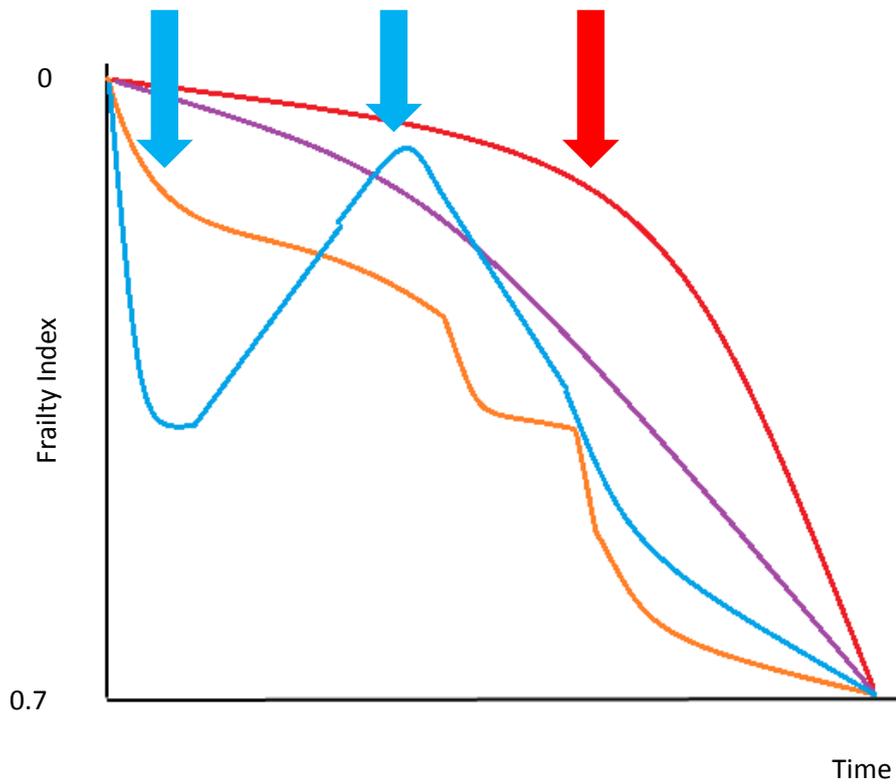
B



Relationship between sarcopenia and frailty. Figure A depicts a simple linear relationship where low muscle mass is a pre-requisite of sarcopenia and sarcopenia is a pre-requisite of frailty. Figure B proposes a more complex relationship where all conditions can exist in isolation but also co-exist with each other in a variety of combinations. The bubble below describes some of the potential drivers of the conditions and the arrows represent the multiple entry points to the frailty cycle.

Frailty as described by the Frailty Phenotype considers frailty a dichotomous event either present or absent. Conversely frailty as described by the Frailty Index is a continuous variable which can be plotted against time to create a trajectory of frailty. Patterns of health and functional decline before death have been described before (416). However, they have often focused on specific diseases, such as cancer or single organ failure, to further our understanding of the process of dying rather than investigate the trigger points of declining function. Accurate characterisation of changes in frailty severity using a Frailty Index to stratify frailty over a period of time would identify trigger points for both increasing and decreasing frailty. Modelling this data with both clinical and immunological parameters would allow for rigorous investigations in to the pathophysiology of the frailty syndrome and clinical outcomes of the syndrome. Figure 7.2 depicts several theoretical examples of trajectories of frailty.

Figure 7.2 – Trajectories and triggers of frailty



Trajectories and triggers of frailty. X axis represents time and Y axis represents Frailty Index. The Y axis is inverse and extends from 0.7 to 0 because a Frailty Index greater than 0.7 is not believed to be compatible with life. Four lines are depicted each representing a different trajectory of frailty. The red line depicts an initial gradual decline and then at the trigger point, denoted with a red arrow, a sharp decline in frailty. The purple line depicts a gradual descent in to frailty with no specific trigger points. The blue line depicts a trajectory with two trigger points: the first where there is a rapid improvement in frailty perhaps during a period of rehabilitation and the second where there is rapid decline in frailty perhaps during a period of acute illness. The orange line represents a step-wise progression in frailty with small incremental increases in frailty over a longer period of time.

The key research questions generated by this thesis can be summarised as:

- 1) Are frailty and sarcopenia separate syndromes, or different manifestations of the same pathophysiological process?
 - a. What are the common pathophysiological pathways with particular respect to immunesenescence and inflammaging?
 - b. Is there a final common pathophysiological intra-cellular pathway in both conditions?
 - c. What are the triggers that result in the accumulation of an additional syndrome, either frailty or sarcopenia?
 - d. What are the protective factors that prevent an individual from accumulating an additional syndrome?

- 2) What are the protective factors and triggers that result in a change in frailty state?
 - a. Is it possible to predict from biomarkers or measures of physical or cognitive health the trajectory of frailty state over time?
 - b. Does a state of inflammation and immunesenescence mirror the progression or trajectory of a frailty state?

To consider these two areas of investigation further work could be conducted using a longitudinal study design comparing multiple cohorts. The cohorts that should be actively recruited to are: healthy older adults, healthy older adults with low muscle mass, healthy older adults with sarcopenia, frail older adults without physical frailty (Frailty Index positive but not Frailty Phenotype positive), frail older adults with physical frailty (both Frailty Index and Frailty Phenotype positive). It could be argued it would be unnecessary to include a

healthy younger adult group as the important comparison is between different states of health and function in old age rather than with changes with ageing itself.

All individuals in these groups would undergo extensive characterisation as described in Chapter 2. The results from the thesis have highlighted a few potential modifications to the original protocol which would improve the quality of future data.

One, abdominal circumference measured in centimetres with a tape measure. Previous research has highlighted the importance of obesity in frailty and sarcopenia and suggested that central abdominal obesity is associated with poorer outcomes (388, 389). Data presented in Chapter 6, although inconclusive, suggest changes in body morphology with frailty and sarcopenia are different in men and women which may be related to hormonal changes. The inclusion of abdominal circumference will allow more complex analysis of changes in body morphology particularly when reviewed in conjunction with the ratio of subcutaneous tissue to muscle thickness and BMI.

Two, ensuring all individuals complete the Stroop Interference assessment. This assessment was incomplete in the majority of frail older adults due to time constraints and perceived burden. However, it is quick to administer and can be completed in five minutes and should be prioritised within the assessment. As demonstrated in Chapter 6 the Stroop Interference test was sensitive in eliciting differences in cognition between healthy younger and healthy older adults. This suggests it may be an excellent test for describing small changes in cognitive ageing which are not apparent on less sensitive tests such as the Addenbrooke's cognitive examination. It therefore may also be better able to describe change in cognition over a period of time which is essential in longitudinal studies.

Three, a structured approach to the categorisation of socio-economic status, diet and exercise. Diet, activity habits and lifestyle are important contributing and ameliorating factors in both frailty and sarcopenia. It is therefore essential to document and consider these factors when analysing the data. There are inherent difficulties in measuring both diet and exercise as they are often inaccurately self-reported. There is also a lack of validated tools reviewing both these variables over an individual's preceding lifetime rather than as a single cross-sectional assessment. Therefore, the most appropriate tool for measuring these three variables would need to be first carefully considered.

It is anticipated the individuals recruited to the different groups would all be regularly reassessed using a combination of telephone interviewing and face to face comprehensive geriatric assessment. Telephone interviewing would use structured assessments such as PRISMA 7, Katz and Lawton to gather information on health state. It could also be possible to devise a Frailty Index comprised entirely of self-reported information and this would ensure a standardised regular assessment of frailty throughout the study period. Face to face assessment would follow the comprehensive geriatric assessment described in Chapter 2 with the addition of the suggested modifications detailed above.

Data from both the local electronic hospital system and primary care electronic health records could be included to review changes in frailty state. This would allow for more subtle assessment of trigger points and drivers of frailty and sarcopenia. Certain events, such as change in the eFI or admission with delirium, could trigger a more detailed assessment.

Alongside the rigorous clinical characterisation there would be regular assessment of biological and immunological health. This would include; assessment of endocrine health

using previously suggested biomarkers such as DHEA, DHEAS, GH and IGF-1 (417).

inflammatory burden, the data presented in this thesis in Chapters 5 and 6 could be used to inform a unique frailty inflammatory ELISA kit. This would include all the cytokines with a significant difference between either the three or four groups analysed in Chapters 5 and 6: IL-1ra, IL-1 β , IL-4, IL-8, IL-17, IP-10, MIP1 α , Eotaxin, FGF, MCP-1. It will also include the cytokines, TNF α , IL-6 and IL-10; in this thesis there was no significant difference in the concentration of these three cytokines but previous work has demonstrated an association of these three cytokines with frailty and sarcopenia (169, 178, 185, 187, 195, 302).

Characterisation of immunesenescence would be of importance. In the quantification of immunesenescence it is impossible to study all the immune functions in each individual but it is important to consider the interaction and cross-talk between immune cells. A practical biomarker of immunesenescence which allows serial testing of multiple samples in a short time-frame would be ideal for this type of longitudinal research but is as yet uncharacterised. Neutrophil migration has been proved in this thesis to be compromised in frail individuals and is therefore a validated technique in frail older adults but it is a flawed choice because it is neither physiological nor time-efficient.

The longitudinal study design including both rigorous clinical and biological characterisation of individuals will allow for complex modelling of the data to investigate patterns of change in severity of frailty and sarcopenia plus importantly identify the trigger points of descent in to more severe disease.

This thesis has précised some of the difficulties with recruiting frail individuals to a cross-sectional study investigating immunesenescence. The complexities of recruiting frail older adults to a longitudinal study are likely to be more extensive but also far more rewarding.

To capitalise on the potential for novel research the longitudinal study design could be used as a platform to perform spin-out cross-sectional research. Important potential spin-out projects include comparing ultrasound with a gold standard diagnostic tool in the diagnosis of sarcopenia. This would hopefully confirm the accuracy of ultrasound and the BATT as a tool in the identification of low muscle mass in sarcopenia and therefore allow the adoption of both these methods in research and clinical practice. Another essential project would be in depth immune-phenotyping in frailty and sarcopenia. This would focus on not just neutrophils but also include other innate and adaptive immune cells. This research could initially investigate whether previously reported effects with ageing are actually an effect of frailty rather than ageing per se. The longitudinal research study would also offer a well-characterised population amenable for intervention studies. It would be particularly suited to intervention studies requiring mechanistic work investigating the effect of the intervention.

7.4 Concluding remarks

This study has proved it is feasible to recruit a well characterised frail older adult population for mechanistic immunosenescence work and relate parameters of physical health with immune health. It has also reported some of the first evidence of the association of immunosenescence with parameters of frailty and demonstrated this effect is independent of age. The breadth of the data reported in this thesis reflects not only geriatrics as a speciality but the lack of rigorous research into frailty. The general discussion has not only highlighted some of the key findings of this work but has also suggested avenues for future investigation to further our understanding of both frailty and sarcopenia.

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9. Appendices

9.1 Publications arising from this thesis

Papers

Jackson T, **Wilson D**, Grieg C. *Frailty and Ageing in The Ageing Immune System and Health*. Springer October 2017: 141-159.

Drew W, **Wilson D**, Sapey E. *Frailty and the immune system*. Journal of Ageing Research and Healthcare. June 2017

Wilson D, Jackson T, Sapey E, Lord J. *Frailty and Sarcopenia: The potential role of an aged immune system*. Ageing Research Reviews. Feb 2017

Oral Presentations

Wilson D. Neutrophils exhibit a frailty related decline in migratory accuracy. June 2017. MRC-ARUK Centre for Musculoskeletal Ageing Research Annual Conference.*¹

Wilson D. Is cognitive frailty 'functional sarcopenia'? October 2016. European Union Geriatric Medicine Society. Lisbon.

Wilson D. Frailty and immunesenescence – not just association. September 2015. West Midlands Geriatrics Society Autumn Meeting.

Poster Presentations

Wilson D, Stringer H, Moorey H, Sahbudhin I, Sapey E, Lord J. Ultrasound as a diagnostic tool for the identification of low muscle mass and poor muscle quality in sarcopenia. November 2017. British Geriatrics Society Autumn Conference, London.

Wilson D, Drew W, Sapey E, Lord J. Neutrophils exhibit a frailty related decline in migratory accuracy which can be improved with PI3K inhibition. November 2017. British Geriatrics Society Autumn Conference, London.*²

Wilson D, Jackson T, Sapey E, Lord J. Is cognitive frailty 'functional sarcopenia'? October 2016. European Union Geriatric Medicine Society. Lisbon.

Wilson D, Jackson T, Sapey E, Lord J. Ultrasound echogenicity is a better predictor of strength and speed than age. October 2016. European Union Geriatric Medicine Society. Lisbon.

Wilson D, Sapey E, Lord J. Frailty is associated with immunesenescence. Neutrophil migration is slower and less accurate in frail older individuals. October 2016. European Union Geriatric Medicine Society. Lisbon.

*1 - 2nd prize in the early career researchers presentation at MRC-ARUK Centre for Musculoskeletal Ageing Research Annual Conference 2017.

*2 – Norman Exton-Smith prize for the best Scientific Presentation post Norman Exton-Smith prize for the best Scientific Presentation poster at Autumn BGS 2017

9.2 Case Report Form

