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## Immune Dysfunction and Inflammation in NAFLD

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## Declaration

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## Summary

With a global prevalence of 25-40%, non-alcoholic fatty liver disease (NAFLD) is fast becoming the commonest cause of liver disease in the developed world [1]. Defined as increased hepatic lipid accumulation, approximately 25% of individuals with NAFLD go on to develop progressive disease with non-alcoholic steatohepatitis (NASH) and fibrosis, leading to cirrhosis, with increased risk of decompensated chronic liver disease and hepatocellular carcinoma[2, 3]. Immune dysregulation with chronic inflammation is central to the pathophysiology of NAFLD [4, 5]. The liver functions as a key immune organ, with a large population of resident leukocytes, including Neutrophils, T lymphocytes and Mucosal associated invariant T (MAIT) cells[6]. Neutrophils, considered first responders, migrate to the liver in early NASH, promoting chemotaxis in addition to M1 (pro-inflammatory) differentiation of macrophages[7]. Both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes have also been shown to promote NASH, similarly stimulating M1 macrophage differentiation in addition to intrahepatic lipid uptake and fibrosis via stellate cell activation[8]. MAIT cells, are innate like T lymphocytes that have been shown to exhibit a Th1 phenotype, capable of producing copious amounts of pro-inflammatory mediators upon stimulation, perpetuating inflammation in both metabolic and chronic liver diseases[9, 10]. It is likely that activation of these resident immune cells in NAFLD, in addition to the preferential hepatic migration of circulating leukocytes, is critical in promoting the inflammation which drives liver disease progression, however, the non-invasive identification of hepatic inflammation remains a diagnostic challenge in clinical practice[2, 11].

Chapter 2 of this thesis details a descriptive analysis of circulating and intrahepatic immune cells with a focus on MAIT cells assessing for correlates of histological severity. The results show that circulating monocytes are significantly reduced and this reduction is associated with NASH, although there were no significant immunophenotypic changes on flow cytometric analysis. Circulating MAIT cells, particularly CD8<sup>+</sup> MAIT cells and CD8<sup>+</sup> T lymphocytes were reduced in comparison to healthy controls with advancing histological inflammation and fibrosis. Interestingly, on review of the intrahepatic compartment, only CD8<sup>+</sup> T lymphocytes were significantly reduced in NASH and advanced fibrosis. Composite quantification of circulating monocytes, neutrophil-lymphocyte ratio, CD8<sup>+</sup> T lymphocytes

and circulating MAIT cells was found to strongly correlate with NASH and significant hepatic fibrosis, with a greater AUC for advanced fibrosis compared to non-invasive methods currently employed in clinical practice to estimate NAFLD fibrosis inclusive of: FIB-4, NAFLD score and VCTE measurements.

In chapter 3, changes in circulating and intrahepatic MAIT cells in association with dietary and exercise-based weight loss interventions were analysed. A 12-week dietary intervention program resulted in clinically and statistically significant weight reduction, which correlated with reductions in expression of the activation marker CD69 on circulating MAITs. Within the liver, weight loss correlated with a reduction in hepatic steatosis but was not associated with changes in histological features of inflammation or fibrosis. Weight loss was also not associated with any changes in the number or activation marker expression of intrahepatic MAIT cells. In contrast, despite more modest reductions in weight, the exercise intervention led to significant improvements in histological inflammation (as defined by hepatocyte ballooning) and fibrosis. These histological improvements were associated with increased expression of the terminal activation marker CD95 and reduction in the percentage of intrahepatic MAIT cells, suggesting that exercise has unique immunomodulatory effects promoting accelerated apoptosis of MAIT cells. These results support a potential pathogenic role of MAITs, with intrahepatic depletion coinciding with fibrosis regression.

Finally, Chapter 4 investigated clinical features of systemic inflammation and accelerated ageing inpatients with NAFLD by performing frailty measures in a cohort of patients across a spectrum of disease severity. The results confirmed a high prevalence of frailty and pre-frailty amongst individuals with non-cirrhotic NAFLD, most notably amongst female patients. The frailty measures: SRFI, FI-LAB and 30STS were significantly associated and positively correlated with fibrosis stage.

All participants for the studies in this thesis had a clinical diagnosis of NAFLD and were recruited from the Hepatology department at St. James' Hospital in Dublin, allocation to the different intervention groups was performed by convenience sampling.

In conclusion, NAFLD is a state of chronic systemic inflammation resulting from persistent immune cell activation. Alterations in circulating immune cells both correlate with and are predictive of liver disease severity, whilst changes in intrahepatic MAIT cell populations are associated with histological improvements post exercise intervention. This chronic inflammatory state is also a factor in the development of frailty, with high rates of frailty and pre-frailty seen even in individuals with early stage NAFLD.

These findings identify immunological changes that can be used to improve the non-invasive staging of NAFLD severity, in addition to highlighting effective therapeutic strategies and potential novel targets that can be used to improve outcomes for patients with NAFLD.

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### List of Abbreviations:

|              |                                                     |
|--------------|-----------------------------------------------------|
| AASLD        | American association for the study of liver disease |
| ALT          | Alanine aminotransferase                            |
| AST          | Aspartate aminotransferase                          |
| BAFF         | B cell activating factor                            |
| Bcl-2        | B cell lymphoma 2                                   |
| BMI          | Body Mass Index                                     |
| BSA          | Bovine serum albumin                                |
| CAP          | Controlled Attenuation Parameter                    |
| CCL2         | Chemokine (C-C motif) ligand 2                      |
| CCR          | Chemokine receptor                                  |
| CXCL         | Chemokine ligand                                    |
| CI           | Confidence interval                                 |
| CTLA4        | Cytotoxic T lymphocyte associated protein 4         |
| DAMPs        | Damage associated molecular patterns                |
| EASD         | European association for the study of diabetes      |
| EASL         | European association for the study of liver disease |
| EASO         | European association for the study of obesity       |
| F-AST        | Fibroscan-AST score                                 |
| FIB-4        | Fibrosis-4 index for liver fibrosis                 |
| FFI          | Fried frailty index                                 |
| FI-LAB       | Lab based frailty index                             |
| GGT          | Gamma glutamyl transferase                          |
| GLP-1        | Glucagon-like peptide 1                             |
| GLUT-1       | Glucose transporter 1                               |
| HBSS         | Hanks' balanced salt solution                       |
| HDL          | High density lipoprotein                            |
| HIF $\alpha$ | Hypoxia inducible factor $\alpha$                   |
| HLA-DR       | Human leukocyte antigen complex                     |

|            |                                                                |
|------------|----------------------------------------------------------------|
| IFN        | Interferon                                                     |
| IL         | Interleukin                                                    |
| IL 1RA     | Interleukin 1 receptor antagonist                              |
| IQR        | Interquartile range                                            |
| iNKT cells | Invariant Natural Killer T cells                               |
| kPa        | Kilopascals                                                    |
| LAG-3      | Ligand lymphocyte activation gene-3                            |
| LAMP-1     | Lysosomal-associated membrane protein-1                        |
| LFA-1      | Lymphocyte function-associated antigen 1                       |
| LSM        | Liver stiffness measurement                                    |
| MAIT cells | Mucosal Associated Invariant T cells                           |
| MFI        | Mean fluorescence intensity                                    |
| MCP-1      | Monocyte chemotactic factor 1                                  |
| MHC        | Major histocompatibility complex                               |
| MHR        | Monocyte HDL ratio                                             |
| MR1        | Major histocompatibility complex, class I related              |
| MTORC1     | Mammalian target of rapamycin complex 1                        |
| NAFL       | Nonalcoholic fatty liver                                       |
| NAFLD      | Nonalcoholic fatty liver disease                               |
| NASH       | Nonalcoholic steatohepatitis                                   |
| NFκB       | Nuclear factor kappa light chain enhancer of activated B cells |
| NK cells   | Natural killer cells                                           |
| NLR        | Neutrophil lymphocyte ratio                                    |
| NLRP3      | NACHT, LRR and PYD domains-containing protein 3 and cryopyrin  |
| 5-OP-RU    | 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil               |
| OSE        | Oxidation specific epitopes                                    |
| PBA        | PBS/BSA/Sodium azide                                           |
| PBS        | Phosphate buffer solution                                      |
| PBC        | Primary biliary cholangitis                                    |
| PD-1       | Programmed cell death protein 1                                |

|                  |                                                                                                           |
|------------------|-----------------------------------------------------------------------------------------------------------|
| PIVENS           | Pioglitazone versus vitamin E versus placebo for the treatment of non-alcoholic steatohepatitis in adults |
| PNPLA3           | Patatin-like phospholipase domain containing protein 3                                                    |
| PPAR $\gamma$    | Peroxisome proliferator-activated receptor $\gamma$                                                       |
| PSC              | Primary sclerosing cholangitis                                                                            |
| RPMI             | Roswell Park Memorial Institute solution                                                                  |
| ROC              | Receiver operator curve                                                                                   |
| ROS              | Reactive oxygen species                                                                                   |
| SASP             | Senescence associated secretory phenotype                                                                 |
| SGLT-2           | Sodium glucose co-transporter 2                                                                           |
| SLE              | Systemic lupus erythematosus                                                                              |
| SMP 30           | Senescence marker protein 30                                                                              |
| SRFI             | Self-reported frailty index                                                                               |
| 30STST           | 30 second sit to stand                                                                                    |
| TCR              | T cell receptor                                                                                           |
| TGF $\beta$      | Transforming growth factor $\beta$                                                                        |
| TIM-3            | T-cell immunoglobulin domain and mucin domain 3                                                           |
| TM6SF2           | Transmembrane 6 superfamily member 2                                                                      |
| TNF $\alpha$     | Tumor Necrosis Factor $\alpha$                                                                            |
| T <sub>reg</sub> | T regulatory lymphocytes                                                                                  |
| TUG              | Time to up and go test                                                                                    |
| VAP-1            | Vascular adhesion protein-1                                                                               |
| VCTE             | Vibration controlled transient elastography                                                               |
| VLDL             | Very low-density lipoprotein                                                                              |

## Chapter 1: Introduction

# 1 Introduction

## *1.1 Obesity and metabolic syndrome*

Over the last 50 years, the prevalence of obesity and the metabolic syndrome has steadily risen, reaching epidemic proportions, with approximately 1.9 billion people worldwide classed as overweight (with a BMI  $\geq 25$  kg/m<sup>2</sup>) and a further 650 million, classed as obese (BMI  $\geq 30$  kg/m<sup>2</sup>)[12]. Ireland has now become the second most obese country in Europe with 28.6% of the population being overweight and 25.9% obese [12, 13] (*See Fig. 1-1*).

This is of particular concern as obesity represents a complex, multi-system disease with significant associated morbidity and mortality. Obesity is the number one cause of premature death worldwide and has been linked with increased risk of numerous disease states, including: cardiovascular disease, stroke, chronic kidney disease, metabolic liver disease, infertility, dementia, cancer and diabetes, accounting for up to 70% of cases (*See Fig. 1-2*) [2-4].

## Obesity prevalence

### Adults, 2019

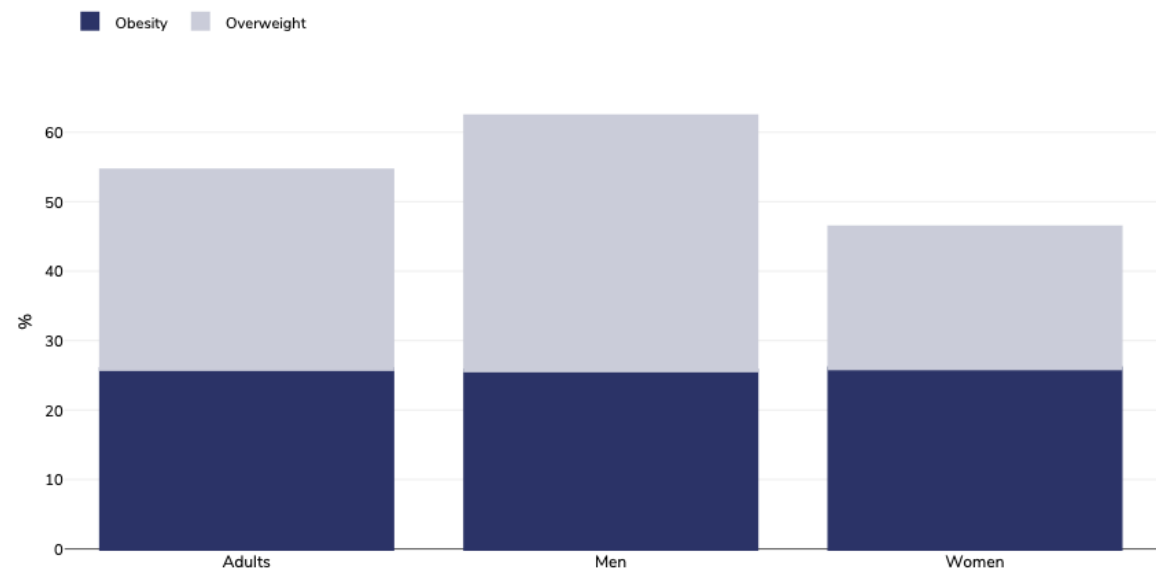


Figure 1-1 Graphical representation of overweight and obesity rates amongst Irish adults 2019  
[https://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=hlth\\_ehis\\_bm1i&lang=en](https://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=hlth_ehis_bm1i&lang=en) (last accessed 09.08.21)

Given its high prevalence and wide spectrum of associated pathology, obesity also has a significant economic impact, costing approximately 2.8% of the global gross domestic product per year [14, 15].

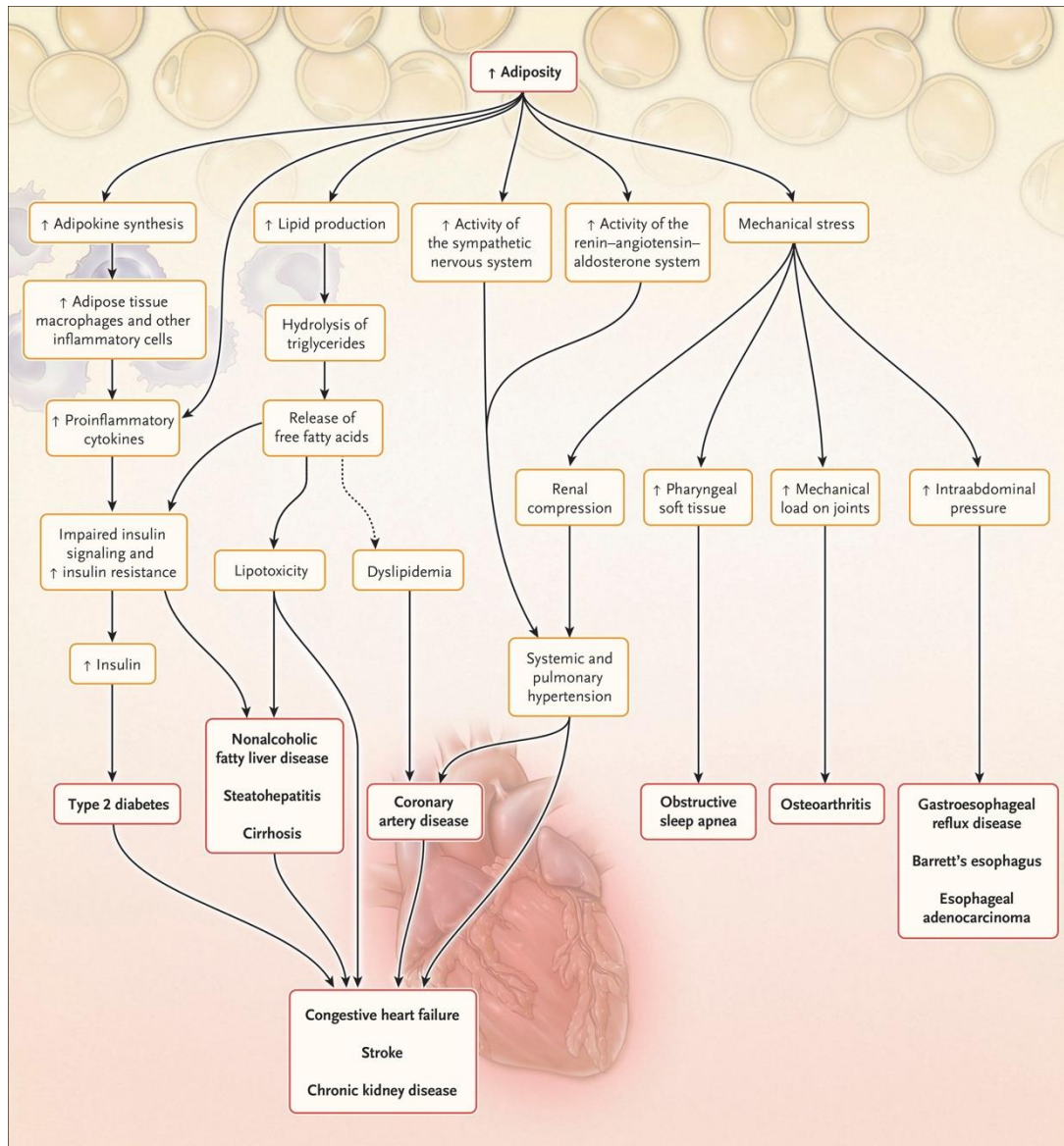


Figure 1-2 Pathways between adiposity in obesity and chronic diseases, Heymsfield et al. [2]

### 1.2 Obesity and Metabolic syndrome as an immune dysregulated, pro-inflammatory state

The pathogenesis of obesity is multifactorial with genetic factors and associated changes in gut microbiome in addition to psychosocial and environmental factors [16-18]. However, at its core, the primary mechanism is centered around an imbalance between energy intake and expenditure.

One of the essential adipokines involved in energy homeostasis is leptin [19, 20]. In healthy individuals, circulating leptin levels increase in response to increased energy stores [19]. Leptin acts on the hypothalamus promoting satiety, reducing further caloric intake and boosting energy expenditure by increasing glucose and fatty acid metabolism by adipocytes, skeletal muscle, and the liver [19, 21]. This signaling pathway is dysregulated in obesity; although high circulating leptin levels persist, leptin resistance develops, resulting in persistently high caloric intake and reduced catabolism [19, 21].

Adipocytes, which act as key regulators of the body's energy stores, attempt to accommodate this energy imbalance by undergoing functional changes to increase their storage capacity for glucose, fatty acids and triglycerides. Such changes include: hypertrophy, increased cell size and hyperplasia, increase in cell number [21, 22]. Adipocytes display a unique ability to expand by more than ten times their original cellular volume, however this does come at a cost [22]. As adipocytes expand, their perfusional surface area decreases, predisposing the tissue to hypoxia and cellular dysfunction resulting in impaired insulin signaling, glucose uptake, and adiponectin release [21, 22]. Reduced adiponectin impairs glucose uptake and  $\beta$  fatty acid oxidation by adipocytes, the liver and skeletal muscle, leading to hyperglycaemia and increased circulating free fatty acids which in turn promotes diabetes, atherogenesis and fat accumulation within the liver [23]. Hypoxic injury also promotes adipocyte apoptosis, with release of damage associated molecular patterns (DAMPs), hypoxia inducible factor  $\alpha$  (HIF $\alpha$ ), pro-angiogenic factors including vascular endothelial growth factor (VEGF), pro-inflammatory cytokines (IL 1 $\beta$ , IL 6, IL 18, IFN $\gamma$ , TNF $\alpha$ ), and chemotactic mediators (MCP-1, CCL2) that promote leukocyte migration, adipose tissue inflammation and fibrosis [21, 22]. Interestingly, visceral adipocytes are more limited in their capacity for expansion, becoming functionally impaired with smaller increases in size compared to subcutaneous adipose tissue [24, 25]. This results in more pronounced upregulation of pro-inflammatory genes and cytokines [24, 25]. Consequently, visceral adipose tissue is considered more 'metabolically active' and its inflammation is more closely linked to clinical outcomes [24].

### *1.3 The immune system in obesity*

Once the cascade of inflammation has been initiated, the activated immune system creates a positive feedback loop, perpetuating the inflammatory response which further exacerbates insulin resistance and weight gain.

Macrophages consist of two distinct functional subsets: M1 and M2. The M1 subtype has pro-inflammatory functions, classically activated by bacterial lipopolysaccharide and/or Th1 cytokines, releasing pro-inflammatory cytokines including IL 1 $\beta$ , IL 6, IL 12, IL 23, and TNF  $\alpha$  [26]. In contrast, the M2 subtype has a more anti-inflammatory and immunoregulatory role, producing IL 10 and TGF  $\beta$  upon stimulation[26]. In obesity, macrophages preferentially migrate to visceral adipose tissue where they account for up to 40% of the cell population in obesity and are predominantly of the M1 (pro-inflammatory subtype) [19, 21]. They upregulate leptin signaling which increases the expression of the pro-inflammatory cytokines, IL 6 and TNF $\alpha$  [19, 21]. In healthy individuals, adiponectin signaling reduces inflammation by promoting the production of IL 10 and the transition of macrophages to inactive foam cells[19, 21]. In obesity, however, the increased circulating IL 6 and TNF $\alpha$  levels further promote inflammation by inhibiting adiponectin release [27]. These changes can be reversed with significant weight loss, with reductions in the concentration of macrophages within visceral adipose tissue and a phenotypic shift from the M1 to the anti-inflammatory M2 subtype seen post bariatric surgery [21].

Circulating neutrophils are increased in obesity, with higher activation marker (CD66b) expression, and increased circulating concentrations of the neutrophil associated proteins myeloperoxidase and calprotectin [20, 25]. Leptin stimulates neutrophil chemotaxis and proliferation, and inhibits neutrophil apoptosis [20, 25], leading to their early accumulation within visceral adipose tissue. As a result, neutrophils are felt to be key initial potentiators of obesity associated inflammation [21, 25, 28], releasing mediators that directly cause tissue injury, encourage macrophage chemotaxis and polarization to the M1 subtype [28]. This inflammatory activity further contributes to weight gain, and in animal models, myeloperoxidase deletion protects against the development of obesity and diabetes [21].

Following weight loss post bariatric surgery, circulating neutrophil associated proteins decline [21].

Dendritic cells and B lymphocytes are also stimulated in obesity and migrate to adipose tissue where they promote the proliferation and activation of macrophages, CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes [21, 22]. In mouse models of obesity, dendritic cells have an altered phenotype with reduced CD40, CD80, CD86, MHC class I and II expression and higher concentrations of CD11c<sup>+</sup> CD1c<sup>+</sup> cells within adipose tissue [21, 29, 30]. They further contribute to inflammation by releasing higher levels of IL 6, TGF  $\beta$  and IL 23, stimulating T cell differentiation to the pro-inflammatory Th17 subtype[21, 29].

In lean individuals, adipose tissue is predominantly populated by IL 10 producing, regulatory B cells[31, 32]. However, in obesity, leptin mediated signaling is believed to influence the switch of B lymphocytes from the regulatory subtype to the mature pro-inflammatory subtype, amplifying the production of IL 6, IL 8 and TNF $\alpha$  [25, 32]. B cells also promote inflammation through their recruitment and activation of M1 macrophages and CD8<sup>+</sup> T lymphocytes[32]. B cell lacking mice fed high fat diets have reduced adipose tissue infiltration by M1 macrophages, CD8<sup>+</sup> lymphocytes and increased regulatory T lymphocytes; they are noted to exhibit better insulin sensitivity despite their elevated BMI [33, 34].

Regulatory T lymphocytes (T<sub>reg</sub>) help to restrict the magnitude of the immune response, preventing tissue damage and promoting immune tolerance[21]. In healthy individuals they are enriched in white adipose tissue and act by reducing macrophage chemotaxis via reduced CCL2 expression, and increased IL 10 production in addition to favoring M2 macrophage polarization [21, 34, 35]. As IL 10 receptors are also expressed on adipocytes, T<sub>reg</sub> cells are believed to modulate adipocyte function, with in vitro studies showing improved adipocyte insulin sensitivity and lipid uptake in the presence of IL 10 [21, 34-36]. In obesity, there is a negative correlation between circulating T<sub>reg</sub> cells and visceral adipose tissue macrophage infiltration, with more aggressive adipose tissue inflammation in Treg knockout mice. Consequently, their relative depletion in obesity is believed to further promote the inflammatory evolution [21, 34, 35].

By comparison, CD8<sup>+</sup> lymphocytes are enriched and activated in the adipose tissue of obese individuals [25, 37]. They recruit M1 macrophages, promoting their differentiation, stimulating pro-inflammatory cytokine release thereby encouraging inflammation [25, 37]. CD8<sup>+</sup> knockout mice exhibit lower levels of circulating IL 6, TNF $\alpha$  and are protected from developing insulin resistance [21, 38].

CD4<sup>+</sup> T lymphocytes are able to differentiate into 4 different subtypes: Th1, Th2, Th17 and Th22 [21]. The Th2 subtype, similar to Treg cells, has anti-inflammatory properties, releasing IL 10 and TGF  $\beta$  [21]. The Th1 and Th17 subtypes however, promote inflammation, secreting IL 2, IL 12, IFN  $\gamma$ , TNF  $\beta$  and IL 17, IL 21, IL 22, IL 24, IL 26 respectively [21, 39]. Th22 lymphocytes also promote inflammation through production of the pro-inflammatory cytokines IL-22, IL-13 and TNF  $\alpha$  [40]. Obesity radically promotes the differentiation and proliferation of the Th17 and Th22 subtypes, increasing circulating levels of IL 17 and IL 22 which correlate clinically with increased risk of diabetes and cardiovascular disease [21, 41, 42]. High IL 17 levels are associated with increased IL 6 production [21]. Circulating IL 6 and TNF $\alpha$  also promote Th22 subtype differentiation, which acts as an additional source of IL 22 [21, 41]. IL 17 and IL 22 not only stimulate macrophages but are also taken up by hepatic and skeletal muscle, impairing their sensitivity to insulin [21, 41].

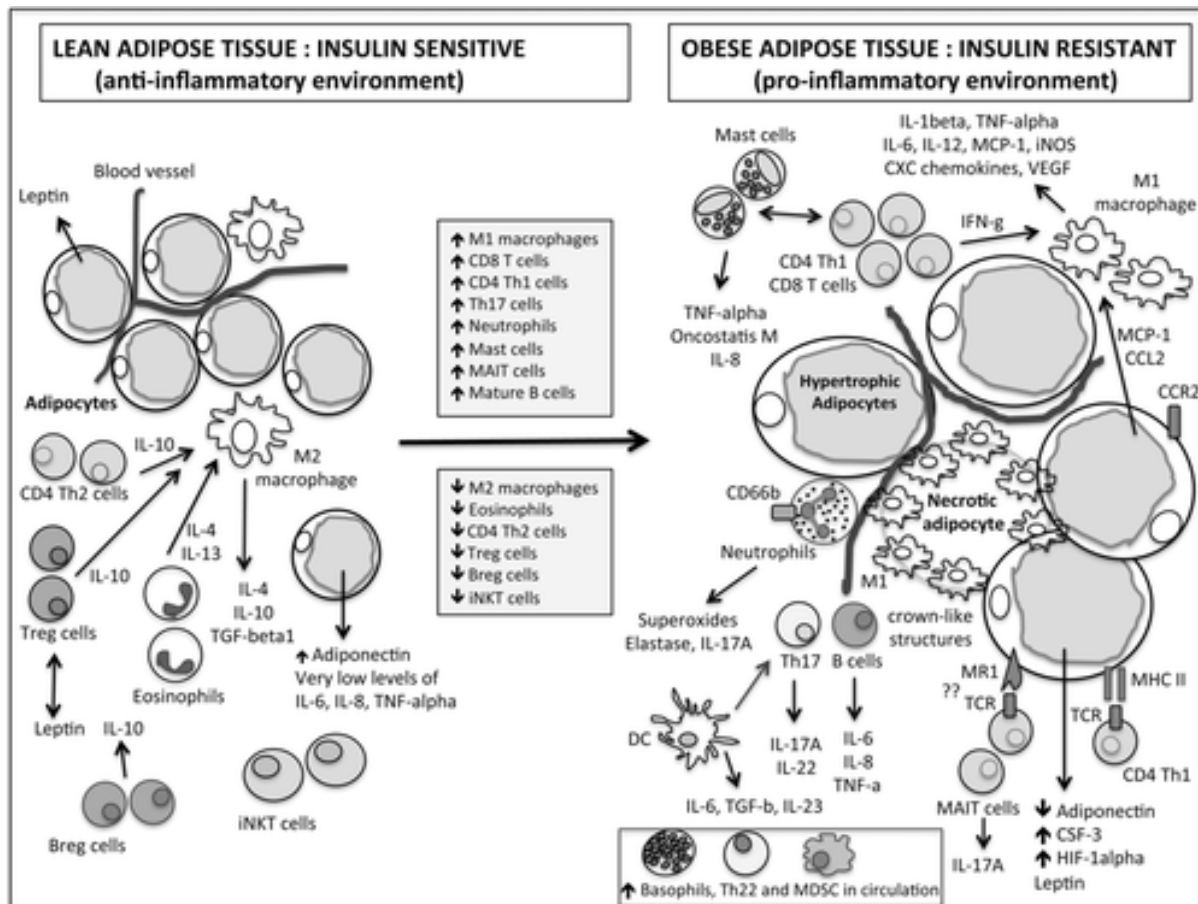


Figure 1-3 The complex interplay of immune cells and adiposopathy in Obesity, Apostolopoulos et al[3]

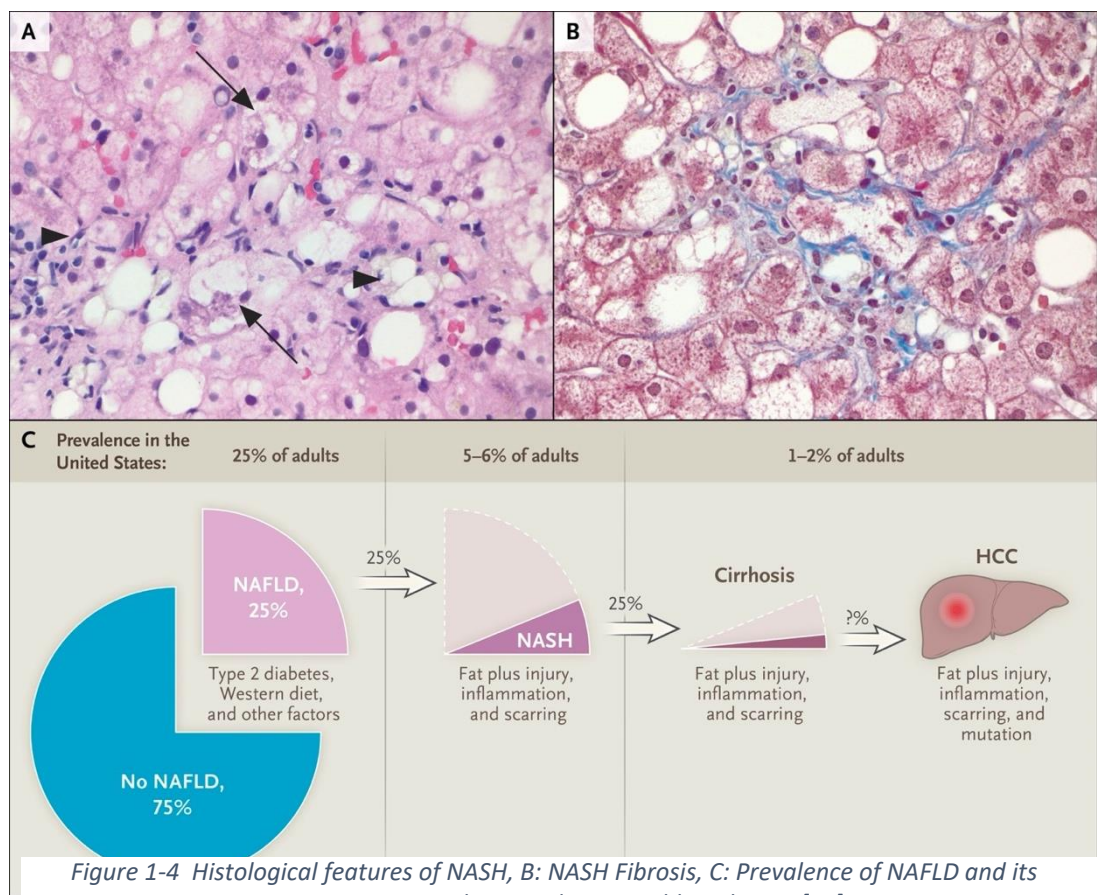
More recently there has been increasing interest in a subset of T lymphocytes, Mucosal Associated Invariant T (MAIT) cells and their role in metabolic diseases. They are innate-like T cells that have the capacity to produce copious amounts of pro-inflammatory cytokines including IL 17, granzyme A and granzyme B when stimulated and appear to undergo functional changes in obesity and diabetes. This cell population will be discussed in further detail in subsection 1.11 (see page 35).

Invariant natural killer T (iNKT) cells are innate-type T cells that are enriched and display distinct properties within the adipose tissue of healthy individuals. They are depleted in obese individuals and have been shown to protect against the development of obesity and diabetes in mouse models.[34, 43, 44] As such, they are believed to play a protective role against the development of metabolic disease, functioning like T<sub>reg</sub> and Th2 cells through their production of IL 10, which promotes M2 macrophage differentiation and by

influencing adipocyte function.[34, 43, 44] Thus, obesity is a state of chronic low-grade inflammation and immune dysregulation driven by adiposopathy (See Fig. 1-3).

1.4 Non-alcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of obesity and the metabolic syndrome. Over the last few decades, the global prevalence of NAFLD has steadily risen in parallel with that of obesity and type 2 diabetes mellitus, such that with a prevalence of 25%, it is now the most common cause of chronic liver disease in developed countries [1]. This figure is notably higher amongst at-risk populations, extending to 68-71% amongst individuals with type 2 diabetes and up to 98% amongst those who are obese [45, 46].



Histologically, NAFLD is defined by greater than 5% fat deposition within the liver [2]. However, the term encompasses a wide spectrum of disease severity, ranging from simple

steatosis, non-alcoholic fatty liver (NAFL), to fat associated inflammation and hepatocyte damage with fibrosis, termed non-alcoholic steatohepatitis (NASH) and ultimately to progressive, advanced fibrosis otherwise known as cirrhosis. Approximately 25% of individuals with a diagnosis of NAFLD will go on to develop NASH, of which a further 25% will develop cirrhosis affecting approximately 2% of the adult population (*See Fig. 1-4*) [1, 3]. The rate of fibrosis progression is accelerated by age, histological stage at diagnosis and the presence of metabolic risk factors[3, 47]. Individuals with cirrhosis are at increased risk of developing hepatocellular carcinoma, decompensated liver disease and liver related mortality[2, 48]. In addition, advanced liver disease signifies a more severe metabolic phenotype, with an increased risk of diabetes, cardiovascular disease, renal impairment and higher all-cause mortality [48]. NAFLD development has been shown to be influenced by environmental factors including: dietary composition, disruptions to circadian rhythm and maternal feeding patterns due to in utero epigenetic effects on insulin sensitivity [49]. Genome wide association studies have also identified several polymorphisms tied to increased risk and severity of NAFLD, most notably PNPLA3 which encodes a lipase involved in cellular triglyceride and retinol metabolism and TM6SF2, which reduces hepatic VLDL secretion[49].

The liver acts as an essential regulator and storer of energy. In the hypercaloric state, hepatocytes like adipocytes, attempt to accommodate the energy imbalance by taking up circulating free fatty acids, triglycerides, glucose, increasing intrahepatic lipid and glycogen reserves[50]. In obesity and the metabolic syndrome, adiposopathy with the resultant increased circulating pro-inflammatory cytokines (TNF $\alpha$ , IL 1 $\beta$ , IL 6, CCL2, NF $\kappa$ B) and adipokines (high leptin, low adiponectin) promote hepatic insulin resistance, thereby stimulating glycogenolysis, gluconeogenesis, lipolysis and lipogenesis [50]. This process further contributes to metabolic dysfunction and hepatic lipid accumulation. Hepatic steatosis alone can exist in the absence of significant liver disease and the degree of lipid accumulation itself does not correlate with NAFLD severity[49]. Although the precise pathophysiology remains to be understood, the key step in disease progression appears to be hepatocyte damage and resultant inflammation [49, 50]. This likely occurs as a result of mitochondrial dysfunction with impaired  $\beta$  oxidation of lipids and associated by-products, leading to lipotoxicity and the generation of reactive oxygen species [49, 50]. Reactive

oxygen species form oxidative stress derived epitopes (OSE), trigger hepatocyte injury, NLRP3 inflammasome activation, apoptosis and inflammation (See Fig. 1-5 and 1-6) [49, 50]. Damaged hepatocytes release DAMPs, stimulating liver resident immune cells, further immune cell migration through release of chemokines CCL1, CCL2, and CCL5 while also attempting repair via activation of hepatic stellate and endothelial cells [50]. This perpetuates a cycle of inflammation, tissue injury and fibrosis.

The liver also serves as the primary site of venous drainage from the gut via the portal circulation and is consequently exposed to translocated pathogens and toxins [50]. Alterations in gut microbial composition and endotoxaemia likely play a causal role in hepatocyte injury and the evolution of NASH through further immune cell activation.

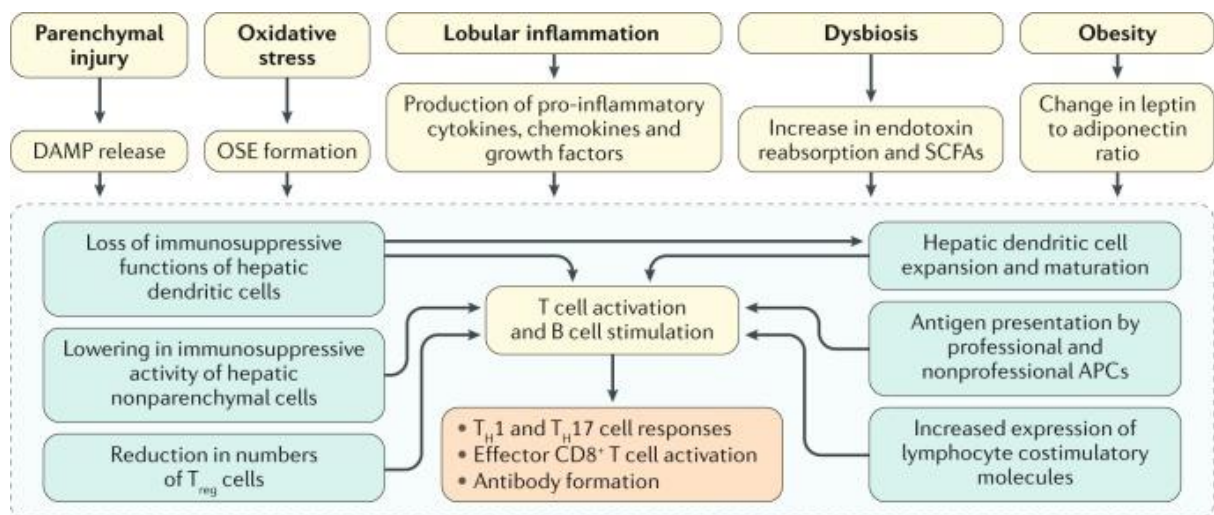
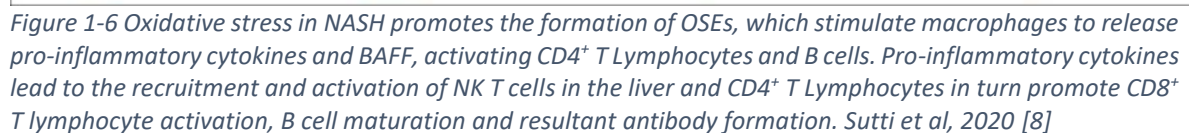


Figure 1-5 Triggers precipitating immune cell activation in NAFLD, Sutti et al, 2020 [76]

Given the common pathophysiology, immune cells also play an important role in the evolution and progression of NAFLD.



capacity to eliminate bacteria and associated products directly via phagocytosis [51, 52]. In NAFLD, they act as early responders within the liver and become persistently activated by DAMPs, bacterial endotoxin and exhibit M1 differentiation [51, 52]. In this state they produce pro-inflammatory cytokines:  $\text{TNF}\alpha$ ,  $\text{IL } 1\beta$ ,  $\text{IL } 12$ , stimulate hepatic stellate cells, promote the chemotaxis and activation of other immune cells further contributing to inflammation, hepatocellular injury and fibrosis [51, 52]. Their pathogenic role is supported by murine models, where Kupffer cell depletion has been shown to improve insulin sensitivity and protect against the development of hepatic inflammation and fibrosis [4].

#### *1.5.2 Neutrophils in NAFLD*

Neutrophils are also thought to be key players in NAFLD, rapidly accumulating in the liver in the early stages of NASH[6, 7]. As previously mentioned, when stimulated, they release myeloperoxidase, a potent inflammatory mediator that causes tissue injury and favours M1 macrophage differentiation[7]. Neutrophil derived elastase also promotes hepatic insulin resistance and inflammation [6, 7]. In murine models both deletion of neutrophil elastase or myeloperoxidase resulted in reduced severity of hepatic inflammation [4]. Neutrophils may also have a role in fibrogenesis, as they produce neutrophil derived peptides which have been shown to promote fibrosis in experimental models [4]. Additionally, circulating neutrophils show a proportionate increase with the severity of NAFLD inflammation and fibrosis, such that the neutrophil-lymphocyte ratio (NLR) has been proposed as a marker of disease severity [53]. As a result the NLR has also been found to be predictive of both NASH with a pooled sensitivity of 78.16% alongside a specificity of 76.93% and advanced hepatic fibrosis, with a sensitivity of 82.62% and a specificity of 81.22% [53, 54].

#### *1.5.3 Natural Killer cells in NAFLD*

Natural killer (NK) T cells are readily activated by lipids and exhibit pro-inflammatory properties upon stimulation. However, NK T cell deplete mice show an increased tendency towards weight gain and hepatic steatosis [4, 8]. In NAFLD, NK T cells have been shown to

be enriched in the liver in association with increased histological severity, as a result further information is required to clarify whether NKT cells play a protective or pathogenic role [4].

#### *1.5.4 Dendritic cells in NAFLD*

Dendritic cells are antigen presenting cells with the potential to produce pro-inflammatory cytokines IL 6, TNF  $\alpha$  and MCP 1 upon activation [4]. However, despite their pro-inflammatory capacity, dendritic cell depletion has been shown to aggravate hepatic inflammation and fibrosis in experimental models, suggesting a possible protective role, although further studies are required [4].

#### *1.5.5 Lymphocytes in NAFLD*

Intrahepatic B and T lymphocytes form aggregates in NAFLD and are independently associated with hepatic fibrosis [55]. In mouse models, the expression of B cell activating factor (BAFF) and resultant activation of B lymphocytes closely mirrors the initiation of NASH, with circulating BAFF levels correlating with the severity of hepatic inflammation and fibrosis indicating a contributory role (*See Fig 1-6*) [8]. B cells release pro-inflammatory cytokines, recruit and activate T lymphocytes in addition to promoting the M1 polarization of macrophages [8]. They also produce antibodies against oxidative stress derived epitopes (OSE) [8]. OSEs are formed by oxidized phospholipids and reactive aldehydes which function as 'self-epitopes' and are generated during lipid oxidation by hepatocytes under conditions of oxidative stress [8]. OSEs are highly immunogenic and likely bolster the inflammatory process in NASH, with anti-OSE immunoglobulins levels correlating with histological severity [8].

CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes are increased both in the peripheral circulation and liver of patients with NASH compared to healthy controls [6, 8]. They likely play a contributory role in the pathogenesis of NAFLD, as in murine models, CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocyte depletion protects against hepatic steatosis and inflammation [8]. In NAFLD, CD4<sup>+</sup> T lymphocytes are predominantly of the Th1 and Th17 subtypes and support the progression to NASH by producing pro-inflammatory cytokines IFN $\gamma$ , TNF $\alpha$ , IL 17 and by promoting M1 macrophage polarization [6, 8]. IL 17, in particular, plays a significant role in the pathogenesis of NASH,

promoting intrahepatic uptake of triglycerides, inflammation, hepatic stellate cell activation and pro-fibrogenic gene expression[6, 8]. Whilst IL 17 depletion is protective against the development of NASH [6].

$T_{regs}$ , in contrast, likely play a protective role in NAFLD, with their ability to suppress the proliferation of  $CD4^+$ ,  $CD8^+$  T lymphocytes and inhibit their actions through the production and release of CTLA4, IL 10, and TGF  $\beta$  [8]. In NAFLD,  $T_{regs}$  are more vulnerable to apoptosis with their resultant depletion in both the peripheral blood and the liver, demonstrating an inverse correlation with histological severity [8, 56].

Dysregulated senescence has been shown to be a factor in the evolution of chronic liver diseases, including NAFLD. In experimental models, provocation of senescence through deletion of telomerase accelerates hepatic fibrosis and the development of cirrhosis [57, 58]. Although the precise mechanism is poorly understood, it is likely that chronic inflammation in NAFLD with mitochondrial dysfunction and increased oxidative stress promotes hepatocyte senescence by upregulating the expression of senescence markers within the liver including p16, p21, p38, p53 and senescence marker protein 30 (SMP 30) [57]. Expression of senescence markers within the liver correlates with the severity of hepatic steatosis and inflammation whilst clearance of senescent cells leads to histological improvement[57, 59].

Similar to obesity, NAFLD is associated with insulin resistance, oxidative damage and chronic inflammation, as such it reflects a state of impaired autophagy [60]. In healthy individuals, autophagy plays a different role depending on cell type [60]. Within hepatocytes it enhances lipophagy reducing hepatic lipid accumulation and also promotes mitophagy, the degradation of dysregulated mitochondria thereby reducing the generation of reactive oxygen species, hepatocyte injury and inflammation [61, 62]. In macrophages, autophagy also plays a protective role, attenuating the activation and reducing the production of pro-inflammatory cytokines [61, 62]. However, in hepatic stellate cells, autophagy can promote fibrogenesis by releasing energy through lipophagy and disrupting cellular quiescence via reduced p62-mediated VDR-RXR signaling [60]. In NAFLD, autophagy is particularly compromised in individuals with NASH, with

overexpression of the autophagy inhibitor, Rubicon [60, 63]. In experimental models, high fat diet mice lacking autophagic machinery develop hepatic steatosis, endoplasmic reticulum stress, hepatocyte injury and inflammation, whereas enhancement of autophagy through Rubicon downregulation reduces hepatic steatosis, endoplasmic reticulum stress and inflammation [60, 64-67].

Given the metabolic dysregulation, persistent inflammation, induced senescence and impaired autophagy, NAFLD has also been linked with frailty. Amongst patients with cirrhosis, NAFLD is an independent predictor of sarcopaenic obesity and frail NAFLD patients have higher all-cause mortality in comparison to those with alcoholic liver disease, although there is paucity of data on frailty in non-cirrhotic individuals [68-70].

### *1.6 The role of MAIT cells in the development and progression of NASH*

#### *1.6.1 MAIT cell definition and phenotype:*

Mucosal associated invariant T (MAIT) cells are innate like T lymphocytes that were first described in 1993 [71]. They develop within the thymus gland and undergo expansion and final maturation following release from the gland. They are described as non-conventional T cells, expressing a canonical T cell receptor  $\alpha$  chain, (V $\alpha$ 7.2-J $\alpha$ 33) which, combined with a T cell receptor  $\beta$  chain forms a semi-invariant T cell receptor ( $i$ TCR) [71]. Mature MAIT cells are defined by the expression of the invariant V $\alpha$ 7.2 T cell receptor and high levels of the C-type lectin, CD161 (CD3<sup>+</sup>V $\alpha$ 7.2<sup>+</sup>CD161<sup>Hi</sup>) [71].

MAIT cells can recognize bacterial and fungal derived vitamin B metabolites presented by the evolutionarily conserved major histocompatibility complex MHC-like molecule, MR1 and exhibit an effector memory phenotype, CD45RA<sup>-</sup>CD45RO<sup>+</sup>CD95<sup>Hi</sup>CD62L<sup>Lo</sup>CD44<sup>Hi</sup> [72, 73]. They account for 1-10% of circulating T cells, reaching peak levels by the age of 25 and declining with advancing age [74]. They are predominantly found at mucosal 'barrier' sites including the lungs, skin, intestines and are particularly enriched in the liver, where they comprise 20-45% of intra-hepatic lymphocytes [74]. Interestingly, in murine models, gut bacteria have been shown to regulate the intra-thymic development of MAIT cells through

release of the antigen: 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU) [75]. Following their exit from the thymus, the integrins and chemokines expressed by MAIT cells (CD69, CXCR6, CCR6, LFA-1, VAP-1) preferentially promote their migration and retention within the liver [75]. This may be beneficial from an evolutionary perspective as the liver is extensively exposed to pathogens, being the primary site of venous drainage from the gut. Therefore, any pathogens or infected cells that are able to successfully translocate through the gut epithelial membrane pass through the 'immune rich' liver and are targeted before they can enter the systemic circulation.

Given their distribution, ability to recognize antigens presented on MHC-1 molecules and effector memory characteristics, MAIT cells are believed to play an important role in the initial defense against infection [74].

#### *1.6.2 Functional Characteristics of MAIT cells:*

The majority of MAIT cells, approximately 80%, are CD8<sup>+</sup>, 15% of MAITs are double negative, CD4<sup>-</sup>CD8<sup>-</sup> and less than 5% are either CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>+</sup>CD8<sup>+</sup> [73, 76]. They can be directly stimulated through an MR1-TCR dependent manner (*See Fig. 1-7*), however, similar to conventional CD8<sup>+</sup> T lymphocytes, they require co-stimulation with either TLR agonists: TLR1, TLR2, TLR6, cytokines including: IL1, IL7, IL12, IL15, IL 18, IL 23 or bacterial and fungal metabolites for maximal effector response [73, 76]. MAIT cell activation and cytokine release is particularly more pronounced when co-stimulated with IL12 and IL 15 [73, 76]. Additionally, they express chemokine receptors, notably CCR5 and CCR6, enabling them to migrate to and concentrate at sites of infection and chronic inflammation [77].

Upon stimulation, MAIT cells upregulate the expression of activation markers CD69, CD25 and the degranulation marker CD107a [73]. They release a variety of pro-inflammatory cytokines including Th1 type cytokines: IFN $\gamma$ , TNF $\alpha$ , IL 12, Th2 type cytokines: IL 4, IL 5, IL 10, IL 13 and Th17 type cytokines: IL 2, IFN $\gamma$ , TNF $\alpha$ , IL 4, IL 5, IL 13, IL 17A, IL 22 and also release the chemokines: CXCL1, CCL3, CCL4 and CXCL16 [73]. MAIT cells also express inducible T cell co-stimulator which is involved in the activation and maintenance of retinoic acid-related orphan receptor  $\gamma$ t (ROR $\gamma$ t) expression [73].

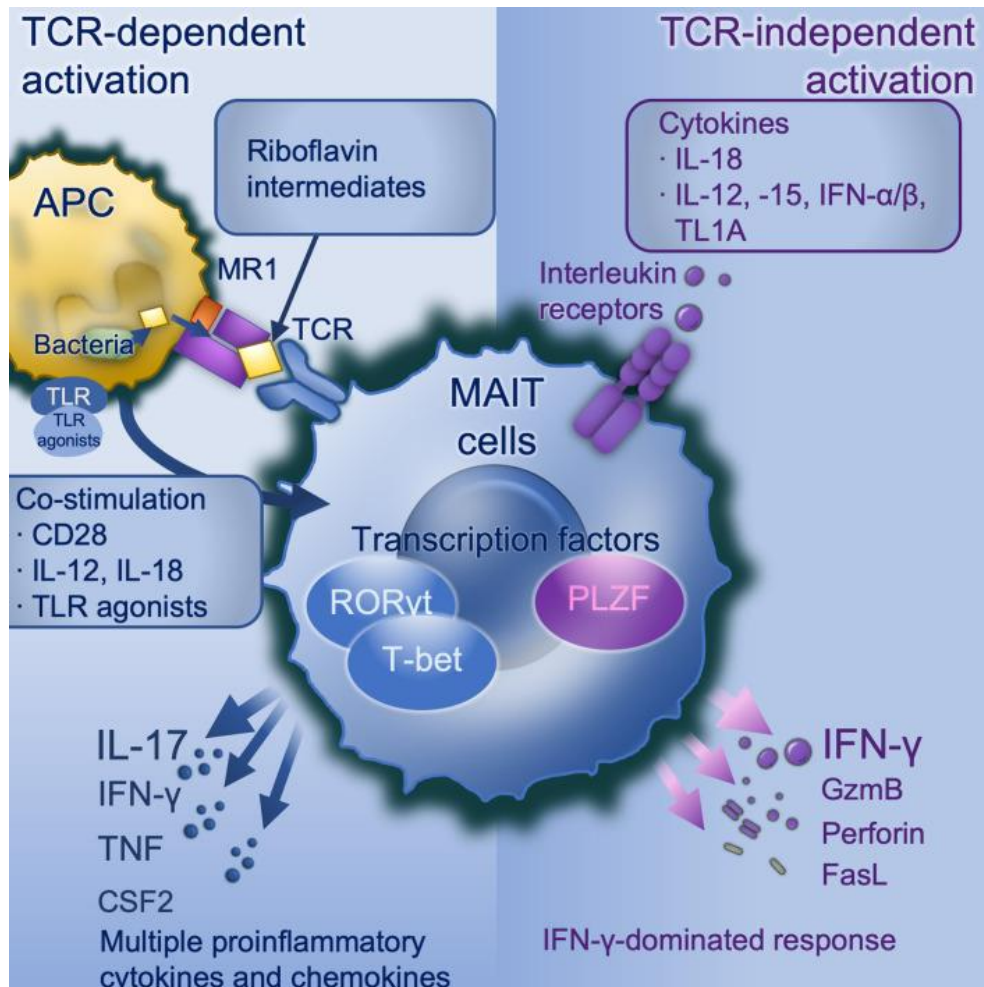


Figure 1-7 MAIT cells activation can occur via a TCR dependent or independent mechanism, the mode of activation influences MAIT cell behaviour and cytokine release, MAIT cell Activation and Functions, Front Immunol 2020[1]

MAIT cell response can vary significantly depending on MAIT cell subset, mode of stimulation and the tissue compartment involved. In the resting state, peripheral blood MAIT cells, particularly CD8<sup>+</sup> and double negative MAIT cells, express granzyme A and K. MR1-TCR, bacterial ligand and cytokine (IL 7, IL 12, IL 15, IL 18) mediated stimulation promote the downregulation of granzymes A and K whilst upregulating the production and release of the cytolytic molecules: granzyme B, perforin, and lysosomal-associated membrane protein-1 (LAMP-1), also known as CD107a[78, 79]. Interestingly, in vitro studies have shown a comparably more muted production of granzyme B and LAMP-1 when CD4<sup>+</sup> peripheral blood MAIT cells are stimulated [79, 80].

Following activation, MAIT cells undergo a metabolic switch from oxidative phosphorylation to glycolysis, upregulating GLUT-1 and increasing glycolytic activity via the MTORC1 signaling pathway [81, 82]. IFN $\gamma$  and granzyme B production have been shown to

be directly linked to glycolysis, whilst  $\text{TNF}\alpha$  and IL 17 production appear to be independent [81, 82]. Thus, the metabolic switch not only enables more rapid energy release for the activated cell, but may also be key in determining its behaviour [81, 82].

### *1.6.3 MAIT cell phenotypes:*

As previously mentioned, tissue compartment also influences MAIT cell characteristics and behaviour. For example, some colonic and intra-hepatic MAIT cells have been shown to constitutively express granzyme B, which can be further increased both through MR1-TCR dependent and independent mechanisms, suggesting that they are primed for more rapid production and release of lytic enzymes in response to bacterial antigens[80]. This may be advantageous for the host defence particularly protecting against the translocation of gut derived pathogens. Similar characteristics are also observed in intervillous and decidua parietalis MAIT cells, potentially protecting the foetus from bacterial infections entering via the maternal circulation[80, 83].

MAIT cells that express NK cell-associated receptors, including:  $\text{CD56}^+$ ,  $\text{CD84}^+$  and  $\text{CD94}^+$  MAITs, have also been found to express higher levels of IL 12 and IL 18 receptors and may consequently have a greater capacity of responding to innate cytokines, making them more efficient in mounting MR1-independent responses during viral and bacterial infections, as well as sterile inflammatory conditions[84, 85]. Interestingly,  $\text{CD56}^+$  MAIT cells are also reportedly more abundant in the liver [85].

As potent producers of Th1 mediators:  $\text{IFN}\gamma$ ,  $\text{TNF}\alpha$ , IL-12, CD40 ligand and CD154, MAIT cells also play in role in stimulating MHC-1 and (CD95) Fas-ligand expression by infected cells thereby facilitating their detection by  $\text{CD8}^+$  cytotoxic T cells, macrophages and NK cells for Fas-Fas ligand mediated elimination[73].

Additionally, MAIT cells play a role in tissue repair. Analysis of the MR1 and 5-OP-RU -TCR activated MAIT cell transcriptome shows upregulation of  $\text{TNF}$ ,  $\text{CSF2}$ ,  $\text{HIF1A}$ ,  $\text{FURIN}$ ,  $\text{VEGFB}$ ,  $\text{PTGES2}$ ,  $\text{PDGFB}$ ,  $\text{TGF}\beta 1$ ,  $\text{MMP25}$  and  $\text{HMGB1}$  genes. This mirrors the transcriptome of dermal  $\text{Tc17}$  cells in murine models when activated by commensal flora that promote

wound repair[73, 86-88]. In addition, they also have capacity to produce T cell derived Furin, CCL3 and TNF $\alpha$  which drive macrophage recruitment, tissue repair and supernatants derived from these activated MAIT cells accelerate wound closure in intestinal epithelial cell line systems [73, 86-88]. Interestingly, this function relies on TCR mediated stimulation and has not been observed in MAIT cells that were stimulated by cytokines alone [73].

Thus, by exhibiting differential phenotypes with particular stimuli, MAIT cells ‘sense’ their tissue microenvironment and ‘respond’ by altering their metabolic pathway. Hinks et al have also proposed that they may play a dynamic role in the evolution of tissue injury and infection, switching from predominantly effector, anti-bacterial function to repair (See Fig 1-8) [73].

#### 1.6.4 The Role of MAIT cells in Acute Bacterial and Viral infections:

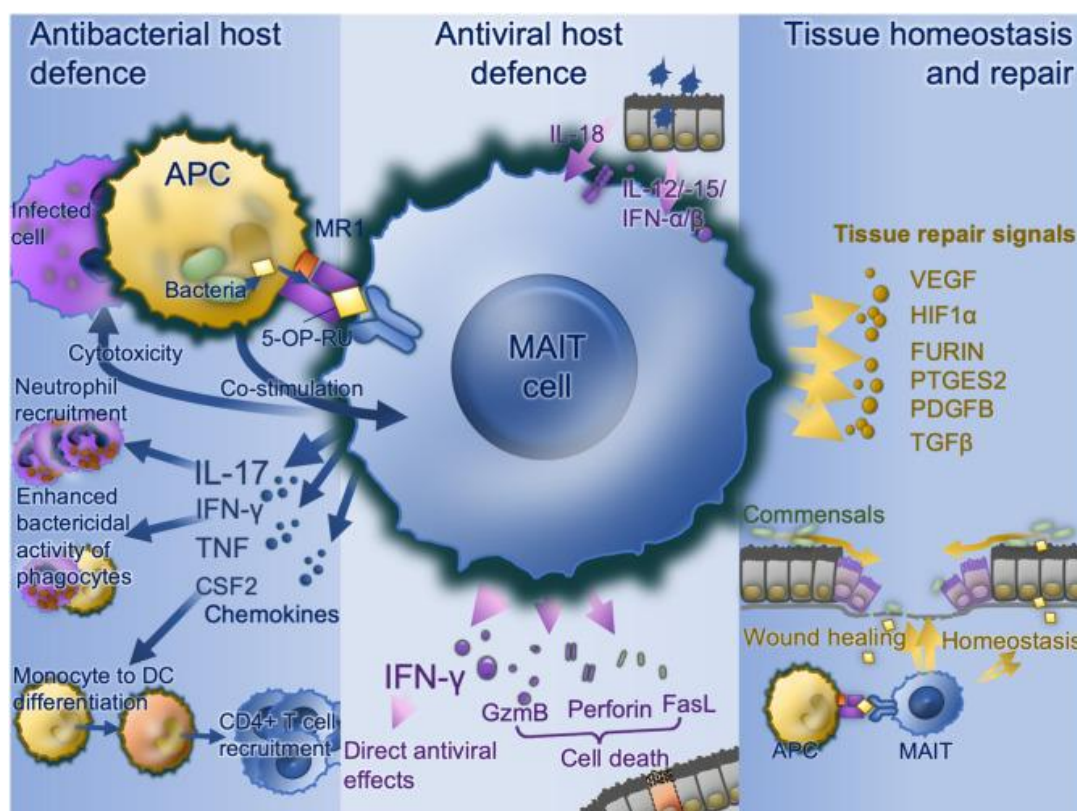


Figure 1-8 MAIT cells are involved in host defense against bacterial and viral infection but also play a role in maintaining tissue homeostasis and repair, MAIT cell Activation and Functions, Front Immunol 2020 [1]

MAIT cells recognize and are stimulated by bacteria and fungi that utilize the RibD riboflavin biosynthesis pathway through the semi-invariant  $\alpha\beta$  T cell receptor, including: *Escherichia*, *Lactobacillus*, *Staphylococcus*, *Shigella flexneri*, *Salmonella*, *Clostridioides*, *Saccharomyces*, *Candida*, *Aspergillus* and also mycobacteria, directly via MR1-TCR

mediated mechanism [73]. This in turn drives the upregulation of transcription factors: ROR $\gamma$ t, T-bet which lead to the synthesis and release of IL-17, IFN $\gamma$ , TNF $\alpha$  and CSF2[73, 89]. This direct pathogen mediated stimulation with polarization of cytokine release indicates that MAIT cells may be able to modulate their cytotoxic response to infected cells that express cognate antigens.

In vitro studies on peripheral blood and hepatic CD8<sup>+</sup> MAIT cells have shown them to be rapid responders to stimulation by bacterial superantigens including *Staphylococcal enterotoxin B* and *Streptococcus pyogenes* [90]. MAIT cells release markedly high levels of IFN $\gamma$  in an MR1-independent manner, through direct stimulation of IL 12 and IL 18 receptors[90]. Production of IFN $\gamma$  has been associated with increased expression of the activation marker CD69 and following superantigen stimulation, MAIT cells upregulate ligand lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulin domain and mucin domain 3 (TIM-3), these genes are markers of cellular exhaustion, rendering them unresponsive to further stimulation [90]. Their markedly potent and rapid response to stimulation in comparison to *i*NKT, conventional effector memory T cells and  $\gamma\delta$  T cells suggests that they may play a significant role in the cytokine storm caused by superantigen-mediated diseases including toxic shock syndrome.

In vivo studies have also demonstrated activation and reduced levels of circulating MAIT cells in patients with active *Mycobacterium tuberculosis* infection, sepsis and in patients post administration of *Salmonella* and *Shigella* vaccines [91].

Viruses lack vitamin B synthesis machinery of their own and activate MAIT cells in a cytokine-dependent manner. IL 12, IL 15 and IL 18 production by myeloid cells such as monocyte-derived macrophages, in response to viral infection has been shown to prime and stimulate MAIT cells, thus increasing their innate cytotoxicity through an NKG2D mediated pathway [92]. This has been demonstrated in hepatitis A, hepatitis B, hepatitis C, HIV infection and in cases of dengue fever[92].

MAIT cells accumulate in the lungs and are activated in murine models of influenza A infection via cytokine mediated stimulation by IL 18 and IL 12, IL 15, IFN $\alpha$  and IFN $\beta$  [93].

This leads to increased surface expression of the activation markers CD25, CD69 and production and release of IFN $\gamma$  and granzyme B, favoring a cytotoxic MAIT profile[93]. MAIT cells play a protective role by enhancing pulmonary T cell accumulation and by production of innate inflammatory cytokines as a result, pulmonary epithelial damage, degree of weight loss and mortality are reduced [93]. Notably, the MAIT cell response was greater and more rapid in comparison with other CD3<sup>+</sup> non-MAIT T lymphocytes, further supporting their role as first responders of the immune system [93].

MAIT cells also alter in response to SARS-CoV-2 infection with significant reductions in the percentage of circulating MAITs, particularly the CD8<sup>+</sup> cohort combined with increased concentration within the airways and high levels of activation marker: CD38, CD69, HLA-DR and exhaustion marker: CTLA-1, PD-1 expression[94, 95]. CD69 expression in particular has been associated with disease severity with significantly higher levels of expression in patients with fatal SARS-CoV-2 infection[94, 95]. Functional studies using MAIT cells obtained from patients have shown high levels of IFN $\gamma$  and TNF $\alpha$  production on stimulation, but failed upregulation of IL-17A, granzyme B and perforin[94, 96]. This may hint that excess stimulation and exhaustion during COVID-19 leads to functional impairments, reducing the cytolytic and consequently antimicrobial activity of MAITs which may contribute to increased susceptibility of patients to bacterial superinfection [94, 96].

#### *1.6.5 The role of MAIT cells in Chronic infections and Inflammatory Conditions:*

MAIT cells have been shown to accumulate in gastric tissue at sites of *Helicobacter pylori* infection and are associated with accelerated gastritis and development of gastric mucosal atrophy, potentiating the pathological process [91].

In HIV infection, circulating MAIT cells are depleted and demonstrate an activated phenotype with increased chemokine receptor activation and exhaustion marker (T cell immunoglobulin and mucin-3 [(TIM-3)] expression [91]. They also exhibit functional impairment, with reduced CD107a, IFN $\gamma$ , TNF $\alpha$  and IL 17 production in response to bacterial challenge with *Escherichia Coli* in association with reduced T-bet and Eomes gene

expression[89]. This functional impairment has been shown to improve in part with the addition of IL 7 and resolves following long-term anti-retroviral therapy [85].

Similarly in chronic inflammatory conditions, circulating MAIT cell frequencies are reduced, with accumulation in affected tissues where they appear to play a pathological role in perpetuating chronic inflammation by being predominantly of the Th17 subtype[97, 98]. In immune mediated arthropathies including rheumatoid arthritis and ankylosing spondylitis, MAIT cells accumulate in synovial fluid, with inverse correlations between circulating MAIT cells and the degree of inflammation[97, 99]. In murine models of arthritis, disease severity appears reduced in MAIT cell deficient mice and is associated with significantly lower levels of IL 17 production[98].

In Crohn's disease and ulcerative colitis, circulatory MAIT cells are reduced and their tissue concentration proportionate to the severity of mucosal inflammation[80]. They exhibit increased NKGD2 and proliferation marker, Ki67 expression with markedly increased IL 17 production upon stimulation *in vitro*[80]. MAIT cells are enriched within psoriatic plaques in patients with psoriasis, accumulate in bronchial fluid in those with asthma and are even found to accumulate in the central nervous system of patients with multiple sclerosis [97, 100, 101].

Circulating MAIT cells are also reduced in multisystem connective tissue diseases including systemic lupus erythematosus (SLE) and Sjögren's syndrome, with expansion of the Th17 subtypes[97, 102, 103]. Disease severity improves in mice upon elimination of IL 17 producing MAIT cells, indicating a contributory role in the pathogenesis of these conditions[97].

#### *1.6.6 The role of MAIT cells in Malignancy*

The role of MAIT cells in the development of cancer remains to be fully understood. MAIT cells have been found within malignant tissue including: melanoma, lung cancer, hepatocellular carcinoma, colorectal cancer, thyroid cancer, renal cancer, cardiac, gastric cancer, oesophageal cancer and also in multiple myeloma [97, 104]. In murine models, MAIT cells appear to promote carcinogenesis with improved survival in MAIT cell-deficient

mice, this is also seen in patients with hepatocellular carcinoma, where patient prognosis inversely correlates with MAIT cell-tumour concentration [97, 105]. Conversely, higher frequencies of MAIT cells appear to be associated with improved outcomes in oesophageal and colorectal cancers, melanoma and multiple myeloma [97, 104]. MAIT cells can exhibit effector function, with the capacity to lyse malignant cells, however IL 17 can, by contrast, promote tumour growth and survival through upregulation of vascular endothelial growth factor and consequent neoangiogenesis [106]. In colorectal adenocarcinoma, there are significantly higher correlates with adverse clinical outcomes[107]. Th17 MAIT cells may also facilitate tumour metastasis through disruption of the epithelial barrier and inhibition of NK cell effector function as demonstrated in murine models of lung cancer [108].

#### *1.6.7 MAIT cells and Metabolic diseases*

Obesity and diabetes reflect a state of chronic low-grade inflammation driven by adiposopathy, dysbiosis and dysregulated immune cell activation. Circulating MAIT cells are reduced in obese adults and those with type 2 diabetes mellitus in comparison to healthy controls[109]. They appear activated with increased CD25, CD69 expression and also upregulate the exhaustion and apoptotic markers: PD-1, cMyc, Casp9 and Bax [110, 111]. Moreover, MAIT cells appear to concentrate in visceral adipose tissue and display a pro-inflammatory Th17 bias, with copious IL 17, granzyme B and reduced IL 10, IFN $\gamma$  and TNF  $\alpha$  production following MR1-TCR mediated stimulation[110, 111]. This Th17 bias appears to be metabolically driven due to the impaired glycolytic capacity, defective mTORC1 signaling and mitochondrial dysfunction in obesity [81] [112]. IL 17 is a key proponent of insulin resistance as it blocks insulin mediated glucose uptake by skeletal muscle, hepatocytes and adipocytes [111, 113, 114]. In murine models, inhibition of IL 17 promotes adipose tissue browning, increasing energy expenditure, suppressing diet induced obesity and insulin resistance [21, 41, 113]. Similarly, MAIT cell deficient mice are protected against the development of obesity [110]. Consequently, the data would suggest that MAIT cells are key contributors to the metabolic dysfunction leading to obesity and diabetes. Significant weight loss following bariatric surgery, has been shown to improve obesity associated inflammation, insulin resistance but also significantly increase circulating MAIT cell frequency, although IL 17 production appears persistently elevated,

even a year after surgery [115]. Notably, significant weight loss following bariatric surgery and the resultant metabolic improvements have been associated with replenishment of circulating MAITs in conjunction with reduced pro-inflammatory cytokine production[116].

#### *1.6.8 MAIT cells in chronic liver disease*

As previously mentioned, the liver is a complex immunological organ, preventing pathogens within the portal circulation from entering the wider systemic circulation. As such, it is host to a large population of resident immune cells including: Kupffer cells, resident macrophages, B lymphocytes, dendritic cells, CD8<sup>+</sup> T lymphocytes, *i*NKT cells,  $\gamma\delta$  cell, NK cells, innate lymphoid cells and is particularly enriched by MAIT cells [10].

Immunofluorescent staining of liver sections from healthy controls has shown approximately 5% MAITs within the portal field, with the majority dispersed in the hepatic sinusoids [75]. Given the ability of MAITs to migrate in response to stimuli, it is likely that their distribution alters in liver disease and studies performed in liver sections of patients with autoimmune liver disease would support this, showing MAIT cells concentrated in inflammatory infiltrates around portal tracts and fibrotic septae [117]. Liver resident MAIT cells display similar phenotypic characteristics to those in peripheral circulation, however, they are noted to express higher levels of CD8, CD56 and CD69, as such, they are function as markers of liver resident MAIT cells [75].

Hepatocytes, biliary epithelium, liver endothelial cells and hepatic stellate cells have all been found to stimulate MAIT cells in an MR1-dependent manner, although maximal MAIT cell response occurs following stimulation by hepatocytes [75]. Additionally, these cells promote the formation of 5-OP-RU Ag, another potent stimulator of MAIT cell activation [75]. Upon stimulation liver resident MAIT cells release pro-inflammatory and pro-fibrogenic cytokines including: IFN $\gamma$ , TNF $\alpha$  and IL 17[75]. Repetitive co-stimulation with the cytokines IL 12 and IL 7, which are also secreted by hepatocytes in response to injury, promotes a large IL 17 response [117]. IL 17, which in conjunction with direct cell to cell contact, has been shown to activate hepatic stellate cells, stimulating proliferation and release of extracellular matrix components [117].

#### *1.6.8(i) MAIT cells in Chronic Viral Hepatitis*

Both circulating and intrahepatic MAIT cell compartments are reduced in liver disease. In patients with chronic hepatitis C infection, peripheral and intrahepatic MAIT cell decline has been associated with severity of hepatic inflammation (HAI) and fibrosis [118]. MAIT cells are also activated exhibiting high levels of CD69, HLA-DR and CD107a expression [118]. In addition, MAIT cell function appears impaired with reduced IFN $\gamma$ , TNF $\alpha$  production in response to bacterial antigen stimulation[118]. Although their ‘anti-viral’ response which is elicited via IL 12, IL 18 mediated stimulation, is preserved and IL 17 production remains unaltered[118]. Interestingly, repletion of intrahepatic MAIT cell population with reductions in activation marker expression are observed within 4 weeks of commencing direct acting antiviral agents[118, 119]. However, production of IFN $\gamma$  and TNF $\alpha$  does not recover with viral eradication[118, 119].

In chronic hepatitis B infection, the circulating MAIT cell population is reduced compared with healthy controls and this is coupled with higher activation (HLA-DR, CD69, CD38) and apoptotic marker expression (PD-1), with normalization of CD38 expression occurring following commencement of antiviral therapy [120, 121]. MAIT cells also exhibit marked cytotoxicity against hepatitis B infected hepatocytes via MR1 dependent pathway [122], although impairment of MAIT cell function has also been observed, similar to chronic hepatitis C, with reductions in IFN $\gamma$ , but not TNF $\alpha$  production with cytokine mediated stimulation via IL 12 and IL 18 [120]. Although intrahepatic MAIT cells are also reduced with increases in PD-1 expression, their activation marker expression and cytokine production has not been shown to differ compared with healthy controls [120]. There has been variability in results with some studies showing reductions in the percentage of intrahepatic MAIT cells in association with liver disease severity similar to hepatitis C and some showing no significant changes[120, 122]. MAIT cells in patients with chronic hepatitis B infection appear to be highly sensitive to conjugated bilirubin, with higher activation (CD69) marker expression, impaired TCR mediated proliferation and IFN $\gamma$  production [122].

#### 1.6.8(ii) MAIT cells in Immune Mediated Liver Disease

In comparison to chronic viral hepatitis MAIT cell behaviour appears similar in immune-mediated liver diseases, with significant reductions in circulating MAIT cells in association with hepatic fibrosis seen in autoimmune hepatitis, primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC) [117, 123, 124]. The percentage of intrahepatic MAIT cells appears to be unaltered in autoimmune hepatitis, but reduced in PSC and PBC when compared to healthy controls [117, 123, 124].

In patients with autoimmune hepatitis, higher expression of activation: CD38, HLA-DR, CTLA-4 and exhaustion markers: CD39, PD-1, TIM-3, are observed with attenuated IFN $\gamma$  production in response to IL 12 and IL 18, although IL 17A production remains unaffected [117]. This is coupled with downregulation of transcription factors which drive cytolytic functions: T-bet and eomesodermin [117]. In vitro repetitive stimulation of MAITs leads to similar functional impairment and surface marker expression, supporting the hypothesis that overstimulation causes exhaustion, diminished effector response and apoptosis [118]. Of particular interest, is the preservation of IL 17 production, which is essential for hepatic stellate cell activation. This suggests that, in autoimmune hepatitis, whilst cytolytic and antibacterial functions are impaired, exhausted MAIT cells continue to produce large quantities of IL 17, promoting hepatic fibrosis and consequently may be key players in the development of cirrhosis [117]. In the setting of autoimmune hepatitis, these functional changes do not show reversal, even following a year of immunosuppressive treatment [125].

Similarly in PSC, circulating MAIT cells are significantly reduced and display an activated phenotype, with higher CD69, CD56, NKG2D and reduced CD28, CD127, CXCR6 expression. Exhaustion marker (PD-1, CD39) expression is also increased with impaired TNF $\alpha$  and IFN $\gamma$  production in response bacterial challenge with *Escherichia Coli* [123]. However, mild impairment in TNF $\alpha$  production alone is seen in response to cytokine mediated stimulation (IL 12, IL 18) [123, 126]. Immunohistochemical staining of explanted livers from patients

with decompensated PSC demonstrates expansion of the CD3<sup>+</sup> intrahepatic compartment with relative depletion of MAIT cells and their accumulation in areas of fibrosis[123, 126].

In PBC, circulating and intrahepatic IL 7 concentrations are measurably increased and have been shown to promote MAIT cell activation and release of pro-inflammatory cytokines [127]. In addition, cholic acid, a constituent of bile, has been shown to enhance IL 7 expression through stimulating Farnesoid X Receptor binding to the IL 7 promoter [127]. This supports the premise that biliary stasis may exacerbate hepatic inflammation and damage in PBC through additive stimulation of MAIT cells. Interestingly, there is reduced discrepancy between circulating MAITs in patients with PBC compared to healthy controls following six months of ursodeoxycholic acid therapy [127].

#### *1.6.8 (iii) The role of MAIT cells in alcohol-related liver disease*

In alcohol-related liver disease, marked reductions in circulating MAIT cells are observed, particularly in patients with severe acute alcoholic hepatitis when compared to those with alcohol related cirrhosis. This depletion is seen in conjunction with increased chemokine receptor expression ( $\alpha 4$ ,  $\alpha e$  integrins and CXCR10), potentially reflecting increased hepatic migration [128, 129]. In this cohort, MAITs express higher levels of CD69, reflecting their activated state and are functionally impaired with marked reductions in granzyme B production, especially in alcoholic hepatitis[128, 129]. However there does appear to be some element of reversibility in alcoholic hepatitis, with increased circulating MAIT cell frequency and reduction of CD69 expression following alcohol cessation [129]. Riva et al showed that circulating MAIT cell frequency correlates inversely with plasma D-lactate concentrations, a surrogate marker of gut permeability and MAIT cell function is not impaired with repetitive cytokine mediated stimulation but following exposure to faecal extracts of bacterial antigens [128]. These findings suggest a link between MAIT cells and gut microbiota.

#### *1.6.8 (iv) The role of MAIT cells in NAFLD*

To date, few studies have explored MAIT cells in NAFLD. Their frequency in peripheral circulation is reduced in comparison to healthy controls with increased expression of activation markers: CD25, CD69 and the chemokine receptor CXCR6 [74, 117, 130]. Mean fluorescence intensity of surface marker expression negatively correlates with MAIT cell frequency, indicating that activation and migration may account for depletion from the peripheral circulation [74, 117, 130]. MAIT cell function is also altered with increased IL 4 and reduced TNF $\alpha$  and IFN $\gamma$  production. They also appear to play a role in macrophage polarization, with Li et al showing that co-incubation of MAITs and macrophages promoted a switch to the 'anti-inflammatory', M2, CD163<sup>+</sup>CD206<sup>+</sup> subtype with more accelerated NASH progression observed in MR-1 deficient mice, suggesting that they may play a protective, immunoregulatory role in NAFLD [130].

Within the intrahepatic compartment, MAIT cells are significantly reduced in NAFLD cirrhosis. Immunofluorescent staining of explants shows accumulation of MAITs within fibrotic septae and in close proximity to hepatic myofibroblasts [74]. Despite reduced frequency, MAIT cells exhibit high levels of the proliferation marker, Ki67 and a negative correlation is seen between the number of MAIT cells and expression of apoptotic markers PD-1 and Bcl-2, suggesting that exhaustion and apoptosis may account for decreased intrahepatic MAIT cell frequency [74]. Functional analysis in NAFLD shows increased IL 17 but unaltered granzyme B, IFN  $\gamma$ , and TNF $\alpha$  production[131]. In contrast to Li et al, Lotersztajn's group have suggested a more pathogenic role for MAITs in NAFLD, with MAIT cell derived TNF $\alpha$  promoting pro-inflammatory cytokine (IL 6, IL 8) production by myofibroblasts and monocytes [74]. This is further supported by their murine models, with MAIT cell enriched mice exhibiting accelerated hepatic fibrosis in carbon tetrachloride models of liver injury, whilst mice lacking in MAIT cells are protected [74]. Paradoxically however, MAIT cells may also have the potential to protect against the evolution of advanced fibrosis by secreting IL 22, which has been shown to promote hepatic stellate cell senescence and IFN  $\gamma$  which enhances NK-mediated hepatic stellate cell cytotoxicity [75, 132].

### 1.6.9 Conclusion

There is significant paucity of data examining MAIT cells in NAFLD, with limited human studies to date. In patients with end-stage liver disease undergoing transplantation for a variety of causes including NASH, MAIT cells numbers have been shown to be reduced both in the peripheral circulation and within the liver [133]. Similarly, in patients with NAFLD, circulating MAIT cells are reduced in comparison to healthy controls [130, 134, 135]. Whether intrahepatic MAIT cells are protectors or protagonists in the context of NAFLD pathogenesis is not yet clear, and may depend on disease stage [136]. Recent studies have shown that liver resident MAIT cells have unique transcriptomic effector profiles and a more polyfunctional phenotype than their blood counterparts [137]. In animal studies, wild-type mice fed a methionine/choline deficient diet had increased intrahepatic MAIT cell number, and MAIT knock out mice had more severe hepatic steatosis and inflammation, suggesting a potential protective role in NAFLD[130]. However, in models of chronic liver injury, MAIT knock out mice exhibited less fibrosis indicating a profibrotic role for MAIT cells [134]. *In situ* staining of paraffin embedded liver tissue from patients with NAFLD (n=40) has suggested an increased number of MAIT cells in NAFLD in comparison to healthy control liver (n=5) [130]. However, a study using flow cytometry and immunophenotyping demonstrated no difference in intrahepatic MAIT cell numbers in liver biopsies from patients with NAFLD (n=15) versus healthy livers (n=3) [135]. No studies to date have investigated changes in MAIT cells in the liver following lifestyle interventions in NAFLD.

### 1.7.1 Accelerated ageing and frailty in NAFLD:

More recently, chronic inflammation and immune dysregulation have been linked with ageing, sarcopaenia (reduced muscle mass and function) and the evolution of frailty[138, 139]. Ageing in healthy individuals without the coexistence of chronic disease or metabolic risk factors is accompanied by immune dysregulation with higher circulating levels of inflammatory cytokines: IL 1, IL 1 receptor antagonist (IL 1RA), IL 6, IL 8, TNF $\alpha$ , TGF $\beta$ , IFN $\gamma$ ,

diminished neuroplasticity, reduced basal metabolic rate and changes in body composition with reductions in lean body mass [138, 139].

In pathological states, particularly central obesity, chronic low grade inflammation has an additive effect, accelerating the ageing process, otherwise known as Inflammageing [140]. This is particularly important as it hastens functional decline, thereby increasing the risk of frailty [140]. Frailty is a clinical syndrome distinguished by reduced physiological function, impaired strength, mobility and increased vulnerability to dependency, hospitalization and death [141, 142]. The adverse clinical outcomes associated with frailty appear to be distinct from age alone and suggest a discrete pathological process [143, 144].

#### *1.7.2 Sarcopaenia in Obesity and NAFLD*

In obesity, as previously described, chronic adiposopathy promotes inflammation by increasing pro-inflammatory cytokine production and release. The circulating concentration of these cytokines, particularly IL 6, positively correlate with adverse clinical events including disability, increased frailty, cardiovascular and neurodegenerative diseases [139, 145-147]. Under these pathological conditions, skeletal muscle undergoes functional changes including reduced muscle mass and strength, otherwise known as sarcopaenic obesity [69, 148]. Total body fat mass is predictive of the accelerated decline of muscle quality and strength [149]. Metabolic dysregulation and inflammation promotes mitochondrial dysfunction, release of reactive oxygen species, insulin resistance and impaired  $\beta$  oxidation of fatty acids resulting in increased uptake of fatty acids by muscle (myosteatorsis) [69, 140, 150]. Lipids accrue in the form of intramuscular adipose tissue or intramyocellular lipids [151]. This lipid accumulation is harmful, not only impairing muscular contractility, but further promoting inflammation and oxidative stress which triggers myocyte apoptosis, resulting in reduced muscle mass [151-153]. Intramuscular lipid deposition also disrupts myokine production, with downregulation of irisin and increase in d myostatin [150-152]. Furthermore, the oxidative stress caused by reactive oxygen species leads to post translational changes within myocytes that compromise muscular protein function [150-152]. Myostatin has been shown to increase reactive oxygen species generation through increased TNF $\alpha$  mediated signalling, promoting inflammation, encouraging adipose tissue insulin resistance and also acts as a negative

regulator of skeletal muscle growth, thereby contributing to the reduction in functional muscle mass through inhibition of myogenesis [152, 154, 155]. Irisin enhances oxidative metabolism and mitochondrial synthesis, reduces  $\text{TNF}\alpha$ , IL 6, promotes M2 macrophage polarization and circulating irisin levels correlate with muscle mass and strength, consequently its downregulation further perpetuates the cycle of inflammation, damage and muscle loss [152, 154, 155]. This effect is visible clinically, with lower irisin levels associated with reduced mobility, increased risk of falls and increased mortality [139, 152, 156-158].

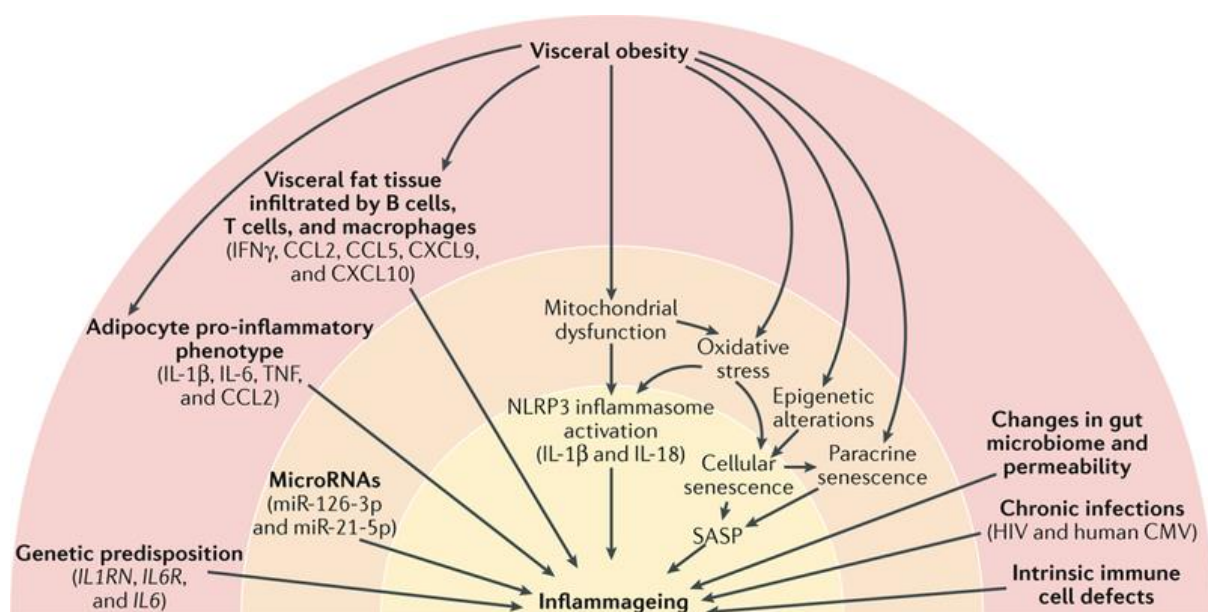


Figure 1-9 The relationship between obesity-associated inflammation, cellular senescence and inflammaging, Ferrucci et al, 2018[40]

### 1.7.3 Senescence

Cellular senescence has also been linked with accelerated ageing. Senescence is a process which essentially acts as a safety mechanism arresting the cell cycle, thereby stopping the proliferation of damaged cells and causing them to acquire a senescence associated secretory phenotype (SASP) [150, 152]. It is triggered by DNA damage, mitochondrial dysfunction and cellular exposure to DAMPs [150]. This switch promotes the release of pro-inflammatory cytokines: IL 1 $\alpha$ , IL 1 $\beta$ , IL 6, chemokines: IL 8, growth-regulated- $\alpha$  protein and growth factors: fibroblast growth factor 2, hepatocyte growth factor and extracellular matrix components to help repair surrounding tissue, further promote senescence and help 'label' damaged cells for immune clearance (See Fig. 1-9) [150, 152, 159]. Senescent

cells have been shown to accumulate in skin, pancreas, vascular endothelium, cardiac myocytes, visceral adipose tissue and the liver in association with advancing age [59, 150, 160-165]. It is likely that in pathological situations such as obesity, with associated inflammaging and increased cellular damage, senescence is exaggerated with excess accumulation in tissue and resultant organ dysfunction. Interestingly, removal of senescent cells delays age related organ dysfunction and improves health span in murine models [152, 166]. Senescent cells have also been linked with sarcopaenia, with an inverse correlation between senescent cell burden in thigh adipose tissue and grip strength, walking speed and perceived mobility [150, 167]. Exercise has also been shown to have a protective effect, preventing the accumulation of senescent cells by inhibiting inflammatory and enhancing metabolic signalling pathways [152, 168].

#### *1.7.4 Autophagy*

Defects in autophagy, a mechanism of cellular repair, are known to encourage inflammaging [169]. Autophagy describes the process by which damaged intracellular products, organelles and accumulated proteins undergo lysosomal degradation [169]. It is triggered by cellular stress such as hypoxia, exposure to reactive oxygen species and pro-inflammatory mediators [169]. In the healthy state, autophagy removes dysfunctional mitochondria and lipid droplets, reducing the generation of reactive oxygen species, endoplasmic reticular stress and helping maintain normal cellular metabolism [62, 169, 170]. It aids the immune system by directly eliminating intracellular pathogens and also acts as a regulator of inflammation, inhibiting the inflammatory cascade by breaking down components of the NLRP3 inflammasome including: pro IL 1 $\beta$  and IL 18 [62, 169]. Through these actions, autophagy protects against the complications of metabolic disease, in murine models pharmacological activation of autophagy and blockade of the NLRP3 inflammasome is protective against the development of non-alcoholic fatty liver disease, improves insulin resistance, reduces frailty through increased mobility, reduced cognitive decline and increases longevity [139, 171-173]. In obesity and metabolic disease, autophagosomes and associated products are seen to accumulate particularly in adipocytes and hepatocytes, despite this, there is evidence of increased NLRP3 inflammasome activity, mitochondrial dysfunction and lipotoxicity [174-177]. This would

point to an impairment of autophagy as another mechanism driving accelerated ageing in obese individuals.

### *1.8 Current treatment options for NAFLD*

As with obesity and associated metabolic conditions, lifestyle changes are the cornerstone of NAFLD management. One of the hallmark interventional studies conducted by Vilar-Gomez et al, showed significant histological improvements amongst individuals with NAFLD, when at least 5% total body weight reduction was achieved and sustained over a one-year period [178]. In this study, the degree of weight loss determined the extent of histological improvement, with maximal improvements (resolution of NASH in 90% with fibrosis regression in 81%), occurring in individuals who achieved a weight loss target of greater than or equal to 10% of their initial body weight [178]. Consequently, a weight loss target of 7-10% has been incorporated into the EASL-EASD-EASO and AASLD NAFLD management guidelines. However, reaching this target remains a challenge, in the study only 10% of individuals were able to achieve that degree of weight loss despite participating in a supported lifestyle intervention program [178].

Amongst dietary interventions, both hypocaloric and Mediterranean diets appear to be equally beneficial, promoting weight loss, reducing visceral fat, thereby improving insulin resistance and hepatic steatosis [179]. Although the Mediterranean diet is favoured given its cardioprotective effects. Currently, the EASL-EASD-EASO and AASLD guidelines recommend a daily calorie deficit ranging from 500 to 1000 kcal [2, 180]. However, it may not simply be a matter of achieving a caloric deficit, as diet quality also plays a role, with red and processed meat, sugar sweetened beverages and fructose intake increasing the risk of NAFLD[179]. Moderate alcohol consumption also has an additive effect further promoting hepatic steatosis, whilst coffee consumption by contrast, is protective, with a dose dependent reduction in risk of NAFLD, advanced fibrosis, hepatocellular carcinoma and liver-related mortality [179, 181].

With regards to exercise, although few studies involve paired liver biopsies, meta-analyses have shown improvements in liver enzymes, insulin sensitivity and reduction in the degree

of hepatic steatosis with no differences between aerobic and resistance-based interventions [182, 183]. In most of these studies, improvements are attributable to the extent of weight loss and more pronounced in individuals with an elevated BMI [179, 182-184]. However, in one meta-analysis, exercise was found to be beneficial even in the absence of significant weight loss.[183] This would suggest that exercise may exert unique anti-inflammatory effects. Interestingly, studies evaluating the impact of exercise on the immune system would support this hypothesis with differences based on exercise intensity, duration and consistency. Maximal intensity exercise has been shown to enhance immunosurveillance, increasing circulating leukocytes, particularly NK cells, CD8<sup>+</sup> lymphocytes and non-classical monocytes, with normalization within hours, regardless of baseline activity levels and fitness [185]. More consistent activity, by contrast, can exert more lasting effects on the immune system, with higher circulating CD8<sup>+</sup> T lymphocytes seen in athletes compared to increased CD4<sup>+</sup> and T<sub>reg</sub> lymphocytes in sedentary individuals [185]. Regular exercise has also been associated with reduced systemic inflammation in obesity, decreasing circulating IL 6 and CRP levels, in addition to lowering the risk of infection [186]. Indeed, regular exercise has been shown to reduce the risk of upper respiratory tract and influenza infections by up to 40-50% [186]. Improvements in immune function and protection against infection may be explained by senescence of the immune system, deemed 'immunosenescence,' which can potentially be modifiable. A study conducted by Spielmann et al, showed that individuals with higher aerobic fitness levels had proportionately lower senescent T cells in circulation [187], with a recent review showing that exercise is 'senolytic' in human studies[188]. Not much literature exists on exercise mediated immunological changes in NAFLD, however, murine models have shown that 4 weeks of voluntary exercise promotes immunological changes within the liver, reducing macrophage activation marker, pro-inflammatory chemokine, MCP-1 with pro-inflammatory cytokine, IL 6 expression in conjunction with metabolic improvements [189].

Bariatric surgery is currently recommended for morbidly obese individuals and is a therapeutic intervention that promotes the greatest degree of weight loss. In individuals with NAFLD, bariatric surgery is not only associated with improvements in hepatic steatosis (66%), but also hepatic inflammation (50%) and fibrosis (40%) [184, 190]. These improvements correlate with the degree of weight loss and are progressive and persistent,

with NASH resolution in 86% and fibrosis regression seen in 54% of individuals five years post-surgery [191]. Furthermore, bariatric surgery reduces the risk of developing cirrhosis, hepatocellular carcinoma, ischaemic heart disease, renal disease and is associated with reduced inpatient mortality [184, 192]. At present there are no licenced pharmacotherapies for NAFLD, however investigations have been conducted with medications that improve insulin sensitivity and promote weight loss.

Amongst insulin sensitising agents, the use of PPAR $\gamma$  agonist pioglitazone has been assessed most extensively, notably in the PIVENS trial, a randomised, double-blinded, placebo-controlled study, alongside the anti-oxidant, vitamin E [193]. PPAR $\gamma$  agonists, promote insulin sensitization, increasing circulating adiponectin levels and redistributing visceral fat to subcutaneous fat [194]. In the PIVENS study, after 96 weeks, non-cirrhotic NAFLD patients in both pioglitazone and vitamin E showed improvements in hepatic steatosis, 69% and 54% respectively, histological improvements in NASH 34% and 43%, with NASH resolution in 46% of individuals in the pioglitazone group [193]. In a systematic review, PPAR $\gamma$  agonists were also shown to delay the progression of hepatic fibrosis [195]. The off label use of pioglitazone has been incorporated in the AASLD and EASL NAFLD management guidelines for use in individuals with NASH and diabetes, although its use does promote weight gain, can exacerbate heart failure and has been associated with increased risk of osteoporosis in women [2, 180]. Vitamin E has also been included in the recommendations for non-diabetic individuals with NASH, with a further study amongst non-diabetic cirrhotics showing reduced risk of decompensation and increased liver transplant-free survival [2, 180, 196]. These improvements were not reproduced in the paediatric cohort and the use of vitamin E in meta-analyses has been associated with increased risk of haemorrhagic stroke, prostate cancer and all-cause mortality [197-200].

Sodium glucose co-transporter 2 (SGLT-2) inhibitors including: canagliflozin , empagliflozin, are licenced for use in diabetes and improve glycaemic control and promote weight loss by enhancing renal glucose elimination [201]. They have both cardio- and reno-protective effects amongst individuals with diabetes, but are associated with an increased risk of urinary tract infections and osmotic diuresis [201]. Amongst individuals with NAFLD, they have been shown to improve liver enzymes and hepatic steatosis, with reductions in

hepatic triglyceride content directly correlating with weight loss [184, 202]. More recently, 24 weeks of therapy has been shown to lead to improvements ballooning stage and fibrosis in NAFLD patients with diabetes[203].

There has been increasing interest around the use of glucagon-like peptide 1 (GLP-1) agonists; liraglutide and semaglutide. GLP-1 agonists act on the pancreatic  $\beta$  islet cells, stimulating insulin and inhibiting glucagon secretion [201]. Through their actions they delay gastric emptying, increase satiety and promote weight loss [184]. The resultant reduction in visceral fat improves insulin sensitivity and reduces lipotoxicity [204]. Their weight loss effects are more pronounced particularly with semaglutide in comparison with the SGLT-2 inhibitors, with an average 5-7% reduction over a 30 week period [205-207]. Amongst individuals with NAFLD, GLP-1 agonists improve hepatic steatosis, with resolution of NASH in 39-40% of participants [205-208]. Additionally, there have been trials using a combined glucose-dependent insulintropic polypeptide and GLP-1 agonist, tirzepatide in the treatment of obesity. This combination, resulted in even more significant weight loss, with over 80% of participants losing greater than 5% of their initial body weight reduced hepatic steatosis over a 72 week period[209]. However, despite the extent of weight loss and resolution of NASH, the trials to date using this combination therapy have not shown any changes in fibrosis stage compared with placebo [208, 210-213]. Although in a small retrospective study the addition of GLP-1 agonists to SGLT-2 inhibitor in diabetic patients with NAFLD did lead to improvements in NASH and fibrosis stage after a 5 year period[214].

Collectively the data would suggest that lifestyle changes including dietary modification and exercise exert a unique beneficial effect extending beyond weight loss and improved insulin sensitivity. This may be related to specific immunological changes, although very few studies have assessed for the impact on lifestyle interventions on the immune system in NAFLD and its associations with histological improvement.

### *1.21 Thesis Outline and Aims:*

There is presently a global epidemic of obesity and as a consequence NAFLD has become the most common cause of liver disease in the developed world. The pathophysiology of

obesity and NAFLD centres around adiposopathy, lipotoxicity and immune dysregulation which perpetuates a state of chronic inflammation. This is associated with significant morbidity and mortality and has more recently been linked to sarcopaenia, accelerated ageing and frailty. Most of the available data investigating immune cells in the evolution and progression of NAFLD has been derived from animal models, therefore, there is a need for further descriptive and functional studies amongst individuals with the condition. MAIT cells have recently been identified as key players in metabolic disease however, there is very little information on their characteristics and role in NAFLD.

The aims of this thesis are:

- 1) To perform a detailed descriptive analysis of circulating and intrahepatic immune cells with a focus on MAIT cells in individuals with NAFLD and correlate with disease severity based on histological markers.
- 2) To evaluate potential alterations in circulating and intrahepatic MAIT cells in association with histological improvements in NAFLD following weight loss interventions.
- 3) To perform frailty measures amongst individuals with NAFLD and correlate with disease severity

#### *1.22 Thesis outline*

|                    |                                                                                             |
|--------------------|---------------------------------------------------------------------------------------------|
| <b>Chapter I</b>   | Introduction and literature review on Obesity, NAFLD, Inflammageing, Frailty and MAIT cells |
| <b>Chapter II</b>  | Materials and Methods                                                                       |
| <b>Chapter III</b> | Study I: Descriptive analysis of circulating and intrahepatic immune cells in NAFLD         |
| <b>Chapter IV</b>  | Study II: MAIT cells and NAFLD: Changes following weight loss interventions                 |
| <b>Chapter V</b>   | Study III: Frailty in NAFLD                                                                 |
| <b>Chapter VI</b>  | General Discussion                                                                          |

## Chapter 2: Materials and Methods

## Materials and methods:

This chapter will discuss the materials, protocols and data analysis methods used throughout this thesis. A number of methods are common to study I and study II, consequently these chapters will refer back to the relevant section in this chapter when reviewing the individual study methodologies.

### *2.1 Ethical Approval:*

This research was carried out in accordance with the Declaration of Helsinki ethical guidelines for medical research involving human subjects. Ethical approval for this study was obtained from the Tallaght University Hospital/ St. James's Hospital joint ethics committee (May 2017). Participants signed informed consent sheets prior to collection of all specimens and all patient data was pseudo-anonymised.

### *2.2 Data Management:*

All collected data was pseudo-anonymised with study codes. Hard copy data was stored in a secure location and was only accessible by the research team. Data was then transferred to password protected databases and was fully anonymised once collection had been completed.

### *2.3 Missing Data:*

The studies incorporated into this thesis have missing variables including some laboratory results, DEXA scans and VCTE measurements when valid readings were not obtainable. Some clinical data points are missing due to patient withdrawal or non-attendance for specific investigations. Due to issues with contamination of the incubator and evaporation of samples, the prepared tissue conditioned media were not suitable for analysis. As there were also issues with the arrival and condition of ordered ELISA plates, ELISAs were not performed on collected serum samples.

The cell surface receptors were cleaved during the preparation of peripheral blood and digested liver tissue samples obtained from obese individuals at the time of their bariatric surgery for functional MAIT cell analysis, as a result no data was obtained from these analyses.

#### *2.4 Study materials*

The materials used in this thesis are arranged in four tables as follows:

*Table 2-1: Consumables and plasticware*

| <b>Item</b>                       | <b>Source</b>      | <b>Catalogue Number</b> |
|-----------------------------------|--------------------|-------------------------|
| 70 µm cell strainer               | Fisher Scientific  | FB35180                 |
| 50 ml centrifuge tubes            | Fischer Scientific | 11819650                |
| 15 ml centrifuge tubes            | Fischer Scientific | 11755075                |
| Flow cytometry tubes - FACS Canto | Fischer Scientific | 12650366                |
| 1.5 ml Eppendorf tubes            | Eppendorf          | T9661-1000EA            |
| Small gloves                      | Shield             | GD055                   |
| Small nitrile gloves              | Fisher Scientific  | FB69262                 |
| p10 pipettor tips                 | Sarstedt           | 70.1130                 |
| p200 pipettor tips                | Sarstedt           | 0.760.002               |
| p1000 pipettor tips               | Fisher Scientific  | FB78084                 |
| 5 ml Pipettes                     | Corning            | PN5E1                   |
| Scalpels                          | Fisher Scientific  |                         |
| 12-well tissue culture plates     | Sarstedt           | 83.3921.300             |
| 6-well tissue culture plates      | Sarstedt           | 83.8920                 |
| Tissue paper                      | Fisher Scientific  | CMC-716-021F            |
| Autoclave tape                    | Fisher Scientific  | 11-889-5                |
| Biohazard bin liners              | Fisher Scientific  | BAJ-560-050U            |
| Cryovials                         | Sarstedt           |                         |

|                                     |                |            |
|-------------------------------------|----------------|------------|
| Cardboard freezer boxes<br>10x10 cm | Sarstedt       | 95.064.997 |
| Trucount™ beads                     | BD Biosciences | 337166     |
| Multitest™ 6-color TBNK reagent     | BD Biosciences | 644611     |

*Table 2-2: General Reagents and Kits*

| Reagent                                      | Source             | Catalogue Number |
|----------------------------------------------|--------------------|------------------|
| BD lysis buffer                              | BD Biosciences     | 349-202          |
| Bovine Serum Albumin (BSA)                   | Fisher Scientific  | BPE-9703-100     |
| Foetal Bovine Serum (FBS),<br>Hyclone        | Thermoscientific   | SH3 071.03       |
| Collagenase IV                               | Sigma Aldrich      | C5138-500MG      |
| Hank's Balanced Salt Solution<br>(HBSS)      | Gibco              | 14025092         |
| Lymphoprep                                   | BD Biosciences     | 1114547          |
| Ionomycin                                    | Sigma Aldrich      | IO634            |
| Monensin                                     | Biolegend          | 420701           |
| Medium 199                                   | BD Biosciences     | BP231-100        |
| BD FACS lysing solution                      | BD Biosciences     | 349202           |
| Dimethyl Sulfoxide (DMSO)                    | Fisher Scientific  | BP231-100        |
| Saponin                                      | Sigma Aldrich      | S7900            |
| RPMI medium 1640 (1x) +<br>Glutamax™-I       | Gibco              | 61870010         |
| Sterile Phosphate Buffered<br>Solution (PBS) | Gibco              | 14190094         |
| 4% Paraformaldehyde                          | Santa-Cruz Biotech | SC-281692        |
| Sodium Azide                                 | Sigma Aldrich      | S8032            |
| 95% Ethanol                                  | Sigma Aldrich      |                  |
| CompBeads Particles                          | BD Biosciences     | 552844           |
| Pharmingen Stain Buffer (FBS)                | BD Biosciences     | 554656           |

*Table 3-3: Fluorochrome-conjugated monoclonal antibodies for flow cytometry*

| <b>Antibody</b>                                | <b>Fluorochrome</b> | <b>Clone</b> | <b>Company</b> | <b>Catalogue Number</b> |
|------------------------------------------------|---------------------|--------------|----------------|-------------------------|
| Anti TCR V $\alpha$ 7.2                        | FITC                | REA179       | Miltenyi       | 130-100-189             |
| CD161                                          | APC                 | DX12         | BD Biosciences | 550968                  |
| CD3 (Leu4)                                     | APC-H7              | SK7          | BD Biosciences | 560176                  |
| CD45<br>(Leukocyte<br>Common<br>Antigen, Ly-5) | PerCP               | 2D1          | BD Biosciences | 345809                  |
| CD69 (Very Early<br>Activation<br>Antigen)     | PE-Cy 7             | FN50         | BD Biosciences | 557745                  |
| CD95                                           | PE                  | DX2          | BD Biosciences | 555674                  |
| CD8                                            | V450                | RPA-T8       | BD Biosciences | 560347                  |
| CD14                                           | APC-H7              | M $\phi$ P9  | BD Biosciences | 560180                  |
| CD16                                           | BV510               | 3G8          | BD Biosciences | 563830                  |
| CD11b/Mac-1<br>(CR3)                           | PE-Cy 7             | ICRF44       | BD Biosciences | 557743                  |
| CD284 (TLR4)                                   | BV421               | TF901        | BD Biosciences | 564401                  |
| CD64                                           | PE                  | 10.1         | BD Biosciences | 558592                  |
| CD68                                           | Alexa Fluor 647     | Y1/82A       | BD Biosciences | 562111                  |
| CD163                                          | FITC                | GHI/61       | BD Biosciences | 563697                  |
| HLA-DR                                         | BB700               | G46-6        | BD Biosciences | 745782                  |
| Zombie Aqua<br>Fixable Viability               | BV510               |              | MSC            | 423102                  |
| CD107a (LAMP-<br>1)                            | PE                  | H4A3         | BD Biosciences | 555801                  |
| IL-10                                          | BV421               | JES3-9D7     | BD Biosciences | 564053                  |
| IL-17A                                         | BV510               | N49-653      | BD Biosciences | 563295                  |
| IFN $\gamma$                                   | PE-Cy 7             | B27          | BD Biosciences | 557643                  |

*Table 3-4: Equipment and software*

| <b>Equipment</b>                                | <b>Model</b>                              | <b>Company</b>                    |
|-------------------------------------------------|-------------------------------------------|-----------------------------------|
| Vortex                                          | Vortex Genie                              | Scientific Industries, NY, USA    |
| Water bath                                      | Grant                                     | Fischer Scientific, PA, USA       |
| Centrifuge                                      | Eppendorf 5810                            | Eppendorf, Hamburg, Germany       |
| Laminar air flow Thermo Class II safety cabinet | CleanAir MSC BSS6-2                       | Fisher Scientific, MA, USA        |
| Flow Cytometer                                  | FACS Canto II                             | Becton Dickinson, Oxford, UK      |
| Flow Cytometry Software                         | FACSDIVA                                  | Becton Dickinson, Oxford, UK      |
| FlowJo                                          | Version 10.2                              | Treestar Incorporated, NJ, USA    |
| Haemocytometer                                  | Kova Slides                               | Hycor Biomedical, IN, USA         |
| CO <sub>2</sub> Incubator                       | Heracell 150i                             |                                   |
| Microscope                                      | Inverted NAO 30                           | Olympus Corporation, Japan        |
| Pipettes:<br>p20, p100, p1000                   | Labmate pro                               | HTL Lab solutions, Poland         |
| -20° Freezer                                    | Low temperature freezer                   | New Brunswick Scientific, CT, USA |
| -80° Freezer                                    | Ultra-low temperature freezer             | New Brunswick Scientific, CT, USA |
| DEXA                                            |                                           |                                   |
| Fibroscan                                       | FibroScan® 502 touch with M and XL probes | Echosens, France                  |
| Graphpad Prism                                  | Version 9                                 | La Jolla, CA, USA                 |
| SPSS                                            | Version 6                                 | IBM, SPSS Inc.                    |

## *2.5 Study 1: Cross sectional study analysing peripheral and circulating immune cells in patients with biopsy proven NAFLD*

The first part of the thesis involved performing detailed baseline analyses of circulating and intra-hepatic immune cells in patients with NAFLD.

### *2.5.1 Patient selection:*

Patients with a clinical diagnosis of NAFLD attending the Hepatology clinic in St. James's Hospital were enrolled into the study.

Definition of NAFLD pre- liver biopsy:

A clinical diagnosis of NAFLD was defined as: elevated liver enzymes in individuals with metabolic risk factors, with evidence of increased hepatic steatosis either by ultrasound or controlled attenuation parameter (CAP) measurements, without excessive alcohol consumption (defined as >30 g/day for males and >20 g/day for females) and medications known to promote NAFLD development [2, 215]

Full study eligibility criteria are listed below:

Inclusion Criteria:

1. Clinical diagnosis of NAFLD
2. Daily alcohol consumption of  $\leq 30$  g for men and  $\leq 20$  g for women
3. Age  $\geq 18$
4. Capacity for giving informed consent
5. Absence of significant hepatic fibrosis

Exclusion Criteria:

1. Unwilling to participate
2. Age  $\leq 18$
3. Pregnancy
4. Significant hepatic fibrosis or cirrhosis ( $\geq$  F3 disease)
5. Alcoholic fatty liver disease

6. Hepatitis C associated fatty liver disease
7. Haemochromatosis
8. Autoimmune hepatitis
9. Alpha 1 anti-trypsin deficiency
10. Coeliac disease
11. Wilson's disease
12. Hypopituitarism
13. Hypothyroidism
14. Inborn errors of metabolism: e.g. Lecithin cholesterol acyltransferase (LCAT) deficiency, hypobetalipoproteinaemia, lysosomal acid lipase deficiency.
15. Exposure to drugs associated with hepatic steatosis including: tamoxifen, amiodarone, corticosteroids, valproate, TPN
16. Patients post pancreatic resection
17. Patients with short bowel syndrome
18. Hepatocellular carcinoma
19. Inability to attend for clinic reviews and dietary intervention sessions
20. Inherited or acquired coagulopathy
21. Thrombocytopaenia
22. Anti-coagulation therapy
23. Haemoglobin level  $\leq 10$  g/dL
24. Immunosuppressive therapy
25. Hereditary or acquired immunodeficiency

#### *2.5.2 Laboratory assessments:*

All participants had baseline laboratory assessments performed which included: full blood count, renal profile, liver enzymes: ALT, AST, alkaline phosphatase, bilirubin, GGT, glycated haemoglobin A1c, lipid profile, urate and vitamin D levels. Glycated haemoglobin A1c is used in clinical practice to assess glycaemic control in individuals with diabetes, elevated levels reflect the degree of insulin resistance and have been shown to correlate with NAFLD severity [216]. Low circulating vitamin D levels have been associated with increased prevalence of NAFLD and NASH although vitamin D levels do not correlate with histological

severity [217, 218]. Elevated serum uric acid levels have also been independently associated with increased NAFLD risk, with a dose-response relationship on meta-analysis [219, 220]. Circulating neutrophil and lymphocyte counts were measured via a Haematology Analyzer. The NLR was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count[221].

Absolute monocyte numbers were measured using the Haematology Analyzer. The monocyte: HDL ratio (MHR) was calculated by dividing the absolute monocyte count ( $10^9/L$ ) by the HDL levels (mmol/L) and multiplying by one hundred [222].

### *2.5.3 Non-invasive methods of assessing liver disease severity:*

Although liver enzymes can be elevated in individuals with NAFLD, they do not correlate with disease severity and can even be normal in individuals with established cirrhosis [223]. As fibrosis is the most important determinant of prognosis, several non-invasive scoring systems, most notably the FIB-4 and NAFLD scores, have been developed and validated in the NAFLD population. Both have been shown to have moderate accuracy and high negative predictive values following meta-analysis [2, 215, 224, 225].

#### *2.5.3.1 Algorithm for lab-based, non-invasive assessment of NAFLD severity:*

**FIB4 score formula:**  $\text{Age [years]} \times \text{AST [IU/L]} / (\text{platelets} [\times 10^9/L] \times (\text{ALT [U/L]})^{1/2})$

A score of less than 1.45 represents no fibrosis or early-stage disease, scores between 1.45 and 3.25 are in the intermediate range and scores exceeding 3.25 represent a high likelihood of advanced fibrosis.[226, 227]

**NAFLD fibrosis score formula:**  $-1.675 + 0.037 \times \text{age [years]} + 0.094 \times \text{BMI [kg/m}^2] + 1.13 \times \text{IFG/diabetes [yes = 1, no = 0]} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelet} [\times 10^9/L] - 0.66 \times \text{albumin [g/dL]}$

A score of less than -1.455 represents no fibrosis or early-stage disease, scores between -1.455 and 0.675 are in the intermediate range and scores exceeding 0.675 represent a high likelihood of advanced fibrosis [228, 229].

#### *2.5.3.2 Vibration controlled transient elastography:*

A vibration controlled transient elastography (VCTE) device (FibroScan® 502 touch, Echosens, France) was used to estimate the degree liver fibrosis (liver stiffness measurement [LSM]) and steatosis (controlled attenuation parameter [CAP]). VCTE is a widely available point of care test for liver fibrosis that has been validated against biopsies in chronic liver diseases including NAFLD. The Echosens device comes with a choice of two probes: the M and XL, that measures a depth of 2.5 cm and 6.5 cm respectively, from the skin based on skin to liver capsule distance. The machine generates mechanical shear waves which are propagated through the liver, measuring a volume approximately 100x that of a standard liver biopsy, using pulse-echo ultrasonic acquisition, which is then converted to a measure of liver stiffness expressed in kilopascals (kPa) using Young's modulus [230]. The device is also able to estimate the presence of hepatic steatosis by measuring a 3.5 MHz ultrasound coefficient of attenuation, known as the Controlled Attenuation Parameter (CAP) score. [230] The CAP is a measure in decibels per meter (dB/m) of the decrease in the amplitude of ultrasound signals in the liver caused by hepatic steatosis [231, 232]. Study participants were categorised into three groups based on LSM cut-off values for NAFLD: no/minimal fibrosis (F0/F1, <8.2kPa); moderate/advanced fibrosis (F2/F3, 8.2-13.5kPa); and cirrhosis (F4, ≥13.6kPa) [233].

#### *2.5.3.3 F-AST score*

The Fibroscan-AST (FAST) score, a composite score incorporating AST levels, VCTE and CAP measurements, has been validated as a non-invasive test of fibrotic NASH amongst individuals with NAFLD with an AUROC of 0.965 when a cut off score of ≥ 0.35 is applied [11, 234].

F-AST scores were also calculated for all study participants.

***F-AST score formula:***  $(\exp(-1.65 + 1.07 \times \ln(\text{LSM}) + 2.66 \times 10^{-8} \times \text{CAP3} - 63.3 \times \text{AST} - 1)) / \{1 + \exp(-1.65 + 1.07 \times \ln(\text{LSM}) + 2.66 \times 10^{-8} \times \text{CAP3} - 63.3 \times \text{AST} - 1)\}$  [234]

#### *2.5.4 Liver biopsy and histological scoring:*

Liver biopsy remains the gold standard for identifying NASH and assessing NAFLD severity. The characteristic histologic features of NAFLD include increased steatosis, lobular inflammation, hepatocellular ballooning and fibrosis.[235] Two scoring systems have been developed by the Non-alcoholic Steatohepatitis Clinical Research Network (NASH CRN) Pathology Committee, the NAS and Brunt Fibrosis scores, which quantify the histological severity of NASH and NAFLD fibrosis respectively.[235, 236]

Following initial evaluation all patients were scheduled for an ultrasound-guided percutaneous liver biopsy. Whole blood, serum and subcutaneous adipose tissue samples were all taken on the same day as the biopsy. Percutaneous liver biopsies were performed in accordance with the AASLD recommendations outlined by Rockey et al.[237] Two cores of liver were sampled, one was used for histological evaluation of NAFLD severity, including NAS and Brunt scoring, the second core was used for analysis of intra-hepatic immune cell populations. Liver and whole blood specimens were collected and transported in adipose transfer medium (500 ml of PBS, 0.5 ml of Gentamicin with 1.1 ml of 45% Glucose). Samples were processed immediately and underwent analysis within 3 hours of sampling.

Slides were routinely stained with Hematoxylin–Eosin, Masson Trichrome or AFOG, Gomori and Perls staining for iron. A single liver pathologist, Professor Stephen P Finn, who was blinded from the clinical data performed the scoring for all specimens.

#### *2.5.5 BMI, percentage body fat and muscle mass measurements:*

Body parametric assessments were made using DEXA scans which provided values for BMI in addition to estimates for percentage total body fat and muscle mass. Body parametric data and laboratory results were subsequently used to calculate the NAFLD and FIB-4 scores for each participant.

### *2.5.6 Principles of Flow Cytometry*

Flow cytometry refers to the process of using laser technology to enumerate and separate different cell populations. Thousands of cells pass each second through a beam of light generated by lasers via a capillary surrounded by detectors and filters.[238] As cells pass through the beam, light is scattered in different directions depending on the size, granularity and shape of the cells.[238] Fluorochrome-conjugated monoclonal antibodies can also be used to 'label' and aid the identification of specific cell populations. Lasers excite the fluorochromes, causing them to emit light which is then focused by emission filters and directed to detectors.[238] This enables not only the identification and enumeration of fluorochrome-labelled cells but also quantification of the intensity of light emitted by the cells (immunofluorescence).

### *2.5.7 Labelling of cells and Immuno-phenotyping using flow cytometry*

Liver tissue was prepared as per the protocol previously described by Conroy et al. [239] Liver tissue was digested enzymatically in a 50 ml tube containing 10 ml of HBSS and collagenase (0.05%) (BD Biosciences). Tissue was incubated for 25 minutes on a shaking incubator, at maximum agitation, at 37°C at 150 RPM before being passed through a 70 µm polypropylene filter (BD Biosciences). Cells were washed twice, initially with HBSS and subsequently with PBS. The pellet was re-suspended in PBS.

Whole blood and intrahepatic mononuclear cells were stained with anti-CD45-PerCP (BD Biosciences), anti-CD3-APC-H7 (BD Biosciences), anti-CD8-450 (BD Biosciences), anti-CD161-APC (BD Biosciences), anti-Vα7.2-FITC (Miltenyi), anti-CD69-PeVio770 (BD Biosciences), Zombie (MSC) and anti-CD95-PE (BD Biosciences). The gating strategies used are outlined in figures (1-3). MAIT cells were defined as CD161<sup>Hi</sup> Vα7.2<sup>Hi</sup> (See Fig 2-1) and all other CD3<sup>+</sup> cells were labelled as non-MAIT T cells. NK cells were defined as CD3<sup>Lo</sup>CD161<sup>Hi</sup> (See Fig 2-2). The mean fluorescence intensity of the acute activation marker, CD69 and the Fas ligand receptor or terminal activation marker, CD95 were measured.

For whole blood, a second tube was also stained to assess peripheral monocyte populations with anti-CD14-APC-H7 (BD Biosciences), anti-CD-16-BV510 (BD Biosciences), anti-CD11b-PE-Cy7 (BD Biosciences), anti-CD64-PE (BD Biosciences), anti-HLA-DR-BB700 (BD Biosciences), anti-CD68-Alexa Fluor 647 (BD Biosciences) and anti-CD163-FITC (BD Biosciences). Followed by red cell lysis (BD lysing solution) and re-suspension in PBA. Red cells were lysed using BD Lysing solution according to manufacturer's recommendations. The monocyte population was divided in to Non-classical ( $CD14^{dim}CD16^{+}$ ), Classical ( $CD14^{+}CD16^{-}$ ) and Intermediate ( $CD14^{+}CD16^{+}$ ) populations(See Fig 2-3).[240] The MFI of surface markers CD11b, CD64, HLA-DR, CD68 and CD163 were analysed. Cells were immediately analysed using a BD FACSCanto II (BD Biosciences) FACSDiva version 6.1.3 and Flow Jo™ (TREESTAR, WINDOWS 7 version 10.0.8).

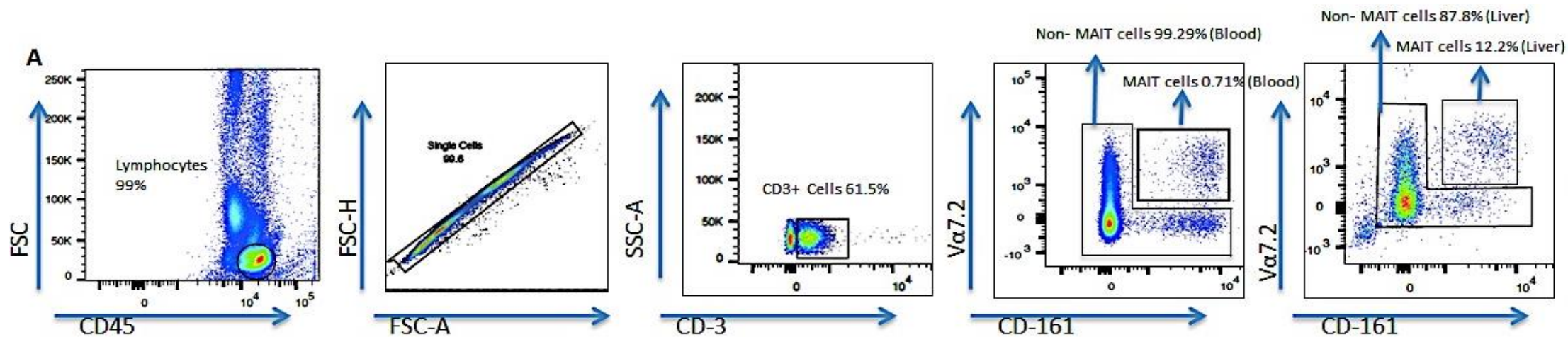


Figure 2-1 The gating strategy for identification of MAIT and non-MAIT T cell populations.

CD45 expression was used to identify lymphocytes in whole blood and in digested liver tissue. Doublets were excluded by gating on single cells in FSC-H and FSC-A dot plot. T cells were isolated by gating on the CD3<sup>+</sup> cell populations. MAIT cells were defined as CD161<sup>Hi</sup> Vα7.2<sup>Hi</sup>, all other cells were labelled as non-MAIT T cells. Mean fluorescence intensity of the activation markers CD69 and CD95 expression on MAIT and non-MAIT T cells were measured.

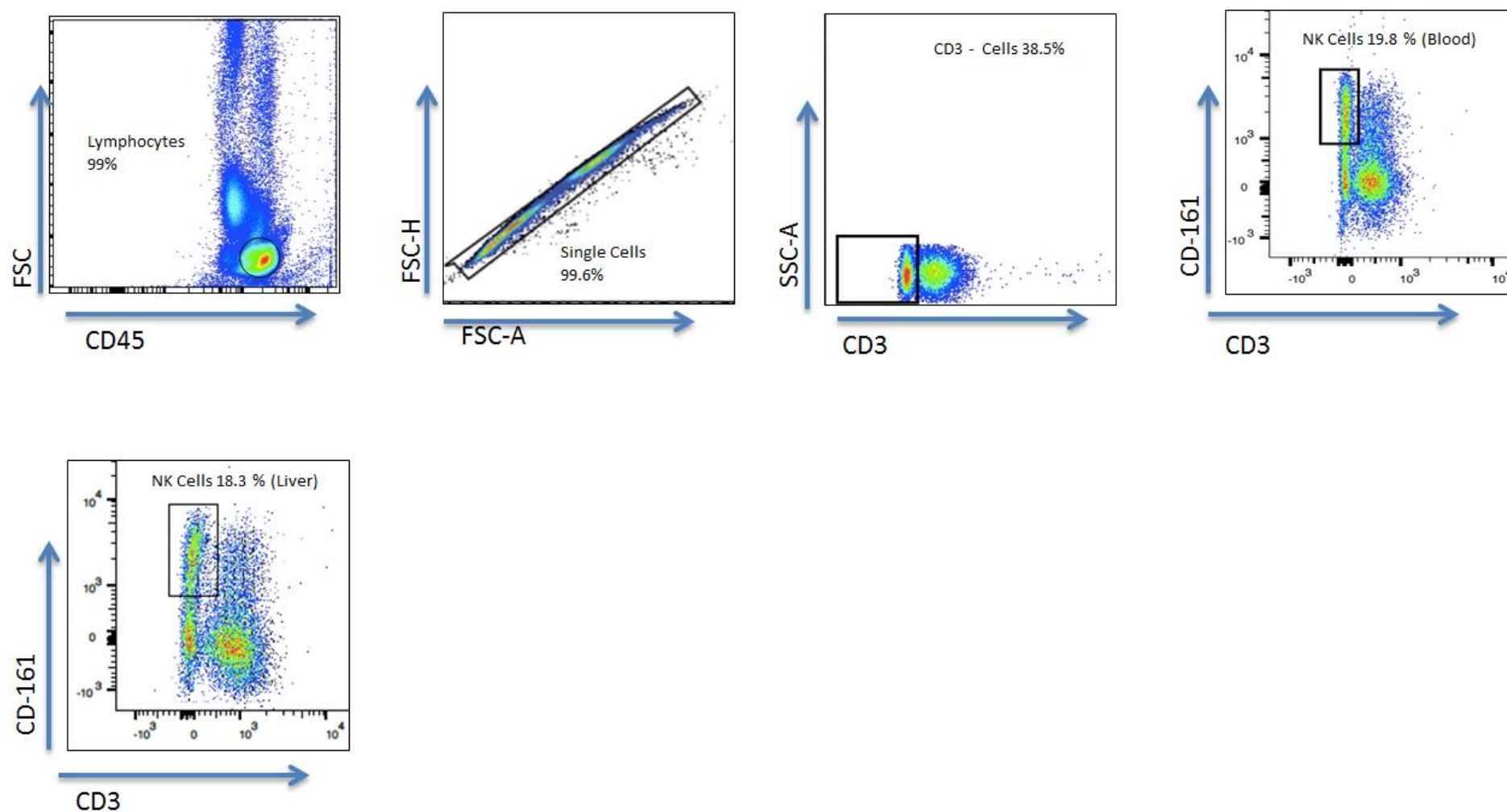
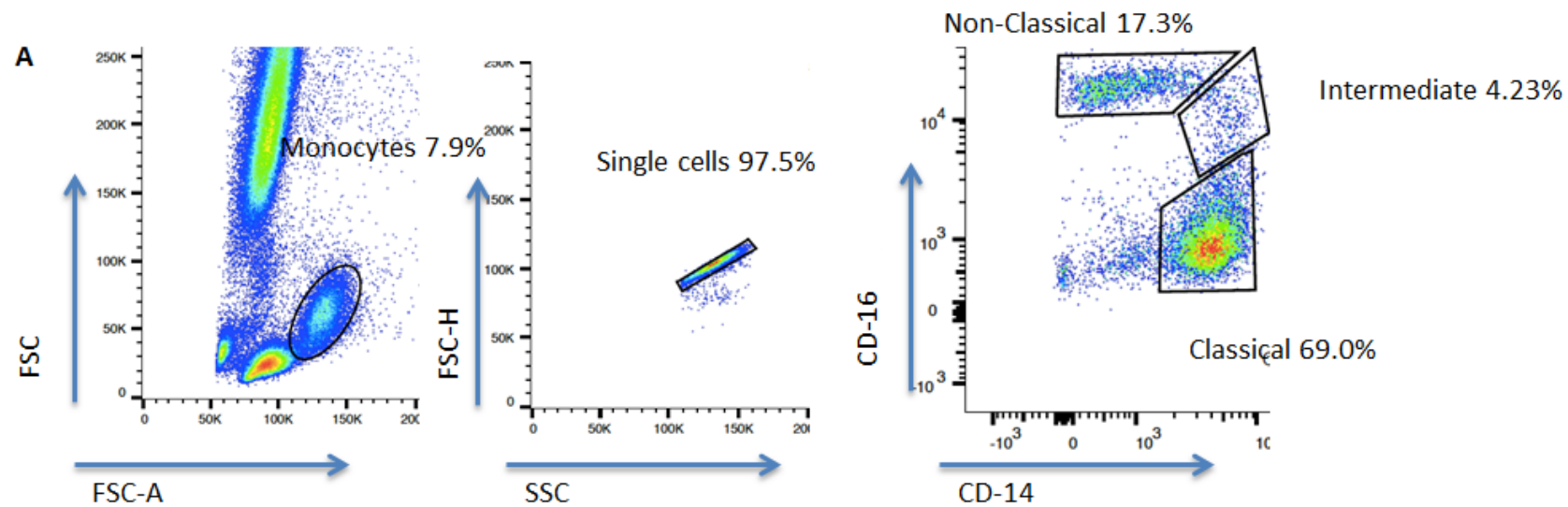


Figure 2-2 The gating strategy for isolating NK cells in whole blood and digested liver tissue.

Lymphocytes isolated using CD45 expression. Doublets were excluded by gating on single cells in FSC-H and FSC-A dot plot. NK cells were defined as CD3<sup>neg</sup>CD161<sup>Hi</sup> population. The mean fluorescence intensity (MFI) of the activation markers CD69 and CD95 expression on circulating and intra-hepatic NK cells were measured.



*Figure 2-3 Gating strategy for isolating monocytes.*

FSC-H and SSC dot plots were used to gate on monocyte populations, doublets were excluded by gating on single cells on FSC-H versus FSC-A dot plots, the monocyte population was divided into non-classical, intermediate and classical using CD16 and CD14 dot plots. Mean fluorescence intensity of CD163, CD11b, TLR-4, CD64, HLA-DR and CD68 expression by different monocyte populations was measured.

Absolute circulating T, B and NK cell populations were enumerated using a single platform method with a BD 6-colour multi-test and BD Trucount beads (BD Biosciences).

#### *2.5.8 Statistical Analysis*

All statistical analyses were conducted using GraphPad Prism v 9.0 (GraphPad Software Inc. La Jolla, CA). Baseline between-group differences were assessed using one-way ANOVA tests for non-normal data. Paired Wilcoxon signed-rank tests were used to assess differences between T0 and T1 measurements within each intervention group. Where appropriate, missing data are noted on each representative table and figure. Statistical significance for all tests was set at  $P < 0.05$  (see table 2-4).

#### *2.6 Study II: Measuring the impact of weight loss interventions in NAFLD on circulating and intrahepatic immune cells*

The second part of the thesis involved assessing the impact of a 12-week nutritional and exercise weight loss interventions on both circulating and intra-hepatic immune cell populations and correlating them with histological changes in patients with NAFLD.

##### *2.6.1 Inclusion criteria changes from study I*

Inclusion criteria for this study were as previously mentioned in Study I, however as this study involved participation in an exercise intervention, additional exclusion criteria were added for patient safety as listed below:

1. Patients with a physical disability or limitation which affects their ability to complete the exercise/ dietary intervention programs
2. Those with a medical history of advanced cardiac or respiratory disease, which is considered to be a contraindication to exercise.
3. Uncontrolled hypertension
4. Uncontrolled asthma
5. Musculoskeletal injury affecting exercise ability

6. Low back pain in the previous three months
7. Rheumatic disorders (e.g. Rheumatoid arthritis, osteoarthritis, fibromyalgia, spondyloarthropathies)
8. Neurological disorders affecting exercise ability (e.g. multiple sclerosis, cerebral palsy)
9. Acute active infections (e.g. cold or flu)
10. Inability to regularly attend exercise sessions

#### *2.6.2 Study design:*

Following the baseline (T0) assessments performed in study I, participants were recruited and allocated to either to a 12-week exercise, nutritional intervention or control group by convenience sampling. The exercise intervention group (EI) comprised of three to five aerobic exercise sessions per week. Two of the weekly sessions were supervised by an exercise specialist with an additional one to three unsupervised sessions per week. The dietary intervention group (DI), consisted of weekly group meetings led by a trained nutritionist focusing on nutritional education, behavioural change promoting a moderately hypocaloric diet, as well as individualised advice and weigh-ins. The control group received standard of care. Following completion of the exercise and nutritional interventions, all participants were reassessed at week 13 (T1).

#### *2.6.3 Dietary Intervention*

As part of the Dietary intervention (DI), participants attended 12 weekly group meetings that focused on nutritional education, behavioural change and group support, as well as weigh-ins and individualised advice from a trained nutritionist. They were asked to keep a weekly food diary that was assessed at each session, with one-to-one feedback and advice offered to each patient. At each meeting a 30 minute presentation was delivered on different aspects of nutrition and dietary change (e.g., the benefits of oily fish or how to read food labels). Participants also received notes to take home, which included information on the fundamentals of nutrition, as well as practical information on portion sizes, how to read food labels when shopping to make healthier dietary choices. Behavioural change support strategies were also discussed. The diet promoted was moderately hypocaloric incorporating aspects of the Mediterranean diet. Low glycaemic load carbohydrates, high fibre content, and

replacement of saturated fat with mono- and polyunsaturated fats was emphasised. Recipes and food plans provided centred on whole foods, fish, nuts, seeds, legumes, vegetables and complex carbohydrates and aimed to reduced reliance on meat and processed foods. No supplements were recommended for the duration of the study. Dietary guidelines were provided to patients in leaflet format at the start of the study (available at: <https://doi.org/10.5518/478>).

At T0, all participants were administered a NAFLD-targeted, validated 48-item food frequency questionnaire [241] in a 20-minute interview with a trained research nutritionist. Participants were then advised on how to complete a detailed written four-day diet diary on two weekdays and two weekend days, for return by post. The four-day diet diary template had three columns for recording meal (i.e., breakfast, lunch, evening meal, snack), time of meal, and the weights and sizes of foods. Participants were encouraged to give as much detail as practical on portion size using information from package information and household measures and to weigh foods whenever possible.

At T1, the same research nutritionist re-interviewed participants and participants were again asked to complete a detailed four-day diary. Nutrient intakes were analysed using the online, myfood24™ dietary assessment tool, which incorporates both the UK food composition dataset of ~3,300 items and an additional >40,000 generic and branded items commonly found in UK and Irish supermarkets[242]. Diaries were inputted as four 24-hour periods and mean intakes per day were calculated for each nutrient for each participant. Overall diet quality was systematically assessed from the food frequency questionnaire data using a scoring system where each question was scored on a scale of 0 to 5 with optimal intakes being allocated a score of 5.

#### *2.6.4 Exercise Intervention*

Exercise intervention (EI) consisted of supervised bi-weekly sessions conducted on treadmills, cycle ergometers and elliptical trainers. Each session consisted of a 5-7-minute warm-up, 21-42 minutes of aerobic exercise (progressively increasing in duration throughout the 12-week EI) and a 5-7-minute cool-down period. The intensity of exercise was individualised using each

participant's heart rate reserve and progressively increased (40-75% heart rate reserve), along with the aerobic duration, throughout the EI. For unsupervised exercise sessions, participants were encouraged (with weekly text messages) to replicate the intensity, type and duration of the prescribed supervised exercise sessions as much as possible. Unsupervised sessions began at weekly for the first 3 weeks and were increased to bi-weekly for weeks 4-7 and thrice weekly for weeks 8-12 of the EI. Adherence to the unsupervised sessions was monitored using exercise diaries.

#### *2.6.5 Cardiorespiratory Fitness Assessment*

Prior to fitness testing, all study participants were screened for risk of cardiovascular disease and readiness for physical activity. Cardiorespiratory fitness was assessed using the modified Bruce sub-maximal cardiopulmonary exercise test protocol on an electric treadmill to give estimates of maximal oxygen consumption ( $\dot{V}O_{2max}$ )[243]. Gas exchange variables were collected using a portable indirect calorimeter and heart rate was continuously measured with a chest strap. Participants performed the exercise protocol until they had achieved 85% of their age-predicted maximal HR or they reached volitional fatigue.  $\dot{V}O_{2max}$  was estimated using the American College of Sports Medicine metabolic equation[243].

#### *2.6.6 Vibration Controlled Transient Elastography*

VCTE and CAP scores were measured at T0 and T1 in all intervention groups (*please see section 2.5.3*).

#### *2.6.7 Histological analysis of liver biopsies*

Liver biopsies were performed on all participants (exercise, nutritional intervention and control groups) at T0 and patients with NASH in the exercise and nutritional groups had repeat biopsies at T1. All liver biopsy specimens were reviewed and scored by a single, blinded histopathologist (*please see section 2.5.4*).

### *2.6.8 Flow cytometry and isolation of MAIT cells*

Analysis of circulating and intra-hepatic immune cell populations were performed as previously described (*please see section 2.5.6*).

## *2.7 Study III: The prevalence of frailty in patients with non-cirrhotic NAFLD*

The third part of this thesis involved clinical measures of frailty amongst patients with NAFLD.

### *2.7.1 Patient selection*

Patients with a clinical diagnosis of NAFLD attending the Hepatology clinic in St. James's Hospital were enrolled into the study. Inclusion and exclusion criteria for this study were as previously listed (*please see section 2.5.1*). All participants had baseline laboratory investigations and VCTE measurements as previously outlined (*please see section 2.5.2-2.5.3.3*)

### *2.7.2 Frailty assessments*

Frailty was assessed using the self-reported frailty index, the Fried frailty index and the FI-LAB score.

#### *2.7.2.1 The Self-reported frailty index (SRFI)*

The SRFI is an investigator administered questionnaire (*see below*), which assesses the presence of 44 health deficits[244]. A minimum completion of 70% is required for a valid SRFI. A SRFI of <0.10 indicates robustness; 0.10–0.249 indicates prefrailty and  $\geq 0.25$  indicates frailty [244].

*Do you suffer from any of the following?*

1. Difficulty walking 100m or 100 yards
2. Difficulty rising from chair

3. Difficulty climbing one flight of stairs
4. Difficulty stooping, kneeling or crouching
5. Difficulty reaching above shoulder height
6. Difficulty pushing/pulling large objects
7. Difficulty lifting/carrying weights  $\geq 10$  pounds or 4.5kg
8. Difficulty picking up a coin from table
9. Difficulty getting dressed or bathing
10. Feeling lonely
11. Mood Problems
12. Constant tiredness
13. Poor Sleep Quality
14. Poor physical health
15. Poor vision
16. Poor hearing
17. Difficulty following a conversation
18. Daytime sleepiness
19. Knee pain
20. A history of 1 or more falls in the past year
21. A history of 1 or more broken bones in the past year
22. Taking more than 5 different medications
23. Poor memory
24. Absent mindedness

*Has a medical professional diagnosed you with any of the following?*

1. Urinary incontinence
2. Gastrointestinal problems
3. Lung or respiratory problems
4. High blood pressure
5. Angina
6. Heart attack
7. Diabetes

8. Hypo/Hyperthyroidism
9. Stroke/Transient ischemic attack
10. High cholesterol
11. Irregular heart rhythm
12. Other heart problems
13. Cataracts
14. Glaucoma/Age related macular degeneration
15. Arthritis
16. Osteoporosis
17. Cancer
18. Varicose ulcer
19. Cognitive Impairment/Cognitive Related Problems
20. Other health problems (please specify):

#### *2.7.2.2 Fried Frailty Index 28 (FFI28)*

The FFI28 comprises of a series of functional tests and self-reported questions that measure frailty through 5 major areas of deficit [143]:

(a) Weakness:

Weakness was assessed by a maximal grip strength test on the participant's dominant hand using a hand-grip dynamometer (Jamar Plus, JLW instruments, Chicago, Illinois) and scores were adjusted for gender and body mass index (BMI). Participants performed three maximum voluntary contractions and the highest score was recorded.

(b) Slow gait:

Gait speed was measured through a walking test to assess average walking speed over 15 feet.

(c) Self-reported exhaustion:

Self-reported exhaustion was measured using two questions relating to experiences with fatigue in the previous week. Questions had four possible responses and were scored on a

four-point Likert scale ranging from 'not at all (0)' to 'a lot (3)'. A score of 2 or more in either question was considered positive for fatigue.

(d) Unintentional weight loss

Unintentional weight loss was assessed by asking participants if they had experienced unintentional weight loss of more than 10 pounds/4.5 kg or  $\geq 5\%$  of their bodyweight in the past year.

(e) Low physical activity

Low physical activity was defined as performing less than 150 min of moderate-to-vigorous physical activity per week in accordance with the WHO physical activity guidelines {Ding, 2020 #2161}. An overall FFI score of 0 indicates robustness; 1–2 indicates prefrailty and  $\geq 3$  indicates frailty.

### *2.7.2.3 FI-LAB score*

The FI-LAB incorporates a combination of standardised blood tests and is based on a cumulative deficits approach using physiological measures[245]. Each blood test was compared with local standardised reference ranges. Tests that were outside of the normal range were considered as a health deficit for the FI-LAB. The FI-LAB used in this study assesses 35 standardised blood tests (please see table 2-5)[245].

*Table 2-1: Variables included in the FI-LAB*

|    |                                            |    |                                             |
|----|--------------------------------------------|----|---------------------------------------------|
| 1  | White cell count                           | 19 | Estimated Glomerular Filtration rate (eGFR) |
| 2  | Neutrophil count                           | 20 | Total Protein                               |
| 3  | Lymphocyte count                           | 21 | Albumin                                     |
| 4  | Monocyte count                             | 22 | Total Bilirubin                             |
| 5  | Eosinophil count                           | 23 | Alkaline Phosphatase                        |
| 6  | Basophil count                             | 24 | Gamma Glutamyl Transferase (GGT)            |
| 7  | Red cell count                             | 25 | Aspartate aminotransferase (AST)            |
| 8  | Haemoglobin                                | 26 | Alanine aminotransferase (ALT)              |
| 9  | Haematocrit                                | 27 | Glucose                                     |
| 10 | Mean Corpuscular Volume                    | 28 | Glycated Haemoglobin (HbA1c)                |
| 11 | Mean Corpuscular Haemoglobin               | 29 | Urate                                       |
| 12 | Mean Corpuscular Haemoglobin concentration | 30 | Total Cholesterol                           |
| 13 | Red Cell Distribution Width                | 31 | High Density Lipoprotein (HDL)              |
| 14 | Platelet Count                             | 32 | Triacylglycerol (TAG)                       |
| 15 | Erythrocyte Sedimentation Rate (ESR)       | 33 | C-Reactive Protein (CRP)                    |
| 16 | Urea                                       | 34 | Insulin                                     |
| 17 | Sodium                                     | 35 | Potassium                                   |
| 18 | Creatinine                                 |    |                                             |

An index was created by dividing the total amount of blood tests outside normal ranges by the total amount of blood tests investigated to give a score ranging from 0 to 1.

### *2.7.3 Functional assessments for frailty*

In addition to the frailty assessments, two additional functional assessments were also conducted: the timed up and go (TUG) test and the 30 second sit-to-stand (30STST) test[246, 247].

#### *2.7.3.1 The timed up and go (TUG) test*

The TUG test is used to assess functional mobility and balance and has been reported to be a sensitive and specific measure of frailty[246]. Patients sat on a standardised chair with armrests in place and were prompted to rise out of the chair, walk around a cone 3m away and sit back on the chair as quickly as possible.

#### *2.7.3.2 30 second sit to stand (30STST) test*

The 30STST test acts as a functional assessment of lower body strength [247]. For the 30STST test, participants sat on a standardised chair and were asked to stand up from the chair and return to a seating position as many times as possible in thirty seconds.

#### *2.7.4 Statistical analysis*

Statistical analyses were performed using SPSS Statistics, version 26 and GraphPad Prism, version 9.1.1 (*see table 2-4*). Between-group differences were assessed using a one-way analysis of variance or the Kruskal-Wallis test for normal and non-normal continuous data, respectively. Tukey or Mann-Whitney post-hoc analysis was performed where appropriate for normal and non-normal continuous data, respectively. For between-group, independent categorical comparisons, Pearson's chi-square test was used. Multivariate linear regression was performed to assess potential outcomes that were associated with SRFI, FI-LAB and 30STST. Statistical significance was set at  $p \leq 0.05$  for all tests.

## Chapter 3: Cross sectional study analysing peripheral and circulating immune cells in patients with biopsy proven NAFLD

### 3 Cross sectional study analysing peripheral and circulating immune cells in patients with biopsy proven NAFLD: Study I

#### *3.1 Introduction*

In NAFLD, as with obesity, the immune system plays a key role in the pathogenesis and progression to advanced liver disease. Whilst the currently available, validated non-invasive scoring systems for fibrosis and NASH rely on clinical parameters, there have been some studies investigating the utility of circulating immune cells as biomarkers for advanced NAFLD[248]. The reliability of existing clinical scores including the NAFLD and FIB-4 scores are confounded by extremes of age, with reduced specificity particularly in those over 65 years whilst the F-AST score has not yet been validated in individuals who fall in a higher BMI bracket ( $\geq 40 \text{ kg/m}^2$ )[11, 249, 250]. Thus, there is still a need to further optimise the non-invasive diagnosis of NASH, particularly to identify those at risk of disease progression.

Neutrophils are the most abundant leukocytes in circulation, comprising up to 70%. They act as first responders in acute infection but also play a pathogenic role in chronic inflammatory conditions with increases in the circulating fraction and concentration of neutrophil derived products in association with disease severity[53]. As a result, the NLR is an effective inflammatory marker. In murine models of obesity, activated macrophages within inflamed adipose tissue promote the hepatic migration of neutrophils and myeloperoxidase released from activated neutrophils stimulate hepatic stellate cells, suggesting that neutrophils play a key role in the progression of NAFLD to NASH and the development of hepatic fibrosis[251, 252]. NAFLD is a chronic inflammatory state driven by adiposopathy, where hepatic inflammation mirrors the degree of systemic inflammation.

The monocyte: HDL ratio (MHR) has also been investigated as a biomarker for NAFLD. Initially developed as marker of cardiovascular disease, the MHR demonstrated utility as an inflammatory marker and has recently reported to predict the presence of hepatic steatosis and fibrosis in women with NAFLD[222]. Monocytes typically comprise 2-8% of circulating leukocytes and consist of 3 different subpopulations: non-classical, intermediate and classical [253]. Classical monocytes are the largest portion of circulating monocytes and exhibit the

greatest capacity for phagocytosis, readily producing large quantities of reactive oxygen species [253]. They release pro-inflammatory cytokines including IL 6, IL 8, CCL2, CCL3, CCL5 and express several chemokine receptors, enabling their swift migration to sites of infection and tissue injury [253]. In addition, classical monocytes are also able to differentiate into monocyte-derived macrophages in response to stimuli, including hepatocyte and Kupffer cell derived DAMPs [253]. The hepatic migration and differentiation of monocytes is central to the progression of NASH and hepatic fibrosis in NAFLD. In mouse models of NAFLD, the liver is infiltrated by CD11b<sup>+</sup>CD14<sup>+</sup> monocytes which swiftly differentiate into potent pro-inflammatory monocyte derived macrophages [254]. Monocyte derived macrophages are the greatest producers of TNF $\alpha$  within the liver and also release growth factors which promote angiogenesis and hepatic fibrosis [254]. These cells express high levels of CD68 and are enriched in the portal areas in NASH and advanced fibrosis [254, 255]. Interestingly, selective inhibition of hepatic monocyte migration has been shown to ameliorate histological NAFLD severity, this has formed the basis of the development of cenicriviroc, an inhibitor of monocyte migration which has been shown to promote fibrosis regression following Phase III trials in advanced NAFLD [255, 256]. By comparison, intermediate monocytes exhibit limited phagocytic capacity, although their role is not fully understood, they are primed for antigen presentation, with the highest surface expression of antigen presenting molecules [240]. Similarly, non-classical monocytes are not effective at phagocytosis and express antigen presenting molecules. They have a role in wound healing and repair, expressing high levels of chemokine receptors and preferentially migrating to sites of tissue injury [240, 257]. Although their role in NAFLD is poorly understood, a few small studies have shown an expansion of the non-classical and intermediate monocyte fractions in comparison with healthy controls, similar changes have been observed with systemic infections and chronic inflammatory conditions [240, 253, 258]. Alterations in monocyte surface marker expression have also been reported in NAFLD, with upregulation of the haemoglobin scavenger receptor and activation marker, CD 163 and downregulation of the antigen presenting marker, HLA-DR [259, 260], although, they have not demonstrated any correlation with NAFLD severity [259, 260].

As previously discussed in Chapter 1 section 1.5.5, circulating T and B lymphocytes have been reported to increase in patients with NAFLD, whilst NK and MAIT cells are reduced with advanced fibrosis. These changes are coupled with intrahepatic immune cell depletion in

cirrhosis. Persistent activation of these cells is believed to be key in the pro-inflammatory cascade driving the development of NASH and evolution of fibrosis[6, 131, 135]. Currently, much of the data on both the innate and adaptive immune system in NAFLD is derived from animal studies, with only small cohort studies on each cell type amongst individuals with the condition[6, 131, 135]. There is a scarcity of data on MAIT cells, table 1 outlines an overview of the current literature (*See table 3-1*). Furthermore, changes in these circulating immune cells have not been evaluated as potential non-invasive markers of advanced fibrosis or NASH.

*Table 3-1: Summary of the current literature on MAIT cells in NAFLD*

| Authors, Year:              | Study design:                                                                                         | N:                           | Summary of findings:                                                                                                                                                                                                                                                                                                                                                                         |
|-----------------------------|-------------------------------------------------------------------------------------------------------|------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Hegde et al, 2018 [74]      | PBMC from cirrhotic NAFLD patients                                                                    | N=13                         | Reduction of circulating MAITs in comparison with healthy controls, with increased expression of activation markers CD25 and CD69                                                                                                                                                                                                                                                            |
| Bottcher et al, 2018, [117] | PBMC from individuals with NASH                                                                       | N=4                          | Peripheral MAIT cells isolated from patients with NASH stimulate pro-fibrogenic gene expression when co-cultured with hepatic stellate cells                                                                                                                                                                                                                                                 |
| Li et al, 2018, [130]       | PBMC and liver immunohistochemistry from individuals with NAFLD. Functional analyses of murine models | N= 60 (PBMC)<br>N=40 (liver) | Elevated chemokine CXCR6 expression with increased IL 4, reduced IFN $\gamma$ and TNF $\alpha$ production by circulating MAITs compared to healthy controls. MAIT cells enriched in the liver with higher NAS scores. In murine models, MAIT cells exhibited (Th2) phenotype. MAIT cell depletion was associated with more severe NASH and increased hepatic infiltration by M1 macrophages. |

This study presented in this thesis investigates changes in these circulating and intrahepatic immune cell populations and their phenotypic features in parallel. Moreover, with the development and validation of non-invasive methods of estimating NAFLD fibrosis and more recently NASH, I evaluate if changes in circulating immune cells can be predictive of histological disease severity and assess their performance alongside established scoring systems currently used in clinical practice.

### *3.2 Hypothesis*

In NAFLD, disease progression is driven by hepatic inflammation resulting from pathological stimulation of components of the innate and adaptive immune system. This stimulation leads to the release of chemokines promoting hepatic migration, persistent immune cell activation may lead to metabolic exhaustion and cell death. This will result in depletion of these cell populations both within the circulation and the liver which may correlate with histological severity. Phenotypic changes in circulating immune cell populations may therefore demonstrate utility as a non-invasive marker or immune signature for NASH and significant fibrosis.

### *3.3 Specific aims*

- To perform a descriptive analysis of circulating and intrahepatic immune cells, especially with an emphasis on monocytes, neutrophils, NK, T and MAIT cells in a cohort of patients with biopsy proven NAFLD
- To evaluate markers of cellular activation and exhaustion expressed by circulating and intrahepatic immune cells
- To assess for phenotypic correlates of circulating and intrahepatic immune cells with histological NASH and Brunt fibrosis stages

### *3.4 Methods*

This was a cross-sectional, descriptive study of circulating and intrahepatic immune cell populations across a range of NAFLD disease severity. 49 participants attending the Hepatology clinic in St James' Hospital with a clinical diagnosis of NAFLD from May 2017 to May 2019 were recruited. Study inclusion and exclusion criteria were as outlined in Chapter 2 section 2.5.1.

#### *3.4.1 Laboratory methods*

Baseline laboratory, body parametric and VCTE measurements were performed as outlined in Chapter 2, section 2.5.2-2.5.3, with the results detailed in Table 3-2. Sample preparation, staining and analyses were performed as previously described in Chapter 2 section 2.5.6-

2.5.7. Circulating MAIT and T cell data from 25 healthy controls, obtained from a prior study by the Department of Immunology at St James's hospital were used for comparison with the study participants. Demographic details were not available. Data on intrahepatic MAIT and T cell populations from 5 donor livers prior to transplantation was obtained from Professor O'Farrelly's team in the Department of Immunology at TCD.

#### *3.4.2 Patient demographics*

Of 49 patients, there was a female preponderance (n=33, 67.3%), with a median age of 58 years (n=27, 55.1%), participants were obese with a mean BMI of  $35.24 \pm 6.14 \text{ kg/m}^2$  and a total body fat percentage of  $41.13 \pm 7.27\%$  (see table 3-2). The majority (n=26, 53.1%) had metabolic syndrome, with (n=27, 55.1%) having a diagnosis of hypertension and (n=29, 59.1%), a diagnosis of impaired glucose tolerance or diabetes. Although most had scores suggestive of advanced fibrosis and severe steatosis on VCTE measurements, following liver biopsy, participants were evenly distributed across different stages of Brunt fibrosis and NAS score. Interestingly, our cohort had vitamin D insufficiency with a median level of 45 nmol/L.

Table 3-2: NAFLD patient demographics

| Parameters:              | N=49          |
|--------------------------|---------------|
| Age (y)                  | 58 (14)       |
| Gender, n (%)            |               |
| Female                   | 33 (67.3%)    |
| Male                     | 16 (32.7%)    |
| BMI (kg/m <sup>2</sup> ) | 35.24 ± 6.14  |
| % Body Fat               | 41.13% ± 7.27 |
| % Muscle Mass            | 58.96% ± 7.67 |
| Hypertension n (%)       | 27 (55.1%)    |
| Diabetes/IGT n (%)       | 29 (59.1%)    |
| Metabolic Syndrome       | 26 (53.1%)    |
| HbA1c (mmol/mol)         | 43 (16.5)     |
| Urate (μmol/L)           | 345.3 ± 87.58 |
| Vitamin D (nmol/L)       | 45 (33)       |
| Triglycerides (mmol/L)   | 1.74 (1.20)   |
| LDL-C (mmol/L)           | 2.30 ± 0.88   |
| HDL-C (mmol/L)           | 1.15 (0.37)   |
| ALT (IU/L)               | 50 (38)       |
| AST (IU/L)               | 33 (28)       |
| GGT (IU/L)               | 69 (87.5)     |
| Fibroscan score          | 12.45 (8.68)  |
| CAP score                | 338.1 ± 42.99 |
| F-AST score              | 0.560 ± 0.23  |
| FIB-4                    | 1.32 (0.66)   |
| NAFLD score              | -0.637 (1.71) |
| Brunt Fibrosis Stage:    |               |
| Stage 0/1                | 15 (30.6%)    |
| Stage 2                  | 13 (26.5%)    |
| Stage 3                  | 13 (26.5%)    |
| Stage 4                  | 8 (16.4%)     |
| NAS score:               |               |
| (NAS < 5)                | 27 (55.1%)    |
| (NAS ≥ 5)                | 22 (44.9%)    |

Data was tested for normality using the Shapiro–Wilks test. Continuous variables are presented as either mean ± standard deviation or as median (interquartile range) as appropriate. Categorical variables are presented as number (percentage). Abbreviations: BMI, body mass index, IGT, impaired glucose tolerance, HbA1c, haemoglobin A1c, LDL-C, low density lipoprotein – cholesterol, HDL-C, high density lipoprotein- cholesterol, ALT, alanine aminotransferase, AST, aspartate aminotransferase, GGT, gamma glutamyl transferase, CAP, controlled attenuation parameter, F-AST score, Fibroscan-aspartate aminotransferase score, FIB-4, fibrosis index for liver fibrosis, NAFLD score, non-alcoholic fatty liver disease fibrosis score, NAS, non-alcoholic fatty liver disease activity score.

### 3.5 Results

There was no demonstrable relationship between vitamin D levels and the degree of hepatic inflammation or fibrosis. For binary logistic regression analyses we divided participants into 2 groups according to histological severity. No significant fibrosis was defined as a Brunt score  $<2$ , whereas a Brunt score of  $\geq 2$  corresponded with moderate/advanced fibrosis as outlined by the NASH clinical research network [261]. NASH was defined as a NAS score of  $\geq 5$  [261]. Following comparison of the existing non-invasive scores for NAFLD, the NAFLD score was the only measure that was predictive of advanced fibrosis in our data set (*see Fig 3-1*). None of the scores, including the F-AST score were predictive of NASH.

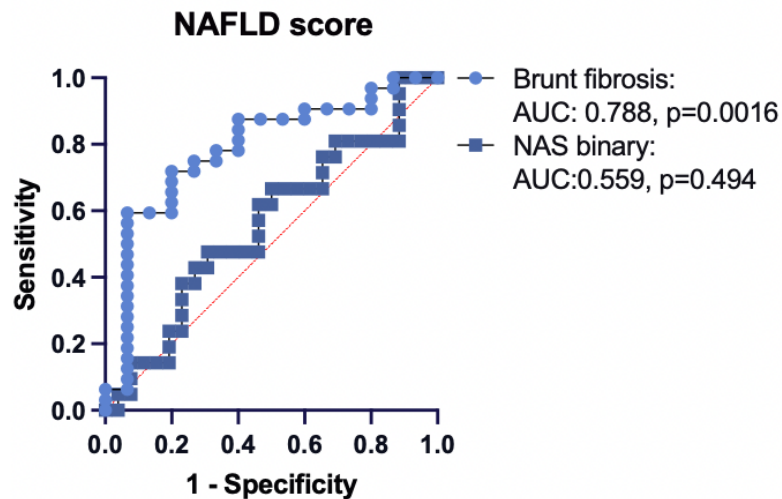


Figure 3-1 The area under the curve (AUC) for the NAFLD score in advanced hepatic fibrosis and NASH. N=49

#### 3.5.1 The Neutrophil: Lymphocyte ratio is predictive of histological inflammation but not fibrosis in NAFLD

For our initial analysis, we assessed the relationship between the neutrophil: lymphocyte ratio (NLR) and histological NAFLD severity. The absolute neutrophil count was not significantly altered across different Brunt and NAS scores (*see Fig. 3-2*) However, following logistic regression analysis, the NLR was found to be predictive of NASH (AUC: 0.701, 95% CI: 0.553-

0.850,  $p=0.0163$ ) but not fibrosis (see Fig. 3-3). There were no other significant correlates between neutrophil count, NLR and other clinical parameters.

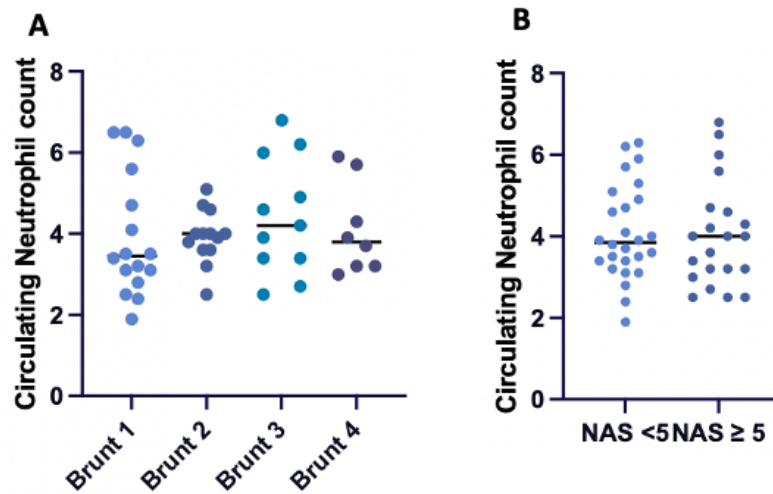


Figure 3-2 Comparison of the circulating (A) neutrophil count across different stages of Brunt fibrosis using a two-tailed Kruskal-Wallis test and (B), according to histological NAS score via the Mann-Whitney U test, medians are depicted graphically,  $N=49$

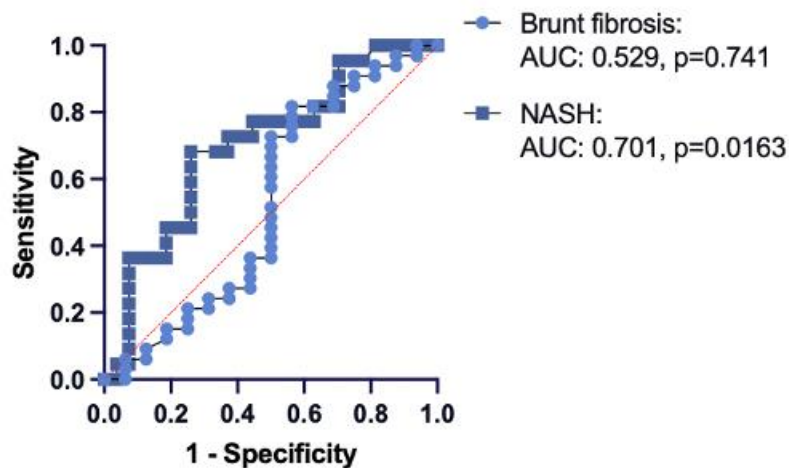


Figure 3-3 The area under the curve (AUC) for NLR in advanced hepatic fibrosis and NASH.

Circulating neutrophil and lymphocyte counts were measured via a Haematology Analyzer. NLR was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. Abbreviations: NLR, neutrophil: lymphocyte ratio.  $N=49$

### 3.5.2 The percentage of circulating monocytes are significantly reduced with hepatic inflammation and are predictive of NASH, although they do not exhibit any phenotypic changes

We subsequently analysed circulating monocytes. We found a weak positive correlation between the absolute monocyte count, age (*Spearman*  $r$ : 0.317, 95% CI: 0.0309-0.555,  $p=0.0264$ ) and CAP scores, (*Spearman*  $r$ : 0.316, 95% CI: 0.0258-0.556,  $p=0.0289$ ), however

there was no significant association between the variables following linear regression analysis. The absolute and percentage of circulating monocytes were unaltered with advancing hepatic fibrosis (*See Fig. 3-4*). However, the percentage of circulating monocytes was significantly reduced with increasing NAS score ( $p=0.0178$ ) and was found to be predictive of NASH (AUC: 0.738, 95% CI: 0.564-0.912,  $p=0.0181$ ) (*See Fig. 16*).

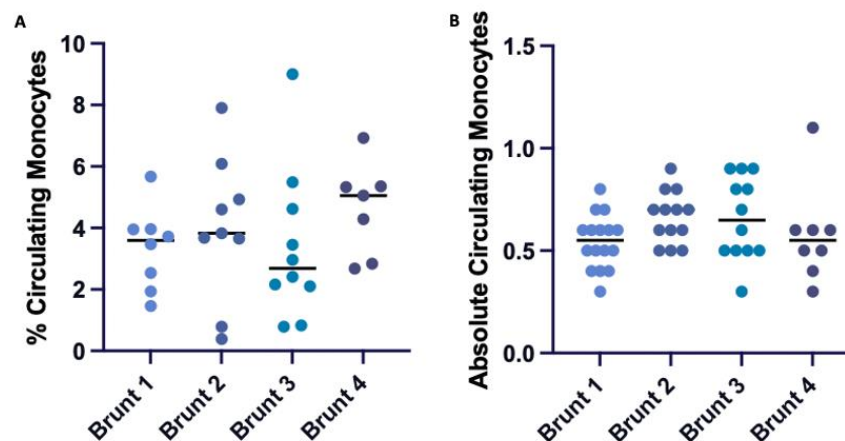


Figure 3-4 Analysis of (A) percentage and (B) absolute circulating monocyte levels across different grades of Brunt fibrosis using a two-tailed Kruskal-Wallis test.

Medians depicted graphically, absolute circulating monocytes were measured using a Haematology analyser, the percentage of circulating monocytes was determined using flow cytometry by gating on FSC-H<sup>Lo</sup> FSC-A<sup>Hi</sup> cells, please see Chapter 2, figure 2-3 for gating strategy. <sup>A</sup>N=34, <sup>B</sup>N=49.

We then examined the different monocyte subpopulations: non-classical, intermediate and classical, to see if there were any alterations in their proportions and surface marker expression in NAFLD. We found no relationship between monocyte subpopulations and clinical parameters. There were no significant changes in the proportion of non-classical (*see Fig. 3-5*), intermediate (*see Fig. 3-6*) and classical (*see Fig. 3-7*) monocytes or their surface marker expression in association with either advancing fibrosis or inflammation (*full data set and analyses in tables 3-3 and 3-4 respectively*).

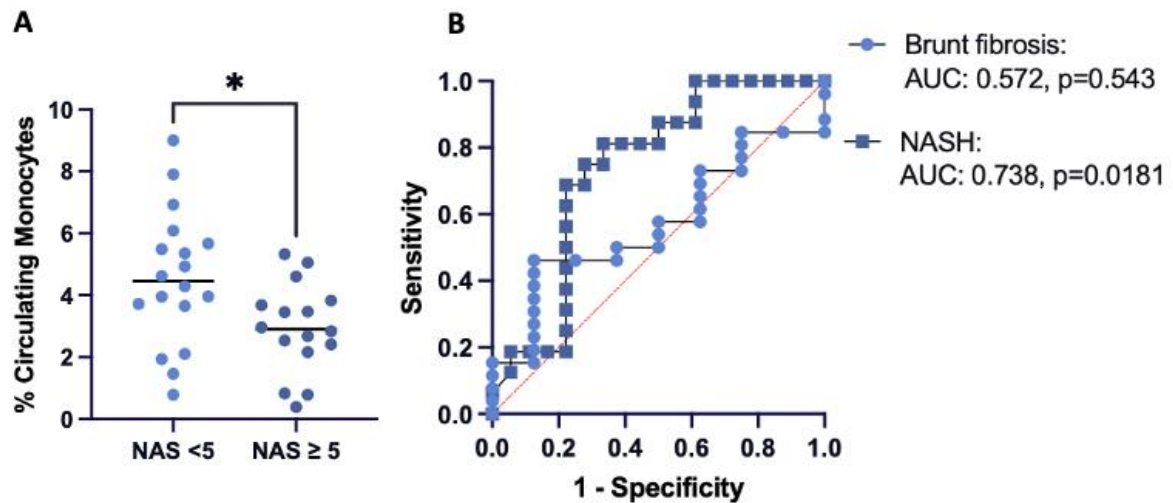


Figure 3-5 Comparison of the percentage of circulating monocytes in individuals with ( NAS ≥ 5) and without NASH (NAS <5) performed using a two-tailed, unpaired t test.

\* p=0.0178, means displayed graphically, N=34. (B) The area under the curve (AUC) for the percentage of circulating monocytes in advanced hepatic fibrosis and NASH. Abbreviations: NAS, NAFLD activity score. N=34.

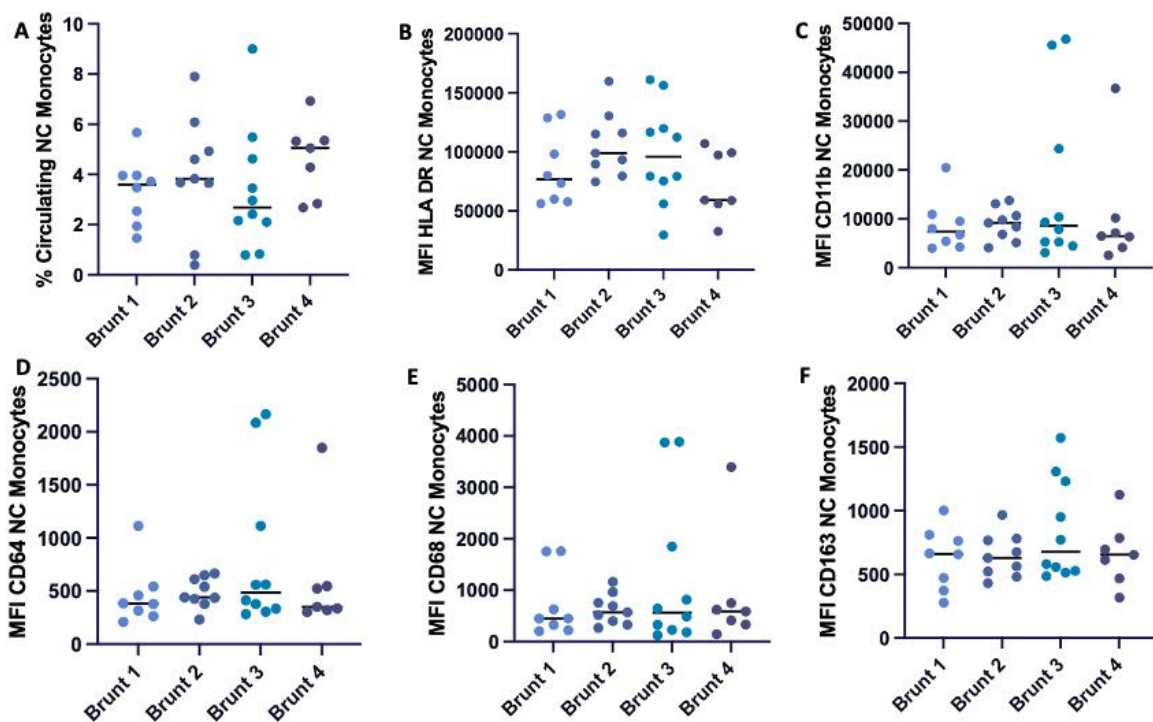


Figure 3-6 Figure 17: (A) The percentage of non-classical circulating monocytes and expression of HLA-DR (B), CD11b (C), CD64 (D), CD68 (E) and CD163 (F) with increasing Brunt fibrosis stage.

Data analysed using the Kruskal-Wallis test, medians displayed graphically, N=34, two-tailed test used. Non-classical monocytes were defined as CD14<sup>dim</sup>CD16<sup>+</sup> cells, please see chapter 2, figure 2-3 for gating strategy.

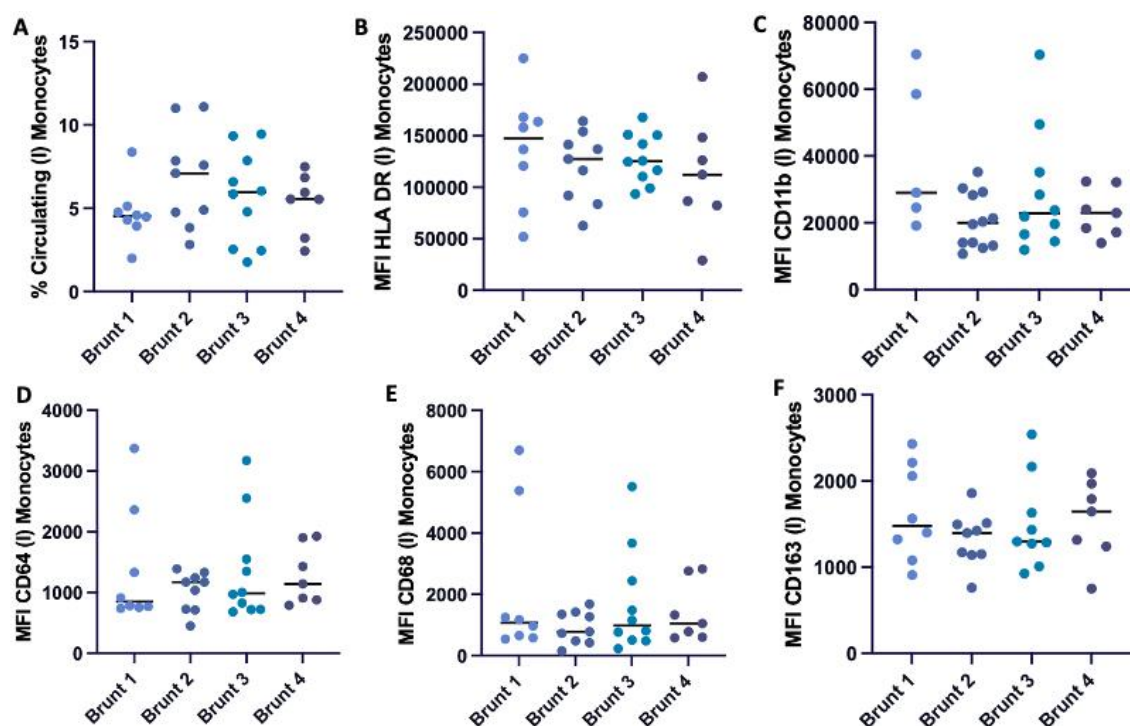


Figure 3-7(A) The percentage of intermediate circulating monocytes and expression of HLA-DR (B), CD11b (C), CD64 (D), CD68 (E) and CD163 (F) with increasing Brunt fibrosis stage.

Data analysed using the Kruskal-Wallis test, medians displayed graphically, N=34, two-tailed test used. Intermediate monocytes were defined as CD14<sup>+</sup>CD16<sup>+</sup>cells, please see chapter 2, page 68 for gating strategy.

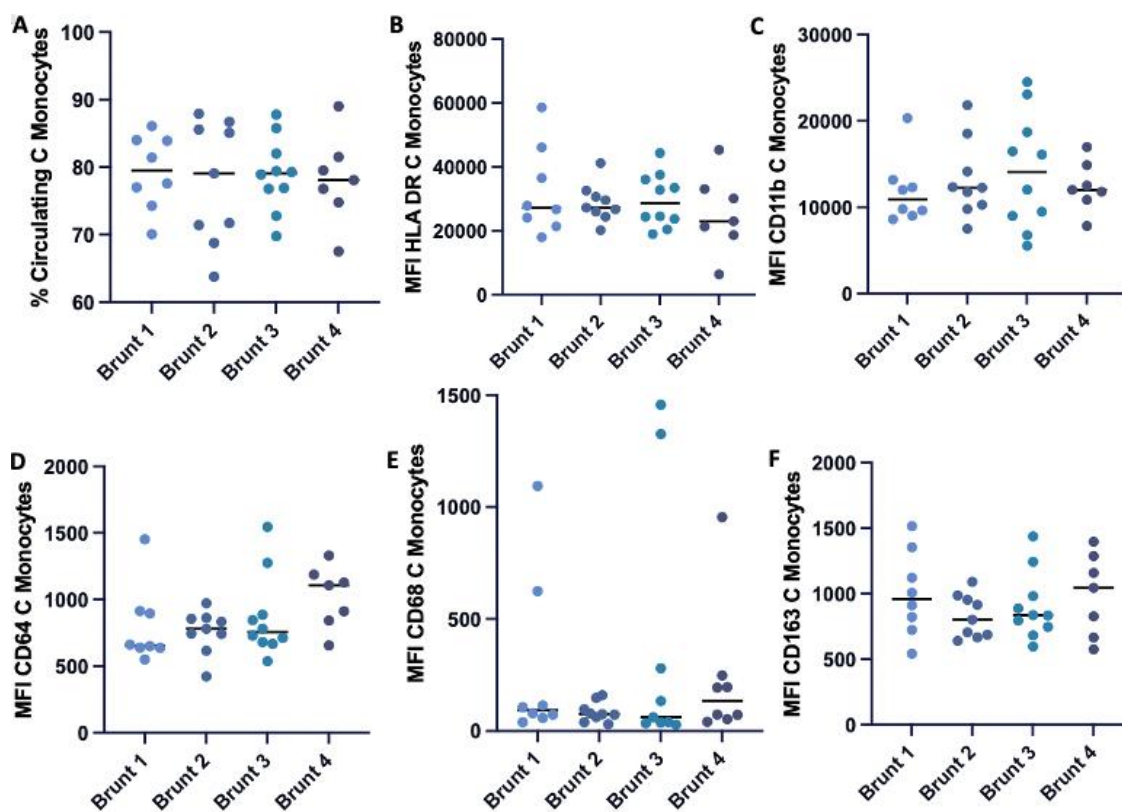


Figure 3-8 (A) The percentage of classical circulating monocytes and expression of HLA-DR (B), CD11b (C), CD64 (D), CD68 (E) and CD163 (F) with increasing Brunt fibrosis stage.

Data analysed using the Kruskal-Wallis test, medians displayed graphically, two-tailed test used, N=36. Classical monocytes were defined as CD14<sup>+</sup>CD16<sup>-</sup> cells, please see chapter 2, figure 2-3 for gating strategy.

Finally, we looked at the Monocyte: HDL ratio (MHR) and its correlates in our cohort. There was a weak positive correlation between the MHR and CAP score (*Pearson*  $r=0.378$ , 95% CI:0.105-0.598,  $p=0.008$ ,  $r^2=0.143$ ), however the MHR was neither predictive of advanced fibrosis nor the presence of NASH.

Table 3-3: Monocyte subpopulations and surface marker expression across different Brunt scores

|                                                                        | Brunt 1           | Brunt 2          | Brunt 3           | Brunt 4           | P value            |
|------------------------------------------------------------------------|-------------------|------------------|-------------------|-------------------|--------------------|
| <b>Absolute Circulating Monocytes<sup>a</sup>, (10<sup>9</sup>/ L)</b> | 0.55<br>(0.18)    | 0.70<br>(0.20)   | 0.65<br>(0.38)    | 0.55<br>(0.18)    | 0.132 <sup>c</sup> |
| <b>Total % Monocytes<sup>b</sup></b>                                   | 3.39 ± 1.34       | 3.98 ± 2.36      | 3.38 ± 2.47       | 4.64 ± 1.51       | 0.692 <sup>d</sup> |
| <b>% NC Monocytes<sup>b</sup></b>                                      | 3.60<br>(1.87)    | 3.83<br>(3.29)   | 2.69<br>(3.06)    | 5.05<br>(2.51)    | 0.369 <sup>c</sup> |
| <b>MFI HLA-DR NC Monocytes<sup>b</sup></b>                             | 85674 ± 30773     | 106317 ± 27024   | 98524 ± 42249     | 72817 ± 28158     | 0.237              |
| <b>MFI CD11b NC Monocytes<sup>b</sup></b>                              | 7398<br>(6024)    | 9182<br>(5877)   | 8589<br>(24584)   | 6461<br>(6021)    | 0.781 <sup>c</sup> |
| <b>MFI CD64 NC Monocytes<sup>b</sup></b>                               | 381.5<br>(245.5)  | 441<br>(227.5)   | 486.5<br>(1029.2) | 352<br>(228)      | 0.617 <sup>c</sup> |
| <b>MFI CD68 NC Monocytes<sup>b</sup></b>                               | 449<br>(1224)     | 568<br>(493)     | 563.5<br>(2138)   | 586<br>(416)      | 0.943 <sup>c</sup> |
| <b>MFI CD163 NC Monocytes<sup>b</sup></b>                              | 660.5<br>(401.3)  | 629<br>(271.5)   | 677<br>(726.2)    | 654<br>(318)      | 0.724 <sup>c</sup> |
| <b>% (I) Monocytes<sup>b</sup></b>                                     | 4.70 ± 1.76       | 6.78 ± 2.96      | 5.68 ± 2.78       | 5.29 ± 1.84       | 0.375 <sup>d</sup> |
| <b>MFI HLA-DR (I) Monocytes<sup>b</sup></b>                            | 137491 ± 54939    | 119814 ± 34130   | 128108 ± 24268    | 113105 ± 56185    | 0.705 <sup>d</sup> |
| <b>MFI CD11b (I) Monocytes<sup>b</sup></b>                             | 29050<br>(42684)  | 19979<br>(15604) | 22810<br>(22698)  | 22959<br>(15011)  | 0.247 <sup>c</sup> |
| <b>MFI CD64 (I) Monocytes<sup>b</sup></b>                              | 847<br>(1346)     | 1171<br>(568)    | 985.5<br>(668)    | 985.5<br>(1078)   | 0.812 <sup>c</sup> |
| <b>MFI CD68 (I) Monocytes<sup>b</sup></b>                              | 1074<br>(3746.2)  | 783<br>(938.5)   | 984.5<br>(2242.7) | 1047<br>(2161)    | 0.808 <sup>c</sup> |
| <b>MFI CD163 (I) Monocytes<sup>b</sup></b>                             | 1482<br>(1034)    | 1397<br>(358)    | 1298<br>(759)     | 1647<br>(729)     | 0.698 <sup>c</sup> |
| <b>% C Monocytes<sup>b</sup></b>                                       | 79.3 ± 5.50       | 77.79 ± 9.03     | 78.95 ± 5.43      | 78.17 ± 6.55      | 0.966 <sup>d</sup> |
| <b>MFI HLA-DR C Monocytes<sup>b</sup></b>                              | 27261<br>(21631)  | 27218<br>(6425)  | 28621<br>(13513)  | 22997<br>(14375)  | 0.767 <sup>c</sup> |
| <b>MFI CD11b C Monocytes<sup>b</sup></b>                               | 10906<br>(3793)   | 12264<br>(6303)  | 14078<br>(11380)  | 12022<br>(4028)   | 0.906 <sup>c</sup> |
| <b>MFI CD64 C Monocytes<sup>b</sup></b>                                | 656<br>(271)      | 780<br>(180)     | 755<br>(305)      | 1108<br>(345)     | 0.192 <sup>c</sup> |
| <b>MFI CD68 C Monocytes<sup>b</sup></b>                                | 92.65<br>(435.55) | 75.4<br>(71.3)   | 60.9<br>(767.4)   | 133.6<br>(177.25) | 0.741 <sup>c</sup> |
| <b>MFI CD163 C Monocytes<sup>b</sup></b>                               | 960<br>(549.5)    | 802<br>(292.5)   | 835.5<br>(316.5)  | 1045<br>(622)     | 0.636 <sup>c</sup> |

Data was tested for normality using the Shapiro–Wilks test. Continuous variables are presented as either mean ± standard deviation or as median (interquartile range) as appropriate. <sup>a</sup>N=49, <sup>b</sup>N=34, <sup>c</sup>Kruskal-Wallis test, <sup>d</sup>One-Way ANOVA, two tailed tests used. Abbreviations: NC, non-classical, MFI, mean fluorescence intensity, I, intermediate, C, classical.

Table 3-4: Monocyte subpopulations and surface marker expression in individuals with and without a diagnosis of NASH

|                                                                      | NAS < 5            | NAS ≥ 5            | P value                   |
|----------------------------------------------------------------------|--------------------|--------------------|---------------------------|
| <b>Absolute Circulating Monocytes<sup>a</sup> (10<sup>9</sup>/L)</b> | 0.6 (0.20)         | 0.60 (0.20)        | 0.499 <sup>c</sup>        |
| <b>Total % Monocytes<sup>b</sup></b>                                 | <i>4.55 ± 2.18</i> | <i>2.94 ± 1.45</i> | <i>0.0178<sup>d</sup></i> |
| <b>% NC Monocytes<sup>b</sup></b>                                    | 7.67 (9.36)        | 4.18 (9.91)        | 0.611 <sup>c</sup>        |
| <b>MFI HLA-DR NC Monocytes<sup>b</sup></b>                           | 41855 ± 16395      | 38904 ± 12002      | 0.558 <sup>d</sup>        |
| <b>MFI CD11b NC Monocytes<sup>b</sup></b>                            | 7939 (7077)        | 7759 (5081)        | 0.721 <sup>c</sup>        |
| <b>MFI CD64 NC Monocytes<sup>b</sup></b>                             | 427.5 (265.5)      | 441 (236.5)        | 0.766 <sup>c</sup>        |
| <b>MFI CD68 NC Monocytes<sup>b</sup></b>                             | 541 (608.5)        | 535.5 (580.7)      | 0.779 <sup>c</sup>        |
| <b>MFI CD163 NC Monocytes<sup>b</sup></b>                            | 613.5 (324.5)      | 655.5 (381)        | 0.251 <sup>c</sup>        |
| <b>% (I) Monocytes<sup>b</sup></b>                                   | 5.78 ± 2.94        | 5.53 ± 1.89        | 0.773 <sup>d</sup>        |
| <b>MFI HLA-DR (I) Monocytes<sup>b</sup></b>                          | 127181 ± 46722     | 122613 ± 36495     | 0.775 <sup>d</sup>        |
| <b>MFI CD11b (I) Monocytes<sup>b</sup></b>                           | 20883 (20816)      | 23354 (13373)      | 0.959 <sup>c</sup>        |
| <b>MFI CD64 (I) Monocytes<sup>b</sup></b>                            | 1089 (614.2)       | 954.5 (684.5)      | 0.597 <sup>c</sup>        |
| <b>MFI CD68 (I) Monocytes<sup>b</sup></b>                            | 980.5 (1136.7)     | 1009 (1012.7)      | 0.878 <sup>c</sup>        |
| <b>MFI CD163 (I) Monocytes<sup>b</sup></b>                           | 1375 (960)         | 1412 (464)         | 0.772 <sup>c</sup>        |
| <b>% C Monocytes<sup>b</sup></b>                                     | 77.54 ± 6.74       | 79.72 ± 6.25       | 0.338 <sup>d</sup>        |
| <b>MFI HLA-DR C Monocytes<sup>b</sup></b>                            | 28965 (12455)      | 25187 (11576)      | 0.297 <sup>c</sup>        |
| <b>MFI CD11b C Monocytes<sup>b</sup></b>                             | 11021 (7728)       | 12305 (4521)       | 0.297 <sup>c</sup>        |
| <b>MFI CD64 C Monocytes<sup>b</sup></b>                              | 813 (259.5)        | 788.5 (410.5)      | 0.986 <sup>c</sup>        |
| <b>MFI CD68 C Monocytes<sup>b</sup></b>                              | 94.35 (94.37)      | 78.85 (195.82)     | 0.878 <sup>c</sup>        |
| <b>MFI CD163 C Monocytes<sup>b</sup></b>                             | 811.5 (498)        | 934.5 (273)        | 0.484 <sup>c</sup>        |

Data was tested for normality using the Shapiro–Wilks test. Continuous variables are presented as either mean ± standard deviation or as median (interquartile range) as appropriate. <sup>a</sup>N=49, <sup>b</sup>N=34, <sup>c</sup>Mann-Whitney U test, <sup>d</sup>unpaired t test -conditions for Chi squared not met, two tailed tests used. Abbreviations: NC, non-classical, MFI, mean fluorescence intensity, I, intermediate, C, classical.

### 3.5.3 Total circulating T, B lymphocytes and NK cells are unaltered with advancing NAFLD inflammation and fibrosis

Absolute circulating T, B and NK cell levels were enumerated using flow cytometry. Although there was a trend towards reduced T lymphocyte levels with increasing hepatic fibrosis (see Table 3-5), this did not achieve statistical significance ( $p=0.197$ ). There were no measurable changes in circulating B lymphocyte ( $p=0.453$ ) and NK cell levels ( $p=0.986$ ) with Brunt fibrosis stage.

Table 3-5: Absolute circulating T, B lymphocytes and NK cell levels across different stages of NAFLD fibrosis

|                      | Brunt 0-1     | Brunt 2       | Brunt 3       | Brunt 4      | P                  |
|----------------------|---------------|---------------|---------------|--------------|--------------------|
| <b>T Lymphocytes</b> | 1523 (771)    | 1445 (466)    | 1649 (1239)   | 1199 (581.5) | 0.197 <sup>a</sup> |
| <b>B Lymphocytes</b> | 250.3 ± 100.2 | 272.1 ± 113.8 | 245.9 ± 105.4 | 318 ± 114.6  | 0.453 <sup>b</sup> |
| <b>NK Cells</b>      | 232 (275)     | 237.5 (130.5) | 273 (180.2)   | 248 (122)    | 0.986 <sup>a</sup> |

N=49. Data was tested for normality using the Shapiro Wilks test. Continuous variables are presented as either mean ± standard deviation or as median (interquartile range) as appropriate. <sup>a</sup>Kruskal Wallis test. <sup>b</sup>One-Way ANOVA. Abbreviations: NK, Natural Killer cells.

We then sought to determine if there were detectable changes in association with hepatic inflammation in individuals with NASH. Following this analysis, a trend was seen towards a reduction of circulating T lymphocytes ( $p=0.071$ ), with increases in B ( $p=0.253$ ) and NK cells ( $p=0.16$ ) with NASH, but this did not achieve statistical significance (see Table 3-6). There were no significant correlates with non-invasive scores and patient demographic factors.

Table 3-6: Comparison of absolute circulating T, B lymphocytes and NK cell levels in participants with and without histological NASH

|                      | NAS <5        | NAS ≥ 5       | P                  |
|----------------------|---------------|---------------|--------------------|
| <b>T Lymphocytes</b> | 1559 (515)    | 1304 (671)    | 0.071 <sup>a</sup> |
| <b>B Lymphocytes</b> | 249.5 ± 108.9 | 284.8 ± 104.3 | 0.253 <sup>b</sup> |
| <b>NK Cells</b>      | 238.5 (141.7) | 265 (163)     | 0.16 <sup>a</sup>  |

N=49. Data was tested for normality using the Shapiro Wilks test. Continuous variables are presented as either mean ± standard deviation or as median (interquartile range) as appropriate. <sup>a</sup>Mann Whitney U test. <sup>b</sup>Unpaired parametric T test. Abbreviations: NK, Natural Killer cells.

### 3.5.4 Total and CD8<sup>+</sup> circulating MAIT cells are significantly reduced in NAFLD compared with healthy controls

Circulating and intrahepatic MAIT cells were analysed as previously described in Chapter 2. As expected, MAIT cells were enriched in the liver compared to the peripheral circulation, with a greater CD8<sup>+</sup> fraction and higher expression of the activation markers: CD69 and CD95 (see Fig. 3-9).

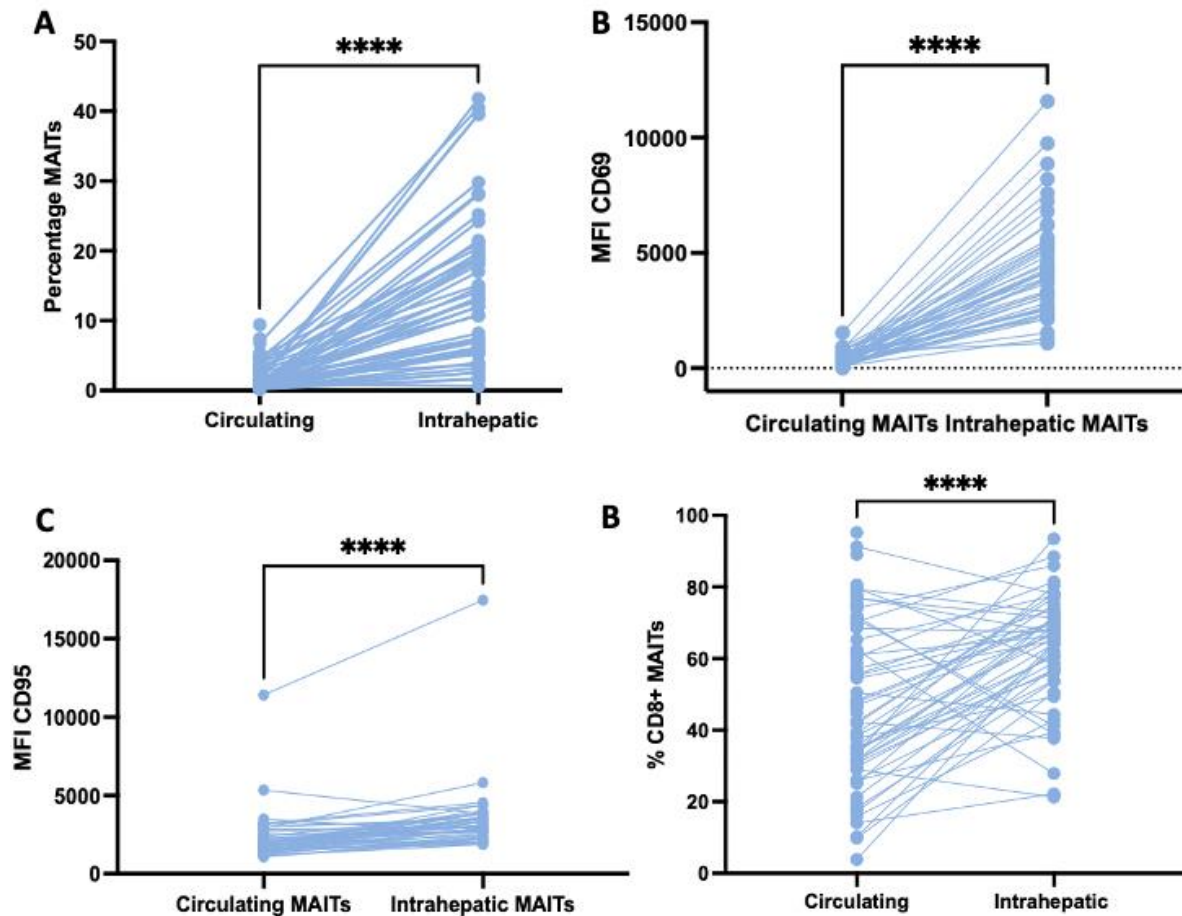


Figure 3-9 Differences between circulating and intrahepatic MAIT cells. Comparison between (A) the percentage of circulating and intrahepatic MAITs, (B) CD69, (C) CD95 expression and (D) the percentage of CD8<sup>+</sup> MAIT cells

Analyses performed using the two-tailed Mann-Whitney U test, \*\*\*\* p<0.00001. Abbreviations: MFI, mean fluorescence intensity, N=46. MAIT cells were defined as CD3<sup>+</sup>Vα7.2<sup>Hi</sup>CD161<sup>Hi</sup>, please see Chapter 2, figure 2-1 for gating strategy.

Following multivariate linear regression analysis, circulating MAIT cells demonstrated a weak inverse relationship with advancing age ( $r^2=0.366$ , 95% CI: -0.121, -0.0536,  $p<0.0001$ ) and NAFLD score ( $r^2=0.291$ , 95% CI: -0.711, -0.257,  $p<0.0001$ ). There were no associations with other clinical parameters.

We found a significant reduction in the absolute and percentage of circulating MAIT cells in NAFLD compared to healthy controls, although there was no intergroup variation across different stages of fibrosis (*see Fig 3-10*), nor with NASH (*see table 3-9*). Additionally, we found no significant difference in the mean fluorescence intensity of the acute activation marker, CD69 or the terminal marker, CD95 (*full data set and analyses in tables 3-7 and table 3-9*). We then wanted to assess if there were any changes in the CD8<sup>+</sup> circulating MAIT cell subset and found that in comparison to controls, the percentage of CD8<sup>+</sup> MAIT cells were significantly reduced in NAFLD, although again there were no changes in CD69 or CD95 expression (*see Fig 3-11, table3- 7*).

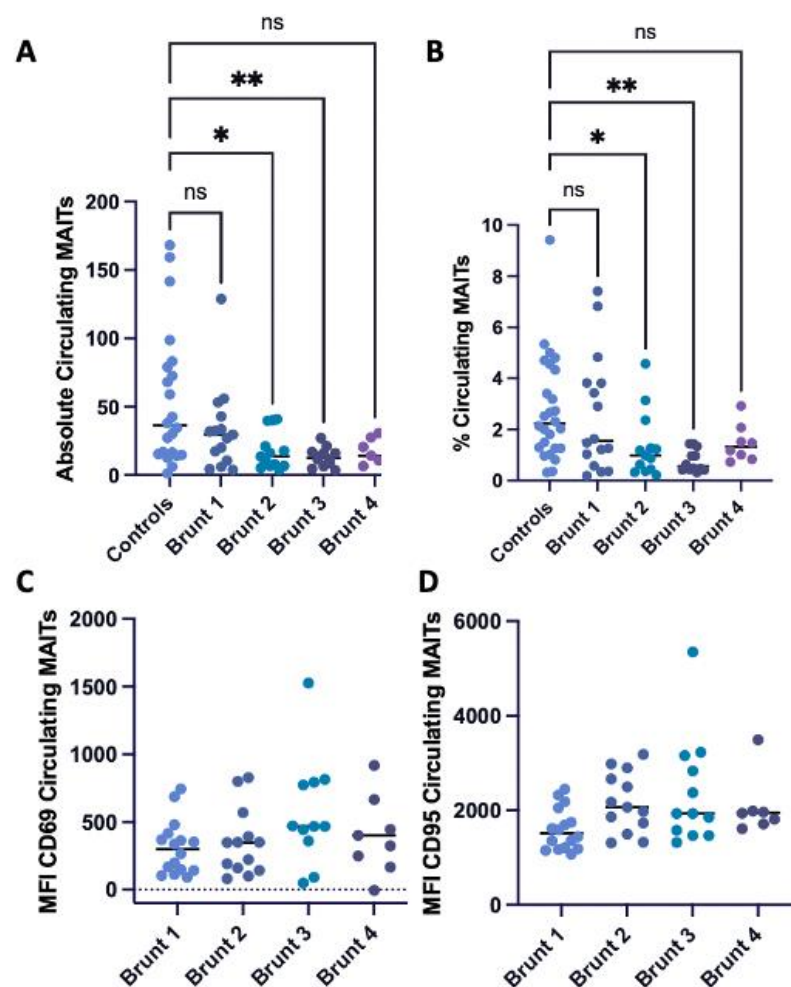


Figure 3-10 Analysis of variance of (A) absolute and (B) percentage of circulating MAIT cells across different grades of hepatic fibrosis, N=68. Comparison between expression of activation markers (C) CD69 and (D) CD95 with advancing Brunt fibrosis stage, N=49.

Data analysed using the Kruskal-Wallis test, medians displayed graphically, P values adjusted for multiple comparisons using Dunn's test, \*  $p < 0.05$ , \*\*  $p < 0.001$ , ns. Abbreviations: ns, non-significant, MFI, mean fluorescence intensity. MAIT cells were defined as CD3<sup>+</sup> Vα7.2<sup>Hi</sup>CD161<sup>Hi</sup>, please see Chapter 2, figure 2-1 for gating strategy.

Table 3-7: Characteristics of circulating MAIT cells across different Brunt stages:

|                                                   | Controls     | Brunt 1       | Brunt 2       | Brunt 3       | Brunt 4      | P value             |
|---------------------------------------------------|--------------|---------------|---------------|---------------|--------------|---------------------|
| <b>Absolute Circulating MAITs</b>                 | 36 (15.25)   | 29.61 (27.22) | 13.61 (23.73) | 12.66 (9.66)  | 14.2 (16.82) | 0.009 <sup>a</sup>  |
| <b>% Circulating MAITs</b>                        | 2.23 (3.12)  | 1.55 (3.13)   | 0.98 (1.42)   | 0.55 (0.83)   | 1.32 (1.06)  | 0.0029 <sup>a</sup> |
| <b>MFI CD69 Circulating MAITs</b>                 | -            | 300.5 (260.7) | 348 (330.5)   | 468 (434)     | 402 (583.5)  | 0.224 <sup>b</sup>  |
| <b>MFI CD95 Circulating MAITs</b>                 | -            | 1515 (784)    | 2065 (1166)   | 1931 (1577)   | 1950 (1378)  | 0.0307 <sup>b</sup> |
| <b>%CD8<sup>+</sup> Circulating MAITs</b>         | 70.2 (17.55) | 38.65 (46.68) | 31.5 (40.05)  | 42.35 (25.82) | 38.85 (35)   | 0.002 <sup>a</sup>  |
| <b>MFI CD69 CD8<sup>+</sup> Circulating MAITs</b> |              | 288.5 (290.3) | 328 (432)     | 494 (498)     | 364 (526.5)  | 0.453 <sup>b</sup>  |
| <b>MFI CD95 CD8<sup>+</sup> Circulating MAITs</b> |              | 1368 (468)    | 1606 (829)    | 1694 (963)    | 1672 (838)   | 0.239 <sup>b</sup>  |

Data was tested for normality using the Shapiro–Wilks test. Continuous variables are presented as median (interquartile range) as appropriate. <sup>a</sup>N=74, <sup>b</sup>N=49, data was analysed using a two tailed Kruskal-Wallis test, P values adjusted for multiple comparisons using Dunn’s test. Abbreviations: MFI, mean fluorescence intensity.

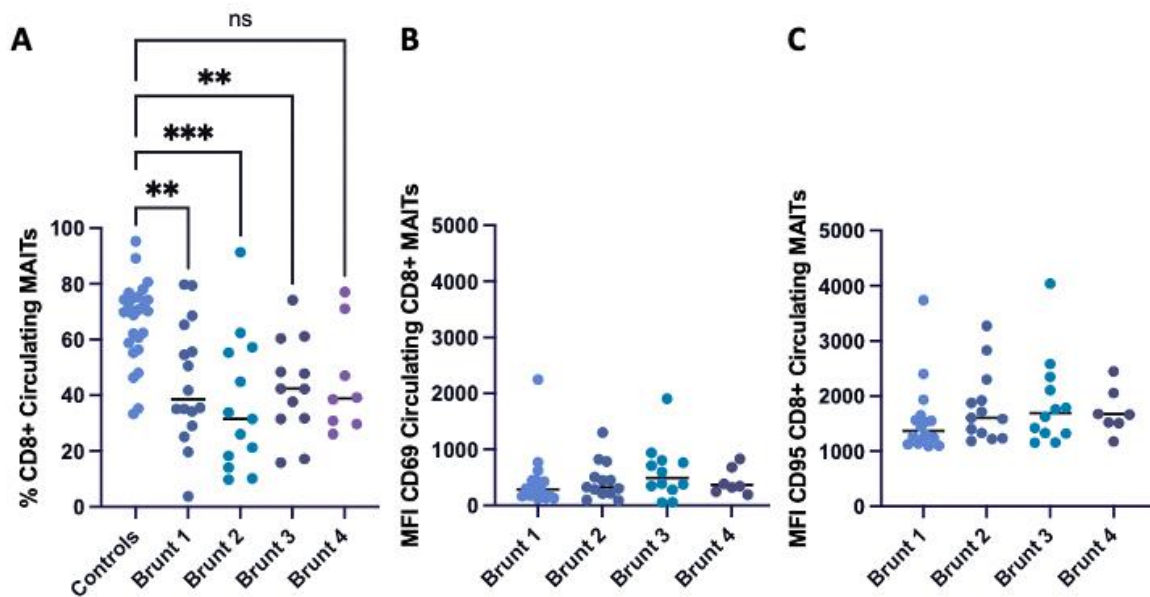


Figure 3-11 Differences in (A) the percentage, (B) MFI of CD69 and (C) MFI of CD95 expression of circulating CD8+ MAITs across different stages of fibrosis, N=68.

Data analysed using the two-tailed Kruskal-Wallis test, medians displayed graphically, P values adjusted for multiple comparisons using Dunn’s test, \*\*p<0.001, \*\*\*p<0.0001. ns. MAIT cells were defined as CD3<sup>+</sup> Vα7.2<sup>Hi</sup>CD161<sup>Hi</sup>, please see Chapter 2, figure 2-1 for gating strategy. Abbreviations: ns, non-significant, MFI, mean fluorescence intensity.

### 3.5.5 The percentage of total and CD8<sup>+</sup> circulating MAIT cells are predictive of both advanced fibrosis and NASH

We calculated ROC curves to determine if circulating MAIT cells exhibited diagnostic utility as non-invasive markers of advanced fibrosis or NASH in NAFLD and compared them to the results obtained from our dataset with the NLR and percentage of circulating monocytes. The percentage of circulating MAIT cells was predictive of both advanced fibrosis and NASH, with an AUC of 0.745, (95% CI:0.631-0.858,  $p=0.0003$ ) and 0.670 (95% CI:0.540-0.799,  $p=0.0218$ ), respectively (see Fig 3-12). Similarly, the percentage of CD8<sup>+</sup> circulating MAITs demonstrated an AUC of 0.730 (95% CI: 0.613-0.846,  $p=0.0007$ ) for advanced fibrosis and 0.678 (95% CI:0.553-0.803,  $p=0.0161$ ) for NASH. Absolute circulating MAITs had the highest sensitivity and specificity for advanced fibrosis with an AUC of 0.754 (95% CI: 0.639-0.869,  $p < 0.0001$ ), although they exhibited the lowest AUC for NASH, 0.648 (95% CI: 0.513-0.782,  $p= 0.0484$ ) (see Fig 3-12). Interestingly, MAIT cells overall had the greatest AUC for advanced fibrosis in comparison to the NLR and percentage of circulating monocytes, whilst the latter were more predictive of NASH.

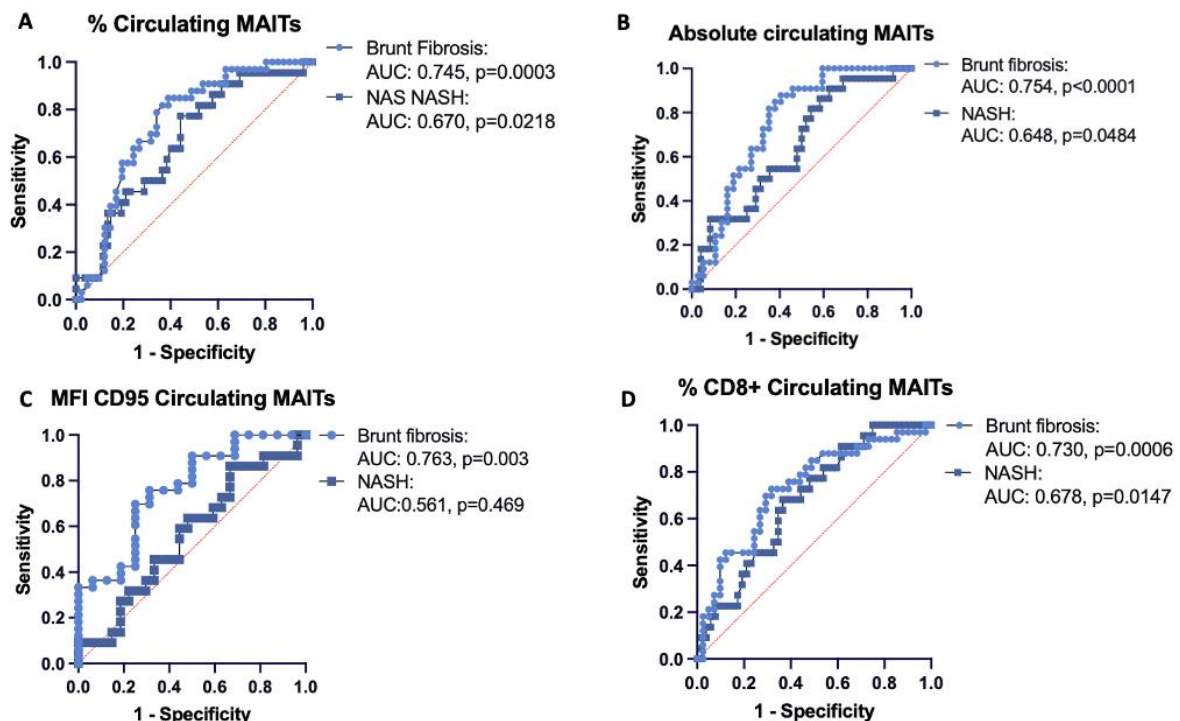


Figure 3-12: ROC curves demonstrating the sensitivity and specificity of: (A) the percentage of circulating MAITs (N=74), (B) absolute number of circulating MAITs (N=74), (C) CD95 expression (N=49) and (C) the percentage of CD8<sup>+</sup> MAIT cells (N=74), for the prediction of advanced fibrosis and NASH. Abbreviations: MFI, mean fluorescence intensity.

3.5.6 The total percentage and fraction of CD8<sup>+</sup> intrahepatic MAIT cells are not significantly altered in NAFLD and do not change in association with NASH nor fibrosis stage

We then explored if the observed changes in circulating MAIT cells were reflected by the intrahepatic MAIT cell population. Liver tissue was processed as detailed in Chapter 3; intrahepatic MAIT cells were identified using flow cytometry. There were no significant changes between the total percentage of intrahepatic MAIT cells and CD8<sup>+</sup> MAIT cells with either advancing fibrosis (see Fig 3-13, table 3-8) or NASH (table 3-9). Similarly, following adjustment for multiple comparisons using Dunn's test, there were no significant changes in the MFI of CD69 or CD95 expressed by intrahepatic MAIT cells with worsening histological severity. Interestingly, we also found no difference between the total percentage of intrahepatic MAITs and CD8<sup>+</sup> MAITs in patients with NAFLD compared with healthy controls (see Fig 3-13A and Fig 3-13D respectively).

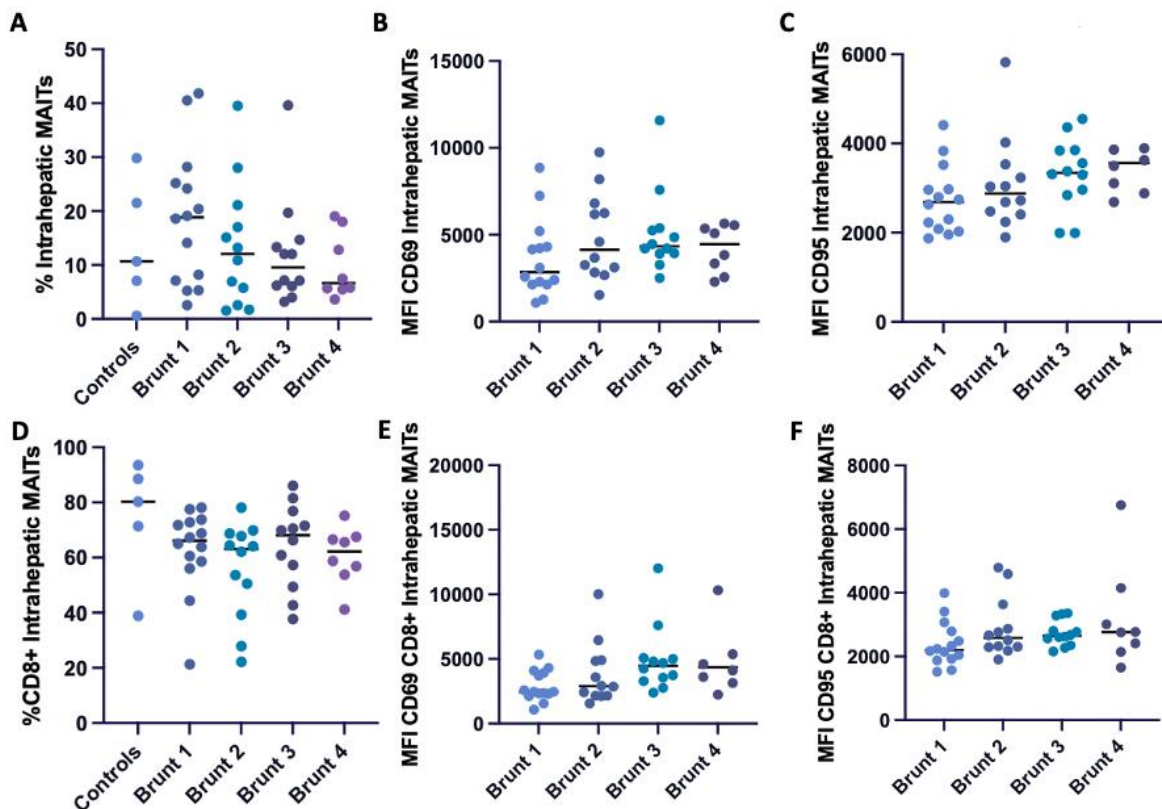


Figure 3-13 Changes in (A) the percentage (N=51), (B) MFI of CD69 and (C) MFI of CD95 expression of intrahepatic MAITs across different stages of fibrosis (N=46). Analysis of the percentage of CD8<sup>+</sup> MAIT cells (D) (N=51), CD69 (E) and CD95 (F) expression (N=46).

MAIT cells were defined as CD3<sup>+</sup> Vα7.2HiCD161Hi, please see Chapter 2, figure 2-1 for gating strategy. Data analysed using the two-tailed Kruskal-Wallis test, medians displayed graphically, P values adjusted for multiple comparisons using Dunn's test, \*\*p<0.001, \*\*\*p<0.0001. ns. Abbreviations: MFI, mean fluorescence intensity.

Table 3-8: Characteristics of intrahepatic MAIT cells across different Brunt stages:

|                                                    | Controls    | Brunt 1            | Brunt 2            | Brunt 3            | Brunt 4            | P value                   |
|----------------------------------------------------|-------------|--------------------|--------------------|--------------------|--------------------|---------------------------|
| % Intrahepatic MAITs                               | 10.7(21.81) | 18.85 (19.28)      | 12.05 (16.72)      | 9.58 (8.24)        | 6.63 (11.16)       | 0.481 <sup>a</sup>        |
| MFI CD69 Intrahepatic MAITs                        | -           | 2840 (2391)        | 4131 (3751)        | 4337(1434)         | 4456 (2742)        | 0.223 <sup>b</sup>        |
| MFI CD95 Intrahepatic MAITs                        | -           | 2687 (1043)        | 2875 (1032)        | 3344 (977)         | 3562 (944)         | 0.0599 <sup>b</sup>       |
| %CD8 <sup>+</sup> Intrahepatic MAITs               | 80.3(35.85) | 66.15 (15.1)       | 63.1 (26.38)       | 68.1 (24.17)       | 62.2 (12.67)       | 0.225 <sup>a</sup>        |
| <i>MFI CD69 CD8<sup>+</sup> Intrahepatic MAITs</i> | -           | <i>2458 (1694)</i> | <i>2887 (2729)</i> | <i>4470 (1693)</i> | <i>4355 (5834)</i> | <i>0.0308<sup>b</sup></i> |
| MFI CD95 CD8 <sup>+</sup> Intrahepatic MAITs       | -           | 2201 (952)         | 2587 (1154)        | 2651 (764)         | 2765 (1652)        | 0.215 <sup>b</sup>        |

Data was tested for normality using the Shapiro–Wilks test. Continuous variables are presented as median (interquartile range). <sup>a</sup>N=51, <sup>b</sup>N=46, data was analysed using a two tailed Kruskal-Wallis test, P values adjusted for multiple comparisons using Dunn’s test. Abbreviations: MFI, mean fluorescence intensity.

Table 3-9: Changes in circulating and intrahepatic MAIT cells in participants with and without a diagnosis of NASH

|                                                    | Controls     | NAS < 5       | NAS ≥ 5       | P value               |
|----------------------------------------------------|--------------|---------------|---------------|-----------------------|
| <b>Absolute circulating MAITs</b>                  | 36.3 (64.83) | 16.31 (23.52) | 15.07 (23.22) | 0.012 <sup>a,e</sup>  |
| <b>% Circulating MAITs</b>                         | 2.25 (3.12)  | 1.17 (2.52)   | 1.08 (1.06)   | 0.0074 <sup>a,e</sup> |
| <b>MFI CD69 Circulating MAITs</b>                  | -            | 360 (308)     | 361 (519.8)   | 0.755 <sup>b,f</sup>  |
| <b>MFI CD95 Circulating MAITs</b>                  | -            | 1747 (964)    | 1931 (981)    | 0.476 <sup>b,f</sup>  |
| <b>%CD8<sup>+</sup> Circulating MAITs</b>          | 70.2 (17.55) | 37.7 (31.1)   | 40.75 (28.3)  | <0.001 <sup>a,e</sup> |
| <b>MFI CD69 CD8<sup>+</sup> Circulating MAITs</b>  | -            | 328 (328)     | 448.5 (547.5) | 0.197 <sup>b,f</sup>  |
| <b>MFI CD95 CD8<sup>+</sup> Circulating MAITs</b>  | -            | 1585 (712)    | 1526 (1004)   | 0.882 <sup>b,f</sup>  |
| <b>% Intrahepatic MAITs</b>                        | 10.7 (21.81) | 14.1 (12.45)  | 6.08 (14.94)  | 0.148 <sup>c,f</sup>  |
| <b>MFI CD69 Intrahepatic MAITs</b>                 | -            | 4213 (3011)   | 4159 (2336)   | 0.690 <sup>d,f</sup>  |
| <b>MFI CD95 Intrahepatic MAITs</b>                 | -            | 3232 (1175)   | 2963 (1527)   | 0.467 <sup>d,f</sup>  |
| <b>% CD8<sup>+</sup> Intrahepatic MAITs</b>        | 80.3 (35.85) | 65.6 (15)     | 64.4 (22.45)  | 0.158 <sup>c,e</sup>  |
| <b>MFI CD69 CD8<sup>+</sup> Intrahepatic MAITs</b> | -            | 2108 (1458)   | 2171 (1175)   | 0.271 <sup>d,f</sup>  |
| <b>MFI CD95 CD8<sup>+</sup> Intrahepatic MAITs</b> | -            | 2605 (1029)   | 2513 (643)    | 0.739 <sup>d,f</sup>  |

Data was tested for normality using the Shapiro–Wilks test. Continuous variables are presented as median (interquartile range). <sup>a</sup>N=74, <sup>b</sup>N=49, <sup>c</sup>=51, <sup>d</sup>=46, <sup>e</sup>data was analysed using two tailed Mann-Whitney U test, <sup>f</sup>data analysed using the two tailed Kruskal-Wallis test. Abbreviations: MFI, mean fluorescence intensity.

### 3.5.7 The percentage of circulating CD8<sup>+</sup> T Lymphocytes is significantly reduced in NAFLD compared with healthy controls and is predictive of advanced fibrosis

In order to see if the changes observed, particularly in the circulation, were unique to MAIT cells or if they were also seen in other T lymphocytes, we analysed circulating and intrahepatic T lymphocytes (excluding MAIT cells, as outlined in Chapter 2) by gating on CD3<sup>+</sup> cells outside of the MAIT gate. There was no difference in the total or percentage of circulating T cells in NAFLD compared to controls, with no significant difference with advanced fibrosis or NASH (*see table 3-10*). Similarly, we found no difference in the MFI of the activation markers CD69 or CD95 expressed by circulating T cells with increasing histological severity (*see table 3-10*). Interestingly however, the percentage of CD8<sup>+</sup> circulating T cells was significantly reduced in NAFLD compared with controls (*see Fig 3-14A*), although there was no intergroup variation across different stages of Brunt fibrosis or NASH and no difference in activation marker expression (*see Fig 3-14B-C*).

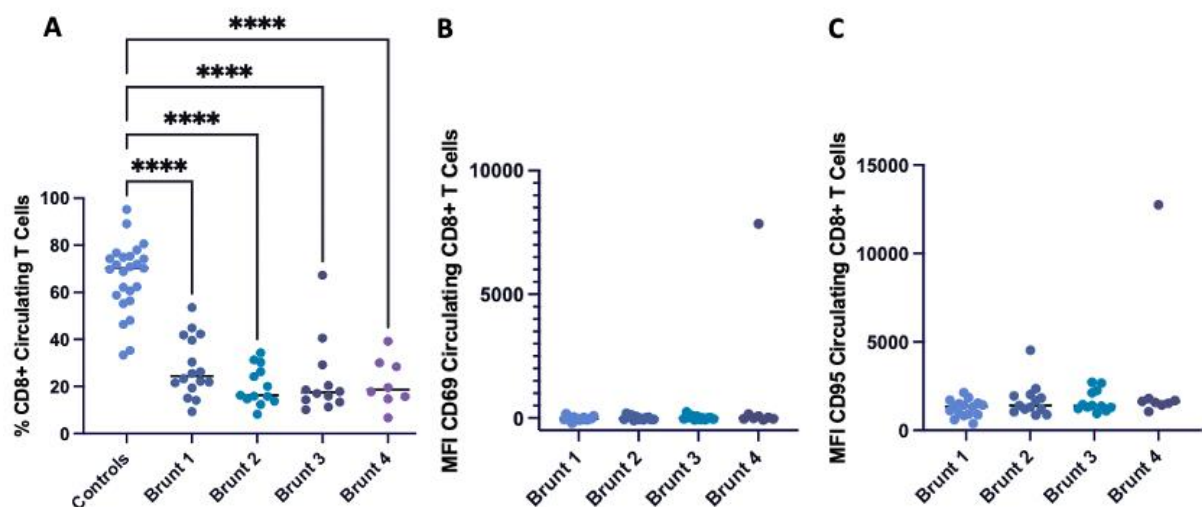


Figure 3-14: Changes in (A) the percentage (N=51), (B) MFI of CD69 and (C) MFI of CD95 expression (N=46) of CD8<sup>+</sup> circulating T cells, MAIT cells excluded, across different stages of fibrosis.

Data analysed using the two-tailed Kruskal-Wallis test, medians displayed graphically, P values adjusted for multiple comparisons using Dunn's test, \*\*\*\*p<0.00001. ns. Abbreviations: MFI, mean fluorescence intensity. T cells excluding MAIT cells were defined as CD3<sup>+</sup> Vα7.2<sup>-</sup>CD161<sup>-</sup>, please see Chapter 2, figure 2-1 for gating strategy. Abbreviations: ns, non-significant, MFI, mean fluorescence intensity.

We subsequently examined the predictive capacity of CD8<sup>+</sup> T cells for advanced fibrosis and NASH. The percentage of circulating CD8<sup>+</sup>T cells performed moderately well for advanced

fibrosis with an AUC of 0.689 (95% CI:0.557-0.820,  $p=0.007$ ), however it was not predictive of NASH ( $p=0.124$ ) (see Fig. 3-15).

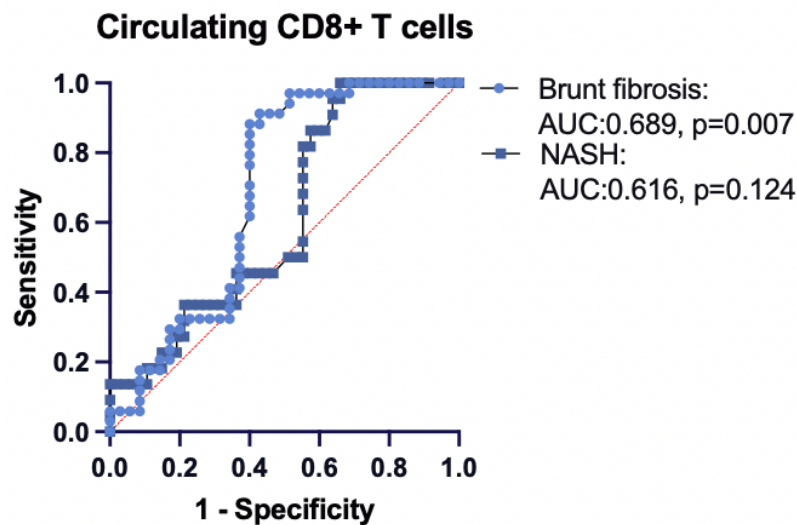


Figure 3-15 ROC curve demonstrating the sensitivity and specificity of: (A) the percentage of circulating CD8+ T lymphocytes MAITs (N=74) for the prediction of advanced fibrosis and NASH. Abbreviations: MFI, mean fluorescence intensity.

### 3.5.8 The percentage of CD8<sup>+</sup> intrahepatic T lymphocytes is significantly reduced with advancing fibrosis and NASH

We next evaluated the intrahepatic T lymphocyte population, excluding MAIT cells. Interestingly, although the total percentage of intrahepatic T lymphocytes were unaltered with advanced fibrosis, our results showed an increase in the MFI of CD69 and CD95 coupled with significant reduction in the percentage of CD8<sup>+</sup> intrahepatic T cells (see Fig 3-16, table 3-10). Similarly, the percentage of CD8<sup>+</sup> T lymphocytes were reduced with NASH, although there were no significant differences in activation marker expression (see Fig 3-16, table 3-11).

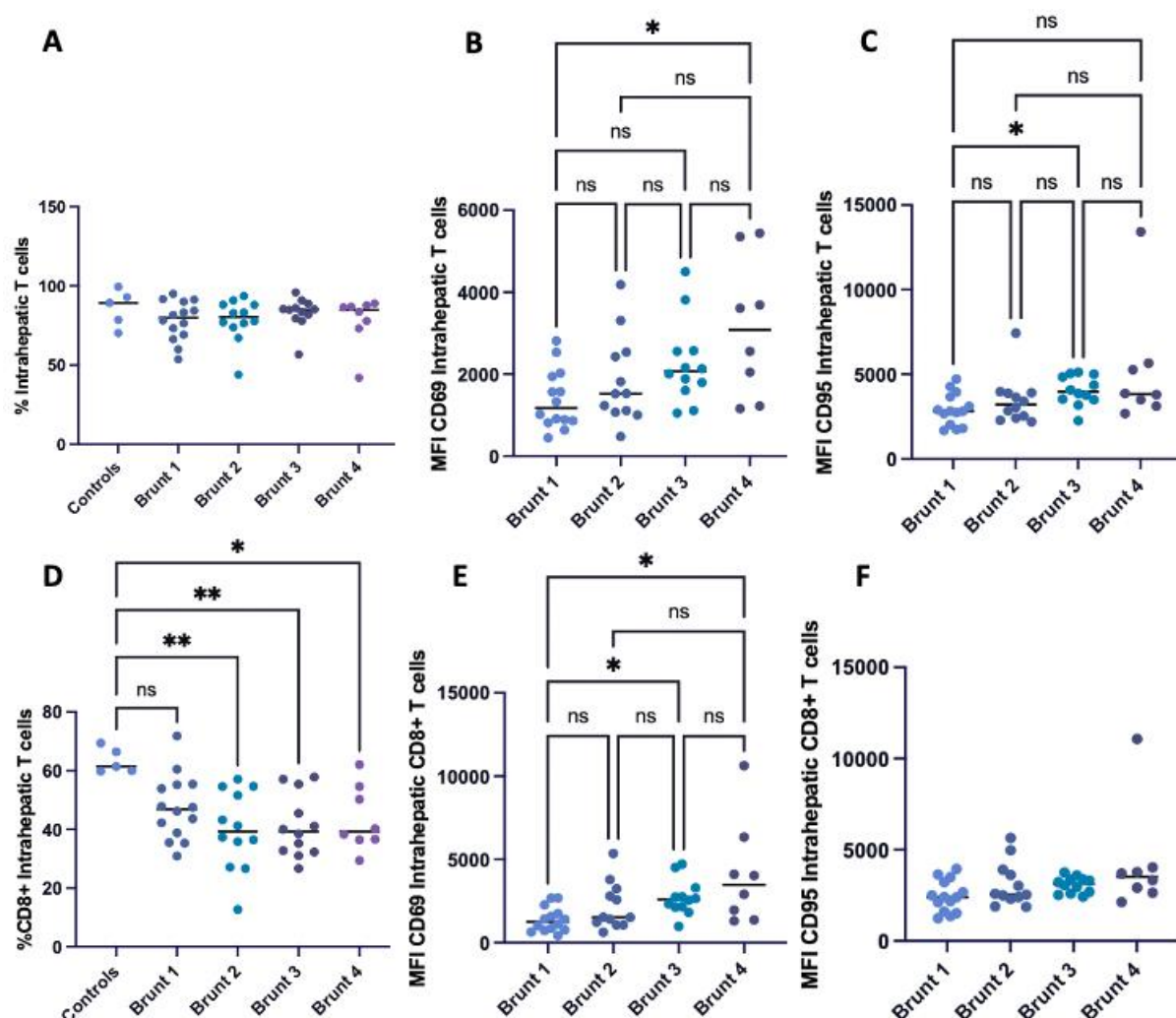


Figure 3-16: Changes in (A) the percentage (N=51), (B) MFI of CD69 and (C) MFI of CD95 expression (N=46) of intrahepatic T cells, MAIT cells excluded, across different stages of fibrosis. (D) Percentage of CD8+ intrahepatic T cells, (E) CD69 and (F) CD95 expression across different Brunt fibrosis stages.

Data analysed using the two-tailed Kruskal-Wallis test, medians displayed graphically, P values adjusted for multiple comparisons using Dunn's test, \*p<0.05, \*\*p<0.001, ns, non-significant. Abbreviations: MFI, mean fluorescence intensity. T cells excluding MAIT cells were defined as CD3<sup>+</sup> Vα7.2-CD161<sup>-</sup>, please see Chapter 2, figure 2-1 for gating strategy

Table 3-10: Characteristics of circulating T lymphocytes across different Brunt fibrosis stages

|                                                     | Controls         | Brunt 1            | Brunt 2           | Brunt 3          | Brunt 4           | P value              |
|-----------------------------------------------------|------------------|--------------------|-------------------|------------------|-------------------|----------------------|
| <b>Absolute Circulating T Cells</b>                 | 1379<br>(5680.6) | 1468<br>(792)      | 1426<br>(460)     | 1637<br>(1233)   | 1091<br>(500.9)   | 0.422 <sup>a</sup>   |
| <b>MFI CD69 Circulating T Cells</b>                 | -                | -6.18<br>(114.78)  | -8.41<br>(111.5)  | -3.49<br>(81.2)  | -19.29<br>(112.4) | 0.737 <sup>b</sup>   |
| <b>MFI CD95 Circulating T Cells</b>                 | -                | 1584 (1271)        | 2190 (896)        | 2544 (1040)      | 2736 (1501)       | 0.0562 <sup>b</sup>  |
| <b>%CD8<sup>+</sup> Circulating T Cells</b>         | 70.2<br>(17.55)  | 24.4<br>(21.4)     | 16.2<br>(14.05)   | 17.45<br>(13.37) | 18.65<br>(14.65)  | <0.0001 <sup>a</sup> |
| <b>MFI CD69 CD8<sup>+</sup> Circulating T Cells</b> | -                | -30.55<br>(102.33) | -16.1<br>(110.25) | 5.15<br>(112.63) | 6.52<br>(165.13)  | 0.605 <sup>b</sup>   |
| <b>MFI CD95 CD8<sup>+</sup> Circulating T Cells</b> | -                | 1349<br>(640.2)    | 1411<br>(860)     | 1388<br>(970)    | 1616<br>(321)     | 0.212 <sup>b</sup>   |
| <b>% Intrahepatic T Cells</b>                       | 89.3<br>(21.81)  | 79.9<br>(21.85)    | 80.25<br>(13.55)  | 85.0<br>(8.1)    | 84.95<br>(13.23)  | 0.593 <sup>c</sup>   |
| <b>MFI CD69 Intrahepatic T Cells</b>                | -                | 1173<br>(1113.5)   | 1524<br>(1427)    | 2070<br>(918)    | 3082<br>(3504)    | 0.0126 <sup>d</sup>  |
| <b>MFI CD95 Intrahepatic T Cells</b>                | -                | 2820 (1760)        | 3224 (1454)       | 3973 (1461)      | 3830 (2355)       | 0.0199 <sup>d</sup>  |
| <b>% CD8<sup>+</sup> Intrahepatic T Cells</b>       | 61.4 (8)         | 46.85 (17.27)      | 39.25 (24.57)     | 39.25 (20.5)     | 39.25 (17.05)     | 0.0094 <sup>c</sup>  |
| <b>MFI CD69 Intrahepatic T Cells</b>                | -                | 1259 (1119)        | 1523 (2025)       | 2608 (1018)      | 3466 (4263)       | 0.0061 <sup>d</sup>  |
| <b>MFI CD95 Intrahepatic T Cells</b>                | -                | 2400 (1706)        | 2558 (1503)       | 3137 (761)       | 3156 (1256)       | 0.0555 <sup>d</sup>  |

MAIT cells excluded. Data was tested for normality using the Shapiro–Wilks test. Continuous variables are presented as median (interquartile range). <sup>a</sup>N=74, <sup>b</sup>N=49, <sup>c</sup>N=51, <sup>d</sup>N=46. Data was analysed using a two tailed Kruskal–Wallis test, P values adjusted for multiple comparisons using Dunn’s test. Abbreviations: MFI, mean fluorescence intensity.

*Table 3-11: Changes in circulating and intrahepatic T cells in participants with and without a diagnosis of NASH*

|                                                     | Controls         | NAS < 5         | NAS ≥ 5            | P value |
|-----------------------------------------------------|------------------|-----------------|--------------------|---------|
| <b>Absolute Circulating T Cells</b>                 | 1379<br>(5680.6) | 1296<br>(616)   | 1544<br>(705)      | 0.480   |
| <b>MFI CD69 Circulating T Cells</b>                 | -                | -10.8<br>(101)  | 10.75<br>(130.63)  | 0.0848  |
| <b>MFI CD95 Circulating T Cells</b>                 | -                | 2381 (1484)     | 2337 (1103)        | 0.507   |
| <b>%CD8<sup>+</sup> Circulating T Cells</b>         | 70.2<br>(17.55)  | 17.9<br>(8.6)   | 25.85<br>(16.55)   | <0.0001 |
| <b>MFI CD69 CD8<sup>+</sup> Circulating T Cells</b> | -                | -14.1<br>(98.2) | -11.24<br>(131.03) | 0.479   |
| <b>MFI CD95 CD8<sup>+</sup> Circulating T Cells</b> | -                | 1433<br>(596)   | 1389<br>(881)      | 0.834   |
| <b>% Intrahepatic T Cells</b>                       | 89.3<br>(21.81)  | 82.9<br>(12.2)  | 84.9<br>(20)       | 0.461   |
| <b>MFI CD69 Intrahepatic T Cells</b>                | -                | 1795<br>(1913)  | 1888<br>(1279)     | 0.627   |
| <b>MFI CD95 Intrahepatic T Cells</b>                | -                | 3639 (1684)     | 3408 (1129)        | 0.671   |
| <b>% CD8<sup>+</sup> Intrahepatic T Cells</b>       | 61.4 (8)         | 43.2 (18.95)    | 38.8 (19.5)        | 0.0037  |
| <b>MFI CD69 Intrahepatic T Cells</b>                | -                | 1743 (2006)     | 2160 (1399)        | 0.526   |
| <b>MFI CD95 Intrahepatic T Cells</b>                | -                | 3020 (1315)     | 2645 (1001)        | 0.418   |

Data was tested for normality using the Shapiro–Wilks test. Continuous variables are presented as median (interquartile range). <sup>a</sup>N=74, <sup>b</sup>N=49, <sup>c</sup>=51, <sup>d</sup>=46, <sup>e</sup>data was analysed using two tailed Mann-Whitney U test, <sup>f</sup>data analysed using the two tailed Kruskal-Wallis test. Abbreviations: MFI, mean fluorescence intensity.

### *3.5.9 Circulating and intrahepatic NK cells are unaltered in advanced NAFLD fibrosis and NASH*

We next analysed the percentage of circulating and intrahepatic NK cells. In contrast to MAIT cells and T lymphocytes, NK cells were unaltered with either advancing brunt stage fibrosis and NASH (see Fig. 3-17, table 3-12 and 3-13). There were no significant changes in activation marker expression on circulating and intrahepatic NK cells in association with worsening histological severity (see Fig. 3-17, table 3-12 and 3-13 ).

Table 3-12: Changes in circulating and intrahepatic NK cells with advancing Brunt stage:

|                                       | <b>Brunt 1</b>  | <b>Brunt 2</b>   | <b>Brunt 3</b>  | <b>Brunt 4</b>   | <b>P value</b> |
|---------------------------------------|-----------------|------------------|-----------------|------------------|----------------|
| <b>% Circulating NK Cells</b>         | 16.8<br>(7.35)  | 19.1<br>(18.85)  | 20.9<br>(10.62) | 24.0<br>(13.88)  | 0.435          |
| <b>MFI CD69 Circulating NK Cells</b>  | 1501<br>(941)   | 1420<br>(1093.7) | 1854<br>(1877)  | 2392<br>(2326)   | 0.264          |
| <b>MFI CD95 Circulating NK Cells</b>  | 1188 (717)      | 966 (282.5)      | 1154 (421)      | 1369 (371)       | 0.0721         |
| <b>% Intrahepatic NK Cells</b>        | 60.2<br>(17.62) | 60.8<br>(26.55)  | 65.7<br>(21.8)  | 32.72<br>(13.23) | 0.733          |
| <b>MFI CD69 Intrahepatic NK Cells</b> | 1428<br>(1023)  | 1263<br>(1125)   | 1927<br>(1819)  | 2367<br>(2228)   | 0.125          |
| <b>MFI CD95 Intrahepatic NK Cells</b> | 1119 (770)      | 952(429)         | 1107 (396)      | 1345 (474)       | 0.150          |

Data was tested for normality using the Shapiro–Wilks test. Continuous variables are presented as median (interquartile range). \*N=49. Data was analysed using a two tailed Kruskal-Wallis test, P values adjusted for multiple comparisons using Dunn’s test. Abbreviations: MFI, mean fluorescence intensity, NK cells, natural killer cells.

Table 3-13: Characteristics of circulating and intrahepatic NK cells in individuals with and without NASH

|                                       | <b>NAS &lt; 5</b> | <b>NAS ≥ 5</b>  | <b>P value</b> |
|---------------------------------------|-------------------|-----------------|----------------|
| <b>% Circulating NK Cells</b>         | 22.7<br>(19.1)    | 16.4<br>(10.5)  | 0.0151         |
| <b>MFI CD69 Circulating NK Cells</b>  | 1791<br>(1621)    | 1602<br>(1322)  | 0.934          |
| <b>MFI CD95 Circulating NK Cells</b>  | 1246<br>(603.5)   | 1055<br>(432.5) | 0.500          |
| <b>% Intrahepatic NK Cells</b>        | 66.4<br>(18.6)    | 56.6<br>(18.5)  | 0.0411         |
| <b>MFI CD69 Intrahepatic NK Cells</b> | 1828<br>(1694)    | 1514<br>(1254)  | 0.674          |
| <b>MFI CD95 Intrahepatic NK Cells</b> | 1200<br>(564)     | 1049<br>(450.7) | 0.640          |

Data was tested for normality using the Shapiro–Wilks test. Continuous variables are presented as median (interquartile range). \*N=46. Data was analysed using a two tailed Mann-Whitney U test, P values adjusted for multiple comparisons using Dunn’s test. Abbreviations: MFI, mean fluorescence intensity, NK cells, natural killer cells.

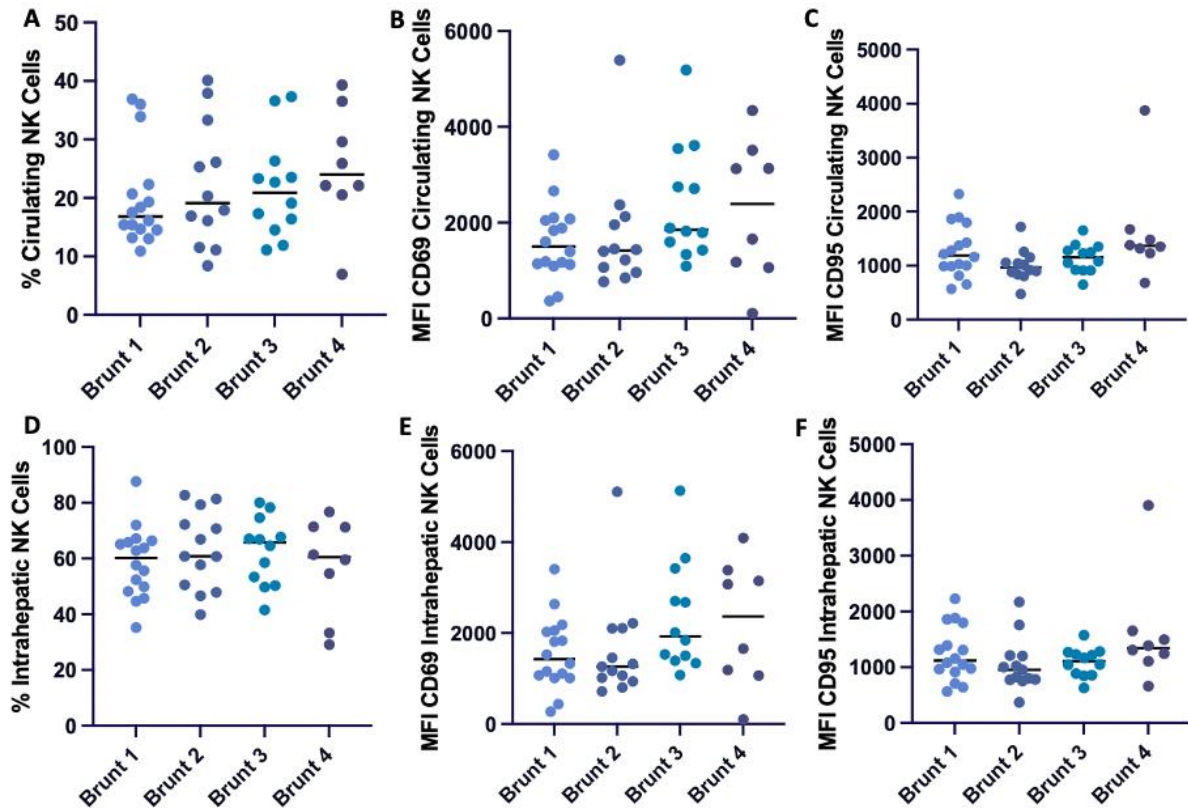


Figure 3-17: Changes in (A) the percentage, (B) CD69 and (C) CD95 expression of circulating NK cells across different stages of Brunt Fibrosis. Comparison of the (A) percentage, (E) CD69 and (F) CD95 expression of intrahepatic NK cells.

Data analysed using the two-tailed Kruskal-Wallis test, medians displayed graphically, P values adjusted for multiple comparisons using Dunn's test, N=49. Abbreviations: MFI, mean fluorescence intensity. T cells excluding MAIT cells were defined as CD3<sup>+</sup> Va7.2-CD161<sup>-</sup>, please see Chapter 2, figure 2-2 for gating strategy.

### 3.6 A combination of the NLR, circulating MAIT cells, monocytes and CD8<sup>+</sup> T lymphocytes resulted in the highest predictive power for both advanced fibrosis and NASH

Finally, we explored the possibility that a combination of these variables would perform better than each in isolation when predicting advanced fibrosis and NASH. The best predictive model was obtained by using a combination of the NLR, percentage of circulating CD8<sup>+</sup> T lymphocytes, monocytes, total absolute number of circulating MAIT cells coupled with the MFI of both activation markers: CD69 and CD95. This resulted in an AUC of 0.979 (95% CI:0.937-1.0, p=0.0003) for advanced fibrosis (*see Fig 3-18A*). For NASH, the addition of the percentage of CD8<sup>+</sup> MAIT cells improved the AUC from 0.747 (95% CI:0.581-0.912, p=0.0143) to 0.813 (95% CI:0.664-0.961, p=0.0019) (*see Fig 3-19B*).

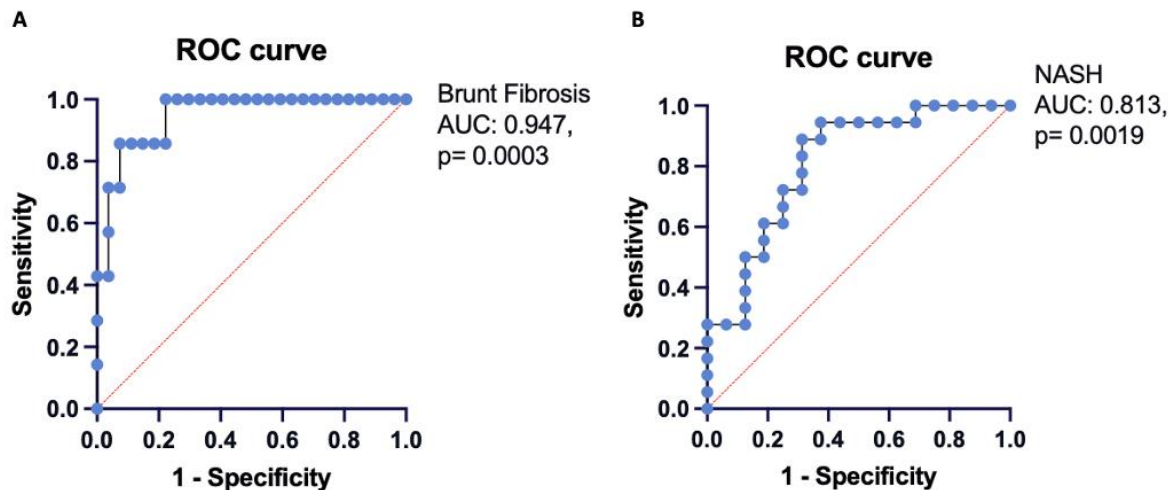


Figure 3-18: ROC curves demonstrating the sensitivity and specificity of: (A) the percentage of circulating CD8+ T lymphocytes MAITs (N=74) for the prediction of advanced fibrosis and NASH. Abbreviations: MFI, mean fluorescence intensity.

### 3.7 Discussion

Following detailed phenotypic analysis of circulating immune cells, it was clear that a composite of the total percentage of circulating monocytes, MAIT cells, CD8<sup>+</sup> T lymphocytes and the NLR was strongly associated and predictive of both NASH and advanced fibrosis. This combination, resulted in a greater AUC than each cell population in isolation, even when compared with existing non-invasive measures including VCTE measurements, the NAFLD and FIB-4 scores.

A significant reduction in the percentage of circulating monocytes in patients with NASH was identified, although, in contrast to other studies, there were no differences in the proportion of non-classical, intermediate and classical monocyte populations or their surface marker expression [240, 253, 258]. This reduction may reflect a proportionate increase in hepatic migration, which has been reported in both murine and human studies[255], as the circulating total lymphocyte and NK cell fractions remained unchanged though this current study did not evaluate chemokine receptor expression or the intrahepatic monocyte population. Monocyte chemotaxis is stimulated via the chemokine receptor, CCR2, by injured hepatocytes and by activated Kupffer cells in NAFLD [255]. Once in the liver, they differentiate into monocyte-derived macrophages, promoting the evolution of hepatic inflammation and

stimulating hepatic stellate cell differentiation into myofibroblasts [255]. The fact that circulating monocytes were reduced in association with hepatic inflammation, but not advanced fibrosis may reflect their involvement in the earlier stages of NAFLD progression. Although differences in monocyte subpopulations in peripheral circulation was not observed, it may be that phenotypic alterations occur following migration, in response to stimuli at a more local level within the liver.

Total and CD8<sup>+</sup> circulating MAIT cells were reduced in patients with NAFLD in comparison to healthy controls and lower percentage of circulating CD8<sup>+</sup> and total MAIT cells, with increased CD69 and CD95 expression, were found to be more strongly associated with and predictive of advanced hepatic fibrosis than NASH. Interestingly, there were no significant differences in the intrahepatic MAIT cell compartment across different fibrosis stages or NASH in the study. The observed decrease in circulating MAITs may reflect cellular migration, however given that the percentage of intrahepatic MAIT cells remained unchanged, this may be due to accelerated apoptosis or migration to other sites such as visceral adipose tissue, due to the systemic, obesity related inflammation associated with NAFLD. Reductions in circulating MAIT cells have been shown in the current literature with increasing fibrosis stage in chronic liver diseases from different aetiologies [117, 118, 262]. This has been associated with features of persistent cellular activation and metabolic exhaustion [117, 263]. Given the gut-liver axis, alterations in gut microbial composition may also influence MAIT cell behaviour in NAFLD. NAFLD has been associated with dysbiosis, interestingly experimental models of obesity have shown that dysbiosis in high fat diet fed mice promoted MAIT cell mediated inflammation in visceral adipose tissue and ileal mucosa, it is possible that a similar mechanism may influence inflammation and fibrosis within the liver [110, 264]. Although MAIT cells have been shown to promote hepatic inflammation through release of pro-inflammatory cytokines and modulation of macrophage to the M2 phenotype [130], functional studies have shown that MAIT cells can stimulate hepatic stellate cells directly, promoting fibrogenesis [117]. This may explain why changes in MAIT cell frequencies and surface marker expression were more pronounced with advanced fibrosis rather than hepatic inflammation.

A novel finding in this current study is that the percentage of CD8<sup>+</sup> MAIT cells was particularly reduced in NAFLD. The CD8<sup>+</sup> MAIT cell subset is typically the most abundant both in circulation

and within the liver, including in patients with established cirrhosis [74, 78, 265]. The observed reduction may be either due to expansion of CD4<sup>+</sup> and CD4<sup>+</sup>/CD8<sup>-</sup> MAIT subsets, preferential migration or accelerated apoptosis of the CD8<sup>+</sup> subset. Due to the limitations of our flow cytometer, we were unable to stain for CD4 to fully categorize the CD4<sup>+</sup>, CD4<sup>+</sup>/CD8<sup>-</sup> populations.

Interestingly, significant reductions in the percentage of both circulating and intra-hepatic CD8<sup>+</sup> T cells were observed in this study, with the liver resident MAIT cells expressing higher levels of the activation markers CD69 and CD95 expression in association with increased NAFLD fibrosis. CD95, also known as the Fas-ligand receptor, functions as a terminal activation marker. An increase in its expression may reflect heightened apoptosis possibly in response to chronic stimulation and cellular exhaustion. In advanced NAFLD, CD8<sup>+</sup> intrahepatic lymphocytes may be more vulnerable to excessive activation and accelerated apoptosis via the Fas-Fas ligand pathway. CD8<sup>+</sup> T lymphocytes have been thought to play a pro-inflammatory role within the liver by secreting IFN $\gamma$  in response to IL12 and IL18 [10]. Reductions in the frequency of circulating CD8<sup>+</sup> T lymphocytes have been shown in patients with metabolic disease [34], experimental animal studies have shown increased migration of CD8<sup>+</sup> T lymphocytes in models of liver disease [266] and small studies amongst patients with chronic viral hepatitis have shown an enrichment of CD8<sup>+</sup> T lymphocytes within the liver [10], but there have been no papers evaluating intra-hepatic populations in NAFLD. Notably, MAIT cells and CD8<sup>+</sup> T lymphocytes share a common metabolic pathway, relying predominantly on mTOR-mediated glycolysis [81, 267]. In obesity and diabetes, this pathway has been shown to be dysfunctional leading to metabolic exhaustion [81, 267]. One explanation could be that the combination of persistent activation and impaired cellular energy supply in NAFLD may lead to metabolic stress, making these subsets more vulnerable to apoptosis.

This is the first study to provide a descriptive assessment of these immune cell populations in parallel in individuals with NAFLD of varying severity. The results support the stated hypothesis that phenotypic changes in circulating immune cells correlate with and are predictive of NASH and NAFLD fibrosis. This suggests that there may be potential in the development of a non-invasive, immunological score for assessing histological severity, although further prospective studies are required to validate the developed models.

This study did have several limitations including (i) of a small sample population, (ii) no functional cellular analysis or measure of chemotactic markers, (iii) intrahepatic immune cell assessment was limited to MAIT cells, T lymphocytes and NK cells and (iv) data from healthy controls were not available for the analysis of NK, monocyte and B cell populations.

### 3.8 Study findings

- The percentage of circulating monocytes are significantly reduced with hepatic inflammation and are predictive of NASH
- Circulating total and CD8<sup>+</sup> MAIT cells are reduced in NAFLD compared with healthy controls and are predictive of both advanced fibrosis and NASH
- The total and CD8<sup>+</sup> intrahepatic MAIT cell populations are unaltered with hepatic inflammation and advanced fibrosis
- Circulating CD8<sup>+</sup> T lymphocytes are reduced in NAFLD compared to healthy controls and are predictive of advanced fibrosis
- Intrahepatic CD8<sup>+</sup> T lymphocytes are significantly reduced with NASH and hepatic fibrosis
- A combination of the NLR, percentage of circulating monocytes, CD8<sup>+</sup> T lymphocytes and MAIT cells provided the greatest AUC for both NASH and advanced fibrosis compared with established scoring systems currently used in clinical practice

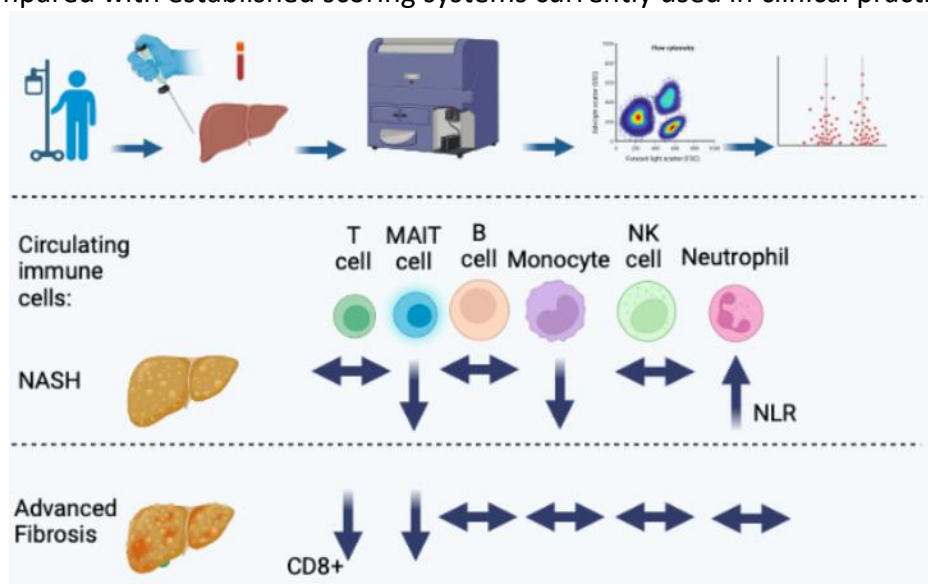


Figure 3-19 Key findings: circulating MAIT cells and monocytes were significantly reduced in NASH in combination with an elevated NLR. Circulating CD8<sup>+</sup> T lymphocytes and MAIT cells were reduced with advanced hepatic fibrosis.

Chapter 4: Measuring impact of weight loss  
interventions in NAFLD on circulating and intrahepatic  
MAIT cells

## 4 The impact of weight loss interventions in NAFLD on circulating and intrahepatic MAIT cells: Study II

### *4.1 Introduction*

Currently, there are no approved specific pharmacological treatments for NAFLD, and given the close association between NAFLD and obesity and consequently current management guidelines are centred around achieving weight loss through dietary and lifestyle intervention [180, 268]. Lifestyle intervention trials focusing on weight loss have reported histological improvements in NAFLD [269], with similar results observed in patients achieving weight loss following bariatric surgery [270]. Resolution of NASH has been observed in individuals achieving 5% or more weight loss, whilst fibrosis regression has been observed in those who lost more than 10% of their initial bodyweight [271]. Lifestyle interventions utilising a combination of dietary changes with exercise have resulted in greater improvements in markers of NAFLD activity than either intervention alone [269].

### *4.2 Hypothesis*

The aim of this study was to examine the relationship between changes in metabolic parameters, histological staging and grading and circulating and intrahepatic MAIT cell populations following either a 12-week dietary or exercise intervention in patients with NAFLD.

### *4.3 Specific aims*

- To assess histological changes following dietary and exercise-based weight loss interventions
- To correlate the histological changes following weight loss interventions with changes in circulating and intra-hepatic MAIT cells

### *4.4 Methods*

Participant enrolment and inclusion criteria are outlined in chapter 2, section 2.6. Sample collection and immunophenotyping was as described in chapter 2, section 2.5.7. Of the 50

subjects enrolled in the study, 45 completed the study (CG, n=14; DI, n=15; EI, n=16; *Figure 4-1*). Details of the DI and EI are described in chapter section 2.6.3 and 2.6.4, respectively. Statistical methods are outlined in chapter 2, section 2.6.5.

#### 4.4.1 Patient Demographics:

At baseline these participants were well matched with no differences in clinical and histological characteristics (*Table 4-1*).

*Table 4-1: NAFLD participant characteristics*

| Parameters               | Control (n=14) | Diet (n=15) | Exercise (n=16) | P value             |
|--------------------------|----------------|-------------|-----------------|---------------------|
| Age (y)                  | 55 (20)        | 58 (14)     | 61 (15)         | 0.3581 <sup>a</sup> |
| Gender, n (%)            |                |             |                 |                     |
| Female                   | 7 (50.0%)      | 8 (53.3%)   | 12 (75%)        | 0.5331 <sup>b</sup> |
| Male                     | 7 (50.0%)      | 7 (46.7%)   | 4 (25%)         |                     |
| Body weight (kg)         | 102.8 ± 33.3   | 97.3 ± 22.3 | 95.6 ± 20.0     | 0.7276 <sup>c</sup> |
| BMI (kg/m <sup>2</sup> ) | 35.9 ± 7.1     | 33.9 ± 5.3  | 36.1 ± 6.9      | 0.5996 <sup>c</sup> |
| Hypertension n (%)       | 7 (50.0%)      | 8 (53.3%)   | 9 (56.3%)       | 0.9431 <sup>b</sup> |
| Diabetes/IGT n (%)       | 10 (71.4%)     | 8 (53.3%)   | 11 (68.8%)      | 0.5391 <sup>b</sup> |
| Metabolic Syndrome       | 7 (50.0%)      | 7 (46.7%)   | 9 (56.3%)       | 0.8630 <sup>b</sup> |
| HbA1c (mmol/mol)         | 46 (19)        | 40 (8)      | 47 (24)         | 0.2126 <sup>a</sup> |
| Urate (μmol/L)           | 365 ± 99       | 351 ± 95    | 324 ± 68        | 0.4362 <sup>c</sup> |
| Vitamin D (nmol/L)       | 33 (20)        | 52 (26)     | 56 (57)         | 0.1632 <sup>a</sup> |
| Triglycerides (mmol/L)   | 1.8 (1.4)      | 1.9 (1.4)   | 1.4 (1.4)       | 0.4797 <sup>a</sup> |
| LDL-C (mmol/L)           | 2.1 ± 0.9      | 2.4 ± 1.0   | 2.3 ± 0.8       | 0.7501 <sup>c</sup> |
| HDL-C (mmol/L)           | 1.3 ± 0.3      | 1.2 ± 0.3   | 1.4 ± 0.5       | 0.1055 <sup>c</sup> |
| ALT (IU/L)               | 66 (37)        | 45 (70)     | 47 (26)         | 0.2763 <sup>a</sup> |
| AST (IU/L)               | 56 (30)        | 33 (47)     | 33 (14)         | 0.1371 <sup>a</sup> |
| GGT (IU/L)               | 113 (148)      | 61 (61)     | 58 (92)         | 0.1420 <sup>a</sup> |
| Brunt Fibrosis           |                |             |                 |                     |
| Stage 0/1                | 4 (28.6%)      | 7 (46.7%)   | 5 (31.3%)       | N/A <sup>d</sup>    |
| Stage 2                  | 2 (14.2%)      | 2 (13.3%)   | 4 (25.0%)       |                     |
| Stage 3                  | 4 (28.6%)      | 4 (26.7%)   | 5 (31.3%)       |                     |
| Stage 4                  | 4 (28.6%)      | 2 (13.3%)   | 2 (12.5%)       |                     |
| Histological scoring     |                |             |                 |                     |
| (NAS <5)                 | 5 (35.7%)      | 8 (56.3%)   | 10 (62.5%)      | 0.6355 <sup>c</sup> |
| (NAS ≥5)                 | 9 (64.3%)      | 7 (43.7%)   | 6 (37.5%)       |                     |

Data were tested for normality using Shapiro-Wilk test and continuous variables presented as either mean ± standard deviation or as median (interquartile range) as appropriate. Categorical variables are presented as number (percentage). ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma glutamyl transferase; HbA1c, haemoglobin A1c; IGT, impaired glucose tolerance; NAS, NAFLD activity score. aKruskall-Wallis test. bChi-square test. cOne-way ANOVA. dConditions for Chi-square not met.

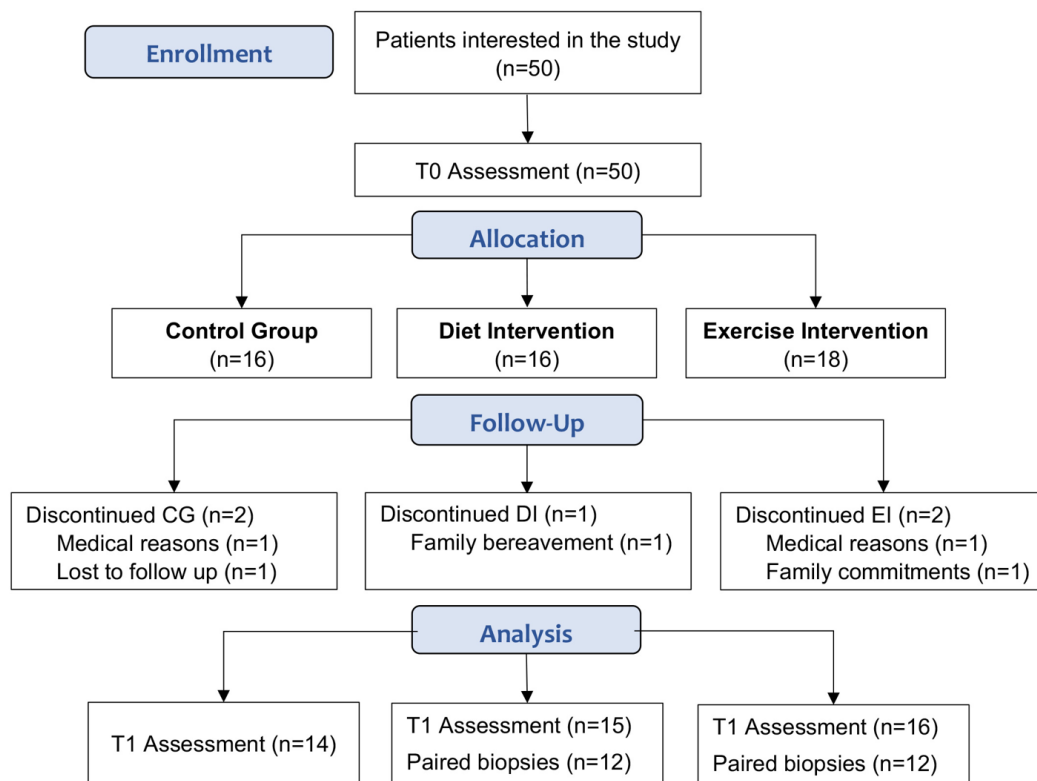


Figure 4-1 Participant recruitment and attrition. T0: baseline assessment, T1: post intervention (week 13)

77% of participants were obese (body mass index, BMI  $\geq 30 \text{ kg/m}^2$ ) and the remaining 23% were overweight (BMI 25-29.9  $\text{kg/m}^2$ ). (64%) had impaired glucose tolerance or type 2 diabetes mellitus, (53%) had an established diagnosis of hypertension (53%) and (51%) fulfilled the criteria for metabolic syndrome. Following review of initial histology, (84%) met the histological criteria for NASH; 64% had advanced fibrosis with Brunt stage  $\geq 2$  and 41% had a NAFLD activity score (NAS)  $\geq 5$ . Six participants (3 in DI, 3 in EI) were categorized as NAFL (steatosis only) on biopsy at T0 and did not have repeat biopsies at T1.

## 4.5 Results

### 4.5.1 Improvements in clinical parameters with diet and exercise

Both the DI and EI participants achieved significant reductions in body weight, BMI, waist circumference, and HbA1c between T0 and T1, however, greater losses were observed in the DI group (see Table 4-2). For details of the DI and EI, please see Chapter 2, section 2.6.3 and

2.6.4, respectively. While the participants in the DI achieved mean weight loss of 7 kg ( $P<0.0001$ ), the EI participants lost a mean of 2 kg ( $P=0.0005$ ). Analogously, mean reductions in BMI (DI:  $-1.9 \text{ kg/m}^2$ ,  $P=0.0002$ ; EI:  $-1.1 \text{ kg/m}^2$ ,  $P<0.0001$ ), and waist circumference (DI:  $-11.6 \text{ cm}$ ,  $P<0.0001$ ; EI:  $-4.0 \text{ cm}$ ,  $P<0.0001$ ), along with median changes in HbA1c (DI:  $-3.0 \text{ mmol/mol}$ ,  $P=0.0027$ ; EI:  $-1.5$ ,  $P=0.0298$ ), were more pronounced in the DI participants in comparison to EI. This modest weight loss with aerobic exercise among NALFD patients in comparison to nutritional intervention has been reported in exercise studies (12, 33). In contrast, no changes were observed in body weight, BMI, weight circumference and HbA1c in participants in the CG between T0 and T1 (*Table 4-2*) in the current study.

While VCTE scores for steatosis and liver stiffness improved significantly in both DI and EI participants, improvements were greater in EI participants. A 12.5% reduction in the mean-controlled attenuation parameter (CAP) score (T0:  $330.4 \pm 47.4$  versus T1:  $289.2 \pm 43.2 \text{ dB/m}$ ;  $P=0.0036$ ) was observed in DI, and a 13.8% reduction (T0:  $334 \pm 43.4$  versus T1:  $288.3 \pm 73.9 \text{ dB/m}$ ;  $P=0.0033$ ) observed in EI participants. While DI participants showed a 20.8% reduction in the median liver stiffness score (T0:  $12.0 (4.5)$  versus T1:  $9.5 (4.8) \text{ kPa}$ ;  $P=0.0154$ ), EI participants had a reduction of 27.6% (T0:  $12.3 (7.1)$  versus T1:  $8.9 (5.4) \text{ kPa}$ ;  $P=0.0038$ ). No changes in CAP or liver stiffness scores were observed in CG participants (*Table 4-2*).

*Table 4-2: Analysis of NAFLD patient characteristics in the Control, Diet and Exercise intervention groups*

| Parameters               | Control (n=14) |              |               | Diet (n=15)  |              |                   | Exercise (n=16) |              |                   |
|--------------------------|----------------|--------------|---------------|--------------|--------------|-------------------|-----------------|--------------|-------------------|
|                          | T0             | T1           | P value       | T0           | T1           | P value           | T0              | T1           | P value           |
| Weight (kg)              | 102.8 ± 33.3   | 102.8 ± 31.6 | 0.9899        | 97.0 ± 22.3  | 90.0 ± 19.9  | <b>&lt;0.0001</b> | 95.6 ± 20.0     | 93.6 ± 19.8  | <b>0.0005</b>     |
| BMI (kg/m <sup>2</sup> ) | 35.9 ± 7.1     | 36.4 ± 7.7   | 0.1422        | 33.9 ± 5.3   | 32.0 ± 5.3   | <b>0.0002</b>     | 36.1 ± 7.0      | 35.0 ± 6.7   | <b>&lt;0.0001</b> |
| WC <sup>a</sup> (cm)     | 105.4 ± 14.0   | 105.2 ± 14.1 | 0.8911        | 119.9 ± 16.0 | 108.3 ± 14.9 | <b>&lt;0.0001</b> | 111.2 ± 15.3    | 107.2 ± 15.7 | <b>&lt;0.0001</b> |
| HbA1c (mmol/mol)         | 46.0 (18.8)    | 43.0 (22.8)  | 0.9629        | 40.0 (8.0)   | 37.0 (6.0)   | <b>0.0027</b>     | 46.5 (23.8)     | 45.0 (23)    | <b>0.0298</b>     |
| CAP (dB/m)               | 325.6 ± 64.7   | 341.0 ± 56.8 | 0.2149        | 330.4 ± 47.4 | 289.2 ± 43.2 | <b>0.0036</b>     | 334.4 ± 43.4    | 288.3 ± 73.9 | <b>0.0033</b>     |
| Liver Stiffness (kPa)    | 17.1 (9.7)     | 13.7 (12.1)  | 0.2166        | 12.0 (4.5)   | 9.5 (4.8)    | <b>0.0154</b>     | 12.3 (7.1)      | 8.9 (5.4)    | <b>0.0038</b>     |
| FAST score               | 0.68 ± 0.22    | 0.57 ± 0.23  | <b>0.0399</b> | 0.54 ± 0.23  | 0.38 ± 0.25  | <b>0.0042</b>     | 0.51 ± 0.21     | 0.43 ± 0.18  | 0.0838            |
| FIB-4 score              | 1.57 (0.72)    | 1.30 (0.61)  | <b>0.0284</b> | 1.34 (0.89)  | 1.44 (0.70)  | 0.2314            | 1.32 (0.67)     | 1.27 (0.81)  | 0.137             |
| ALT (IU/L)               | 66.0 (37.3)    | 47.0 (46.8)  | <b>0.0342</b> | 45.0 (70.0)  | 32.0 (32.0)  | <b>0.0054</b>     | 46.5 (25.5)     | 40.5 (20.5)  | 0.0856            |
| AST (IU/L)               | 56.0 (29.8)    | 34.5 (21.3)  | <b>0.0289</b> | 33.0 (47.0)  | 30.0 (28.0)  | 0.0777            | 33.0 (14.0)     | 33.0 (11.0)  | 0.1682            |
| GGT (IU/L)               | 113.0 (147.8)  | 84.0 (103.5) | 0.5114        | 61.0 (61.0)  | 38.0 (45.0)  | <b>&lt;0.0001</b> | 57.5 (91.5)     | 44.0 (69.8)  | <b>0.0287</b>     |

Data were tested for normality using Shapiro-Wilk test and presented as either mean ± standard deviation or as median (interquartile range), with differences assessed by paired t test or Wilcoxon matched pair signed-rank test as appropriate. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CAP, controlled attenuation parameter; GGT, gamma glutamyl transferase; HbA1c, haemoglobin A1c; FAST score, Fibroscan-AST score, FIB-4, Fibrosis-4 index, WC, waist circumference. aWC control group n=8 only.

Liver enzymes were more variably decreased across groups. Median ALT values (T0: 45.0 (70.0) versus T1: 32.0 (32.0) IU/L; P=0.0054) and GGT (T0: 61.0 (61.0) versus T1: 38.0 (45.0) IU/L; P<0.0001) were significantly decreased in DI participants; changes in median AST levels were not significant (P=0.0777). Only GGT levels decreased significantly in EI participants (T0: 57.5 (91.5) versus T1: 44.0 (69.8); P=0.0287). Although unexpected decreases in ALT and AST levels in the CG were observed between T0 and T1 (*Table 4-2*), this was driven by a single participant who had very high ALT and AST levels at T0 assessment that had decreased by >75% by T1 (*see Figure 4-2*) as a result of the patient's self-motivation to follow the standard of care guidance to lose weight. There were no significant differences in the FIB4 index in the intervention groups, however a significant reduction was seen in the control group (T0: 1.57 (0.72) versus T1: 1.30 (0.61), p =0.0284. Significant reductions in the FAST score were

observed in the control (T0:  $0.68 \pm 0.22$  versus T1:  $0.57 \pm 0.23$ ,  $p = 0.0399$ ) and DI groups (T0:  $0.554 \pm 0.23$  versus T1:  $0.38 \pm 0.25$ ,  $p = 0.0042$ ) but not in the EI group.

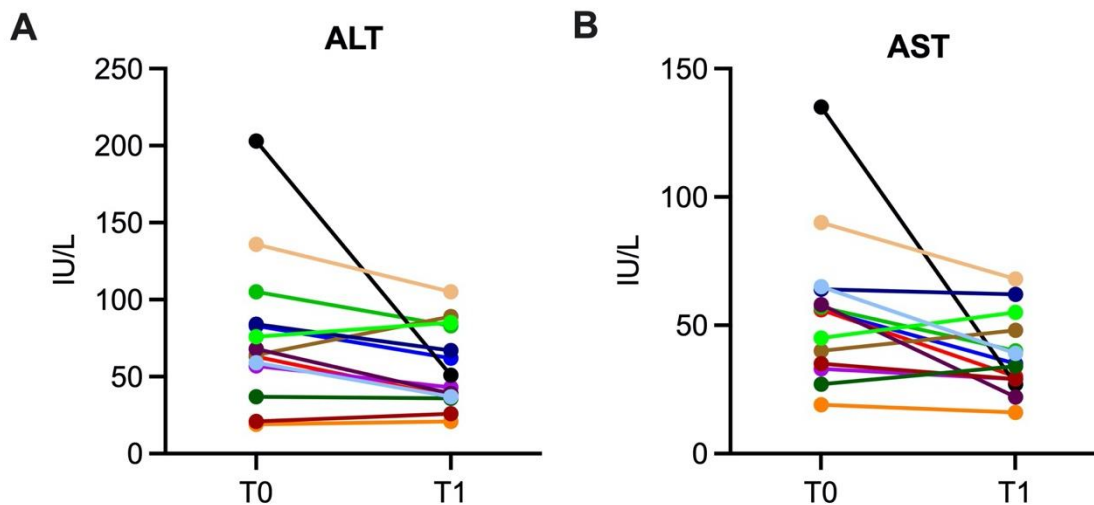


Figure 4-2 Individual liver enzyme changes in control group participants. (A) ALT and (B) AST levels at baseline assessment (T0) and after 12 weeks of standard of care (T1).

Adherence to both the DI (87%) and EI (93%) was high. Significant reductions in energy intakes ( $P=0.0045$ ) and improvements in dietary quality ( $P<0.000001$ ) were observed post intervention in DI, but not EI, participants (Supplementary Figure S2). Importantly, reductions in energy intakes in the DI group were driven by significant decreases in total intakes (g/day) of saturated fat ( $P=0.0260$ ), carbohydrates ( $P=0.0001$ ) and total sugars ( $P=0.0024$ ) (table 4-3). In contrast, there were no changes in dietary quality, or energy or macronutrient intakes in the CG or EI participants (Figures 4-3 and Table 4-3). EI participants significantly improved their cardiorespiratory fitness in response to intervention, as measured by estimated  $\dot{V}O_{2\max}$  during a submaximal cardiopulmonary exercise test. There were no changes to  $\dot{V}O_{2\max}$  observed in the CG, and DI participants did not undergo cardiorespiratory fitness testing.

Table 4-3 Energy and macronutrient intakes calculated from paired 4-day diet diaries

|                                  | Control (n=7)  |                |         | Diet (n=13)    |                |               | Exercise (n=10) |                |         |
|----------------------------------|----------------|----------------|---------|----------------|----------------|---------------|-----------------|----------------|---------|
| Intakes <sup>1</sup>             | T0             | T1             | P value | T0             | T1             | P value       | T0              | T1             | P value |
| Energy (kcal/d)                  | 1869.6 ± 640.8 | 1667.9 ± 692.2 | 0.4342  | 1772.5 ± 479.1 | 1241.7 ± 509.3 | <b>0.0045</b> | 1668.0 ± 440.3  | 1605.0 ± 350.9 | 0.5907  |
| Protein (g/d)                    | 88.3 ± 17.8    | 77.7 ± 23.6    | 0.1563  | 71.8 ± 16.5    | 73.7 ± 21.6    | 0.7501        | 77.4 ± 24.0     | 72.7 ± 18.2    | 0.3579  |
| Protein (%TE)                    | 19.9 ± 4.5     | 19.3 ± 3.3     | 0.5504  | 16.6 ± 2.5     | 24.9 ± 5.4     | <b>0.0003</b> | 18.6 ± 3.5      | 19.3 ± 3.4     | 0.8272  |
| Fat (g/d)                        | 73.3 ± 36.0    | 65.7 ± 43.3    | 0.6250  | 71.1 ± 28.9    | 54.0 ± 27.9    | 0.0803        | 64.1 ± 26.7     | 66.1 ± 19.1    | 0.7648  |
| Fat (%TE)                        | 33.8 ± 8.3     | 33.2 ± 10.0    | 0.8826  | 35.6 ± 8.0     | 38.4 ± 9.6     | 0.3241        | 33.6 ± 7.6      | 36.9 ± 6.5     | 0.2382  |
| SFA (g/day)                      | 26.8 ± 15.9    | 21.6 ± 15.8    | 0.3925  | 24.9 ± 11.9    | 16.5 ± 6.8     | <b>0.0260</b> | 24.4 ± 13.3     | 24.3 ± 9.4     | 0.9636  |
| SFA (%TE)                        | 11.9 ± 4.1     | 10.6 ± 3.6     | 0.3882  | 12.5 ± 4.3     | 12.4 ± 4.4     | 0.9619        | 12.5 ± 4.1      | 13.4 ± 3.2     | 0.4474  |
| Carbohydrate (g/d)               | 213.0 ± 80.0   | 192.7 ± 77.1   | 0.5056  | 184.1 ± 40.1   | 108.9 ± 48.4   | <b>0.0001</b> | 197.2 ± 54.6    | 180.2 ± 45.9   | 0.4045  |
| Carbohydrate (%TE)               | 46.3 ± 9.9     | 47.2 ± 12.0    | 0.8082  | 42.9 ± 9.2     | 35.5 ± 9.6     | <b>0.0475</b> | 47.9 ± 7.4      | 44.9 ± 5.0     | 0.3037  |
| Total sugars (g/d)               | 83.0 ± 42.5    | 61.7 ± 39.5    | 0.2448  | 74.8 ± 30.5    | 41.8 ± 23.9    | <b>0.0024</b> | 80.8 ± 44.0     | 69.8 ± 32.7    | 0.3343  |
| Total sugars (%TE)               | 17.5 ± 5.4     | 14.8 ± 6.5     | 0.3020  | 17.1 ± 5.6     | 13.4 ± 5.3     | 0.0847        | 18.7 ± 6.4      | 17.0 ± 5.4     | 0.3393  |
| Dietary fibre <sup>2</sup> (g/d) | 5.2 ± 4.0      | 7.3 ± 2.8      | 0.1643  | 8.7 ± 5.2      | 7.9 ± 7.3      | 0.4533        | 8.0 ± 3.3       | 7.5 ± 3.4      | 0.5899  |
| Sodium (g/d)                     | 3.1 ± 1.2      | 2.5 ± 1.4      | 0.2099  | 2.4 ± 0.6      | 2.3 ± 0.8      | 0.6770        | 2.6 ± 1.0       | 2.5 ± 0.88     | 0.6441  |

<sup>1</sup>Data were tested for normality using Shapiro-Wilk test and presented as mean ± standard deviation with differences assessed by paired t test. <sup>2</sup>AOAC method. Abbreviations: AOAC, American Association of Analytical Chemists; SFA, saturated fatty acids; TE, total energy.

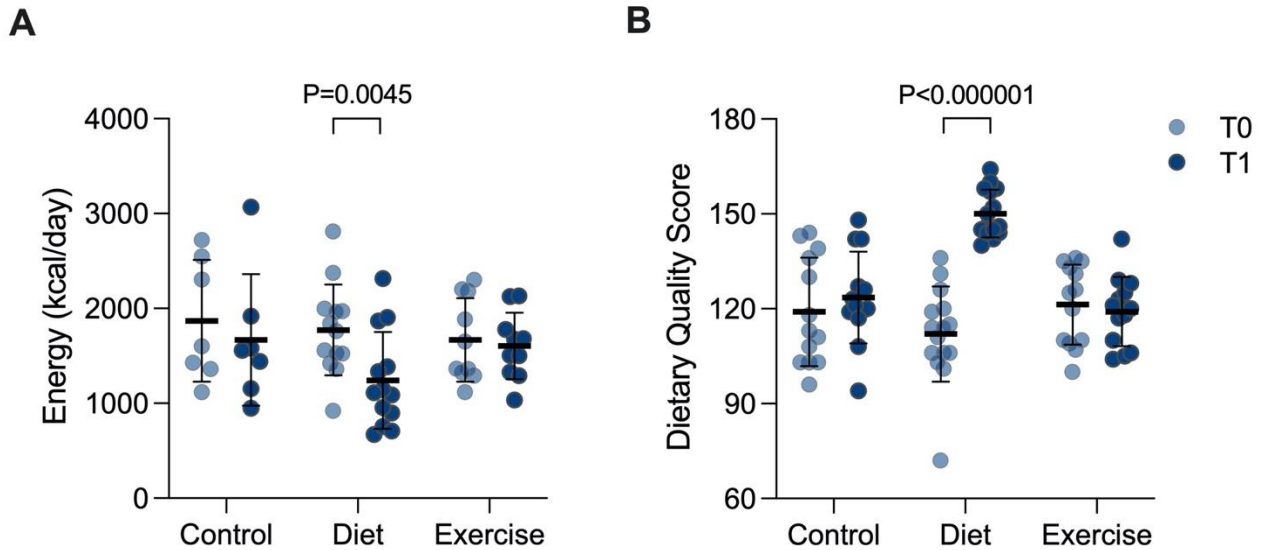


Figure 4-3 Energy intakes and dietary quality before and after diet or exercise intervention.

(A) Average daily energy intakes calculated from detailed four-day diet diaries from control (n=7), nutrition (n=13) and exercise (n=10) group participants who completed diaries both before (T0) and after (T1) 12 weeks intervention. (B) Dietary quality assessed through food frequency questionnaire interviews given to control (n=13), diet (n=15) and exercise (n=13) group participants before and after intervention. Data were assessed for normality and analyzed by multiple paired t tests. Solid black line and whiskers show mean and standard deviation.

#### 4.5.2 Differential improvement in histological outcomes between diet and exercise

At baseline 12/15 (80%) of DI and 13/16 (81%) of EI participants had NASH. Participants with simple steatosis at T0 did not have repeat biopsies at T1 and therefore were excluded from paired histological analyses. In addition, a participant who started methotrexate during the EI intervention was also excluded, resulting in n=12 paired biopsies for examination of histological changes post-intervention for both the DI and EI groups (Figure 4-1).

For each component of NAS and fibrosis grading, the proportion of patients who were stable, improved or worsened was examined (Fig. 4-4) in addition to individual participant changes (Figure 5). EI participants showed significant improvements in Brunt fibrosis stage (58.3% improved, P=0.0352; Fig. 4-4A, 4-5A) and hepatocyte ballooning (66.7% improved, P=0.0195; Fig. 4-4B, 4-5B). 16.7% of EI participants showed an increase in steatosis grade. In contrast, significant improvements in steatosis (66.7% improved, P=0.0039; Fig. 4-2C, 4-5C) and NAS (66.7% improved, P=0.0098; Fig. 4-2D, 4-5D) were observed in the DI group. This differential effect in steatosis grade was associated with changes in dietary quality, particularly

correlating with a reduction in sugar intake ( $r = 0.7534$ ,  $p = 0.0093$ ). Figure 3-4 and Table 4-2 highlight the differential effects of the interventions on dietary quality and macronutrient intakes. There were no significant changes in lobular inflammation after 12 weeks of either DI (*Fig. 4-4E, 4-5E*) or EI (*Fig. 4-4F, 4-5F*).

#### *4.5.3 Alterations in circulating and intrahepatic MAIT cell populations*

Circulating MAIT cells were defined as the  $CD3^+ V\alpha 7.2^{Hi} CD161^{Hi}$  population of  $CD45^+$  lymphocytes, with doublet exclusion part of the gating strategy (*Fig. 4-6A*). Representative histograms of distribution of the CD69 and CD95 surface marker expression in circulating MAIT cells are shown in Fig 4-8. Approximately 1% of the circulating  $CD3^+$  lymphogate were  $V\alpha 7.2^{Hi} CD161^{Hi}$  (*Fig. 4-6B*). There was a trend towards reduced percentage of circulating MAIT cells with advanced NAFLD fibrosis (Brunt stage  $\leq 2$ : 1.260 (1.475) versus Brunt  $> 2$ : 0.960 (0.505) though this did not achieve statistical significance,  $p=0.0829$ . Expression (median fluorescence intensity, MFI) of the acute activation marker CD69 was, in general, quite low in circulating MAIT cells, and DI participants showed decreased CD69 expression (T0: 104 (134) versus T1 27 (114);  $P=0.0353$ ), albeit with notable variance in expression. In contrast, expression of CD95, the Fas death receptor, was at least 10-fold higher than CD69, and significantly increased 1.65-fold in response to EI (T0: 1549 (888) versus T1: 2563 (1371) MFI,  $P=0.0043$ ; Figure 6C).

Intrahepatic MAIT cells were similarly gated (*Fig. 4-7A*), and were notably more abundant in the liver, representing ~5-7% of the  $CD3^+$  population (*Fig. 4-7B*), than in the circulation. Although CD69 was much more robustly expressed in intrahepatic MAIT cells than in circulating MAIT cells (*Fig. 4-7C*), there were no differences observed at T1 for either intervention (*Fig. 4-7C*). However, the percentage of intrahepatic MAIT cells significantly decreased in response to EI (T0: 11.1 [14.4] versus T1: 5.3 [9.3] %,  $p=0.0029$ ; *Fig. 4-7B*). Moreover, this occurred alongside a significant increase in detected CD95 expression in response to EI (T0: 2724 [862] versus T1: 3117 [1622] MFI,  $P=0.0269$ ; *Fig. 4-7D*).

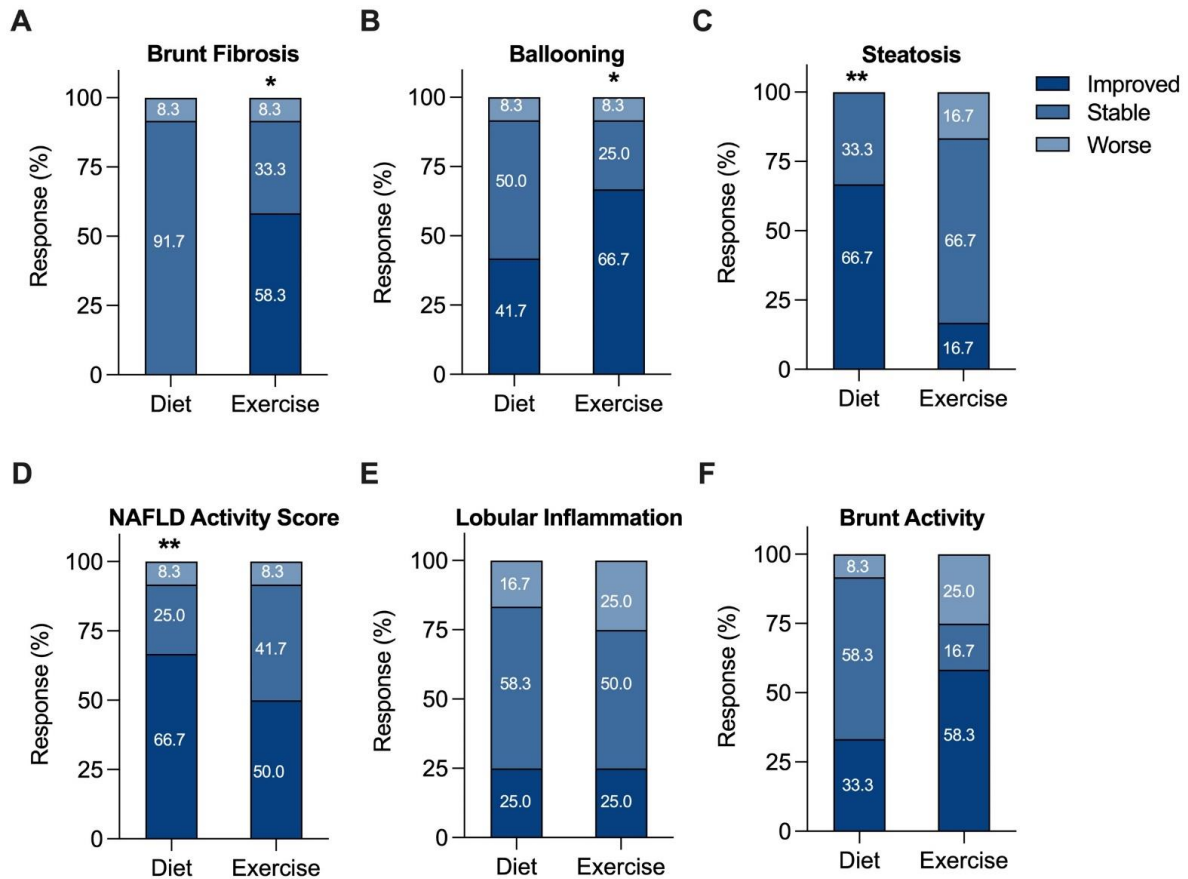


Figure 4-4 Changes in histologic categories (improved, stable, or worse) after a 12-week diet or exercise intervention.

Any increase or decrease in score was considered a change in category. (A) Fibrosis, (B) Ballooning, (C) Steatosis, (D) NAFLD Activity, (E) Lobular inflammation, and (F) Brunt Activity Scores. The percent of patients in each category are shown numerically within each category of the bar graph. \* $P < 0.05$ , \*\* $P < 0.01$  from baseline, Wilcoxon matched-pairs signed rank test.

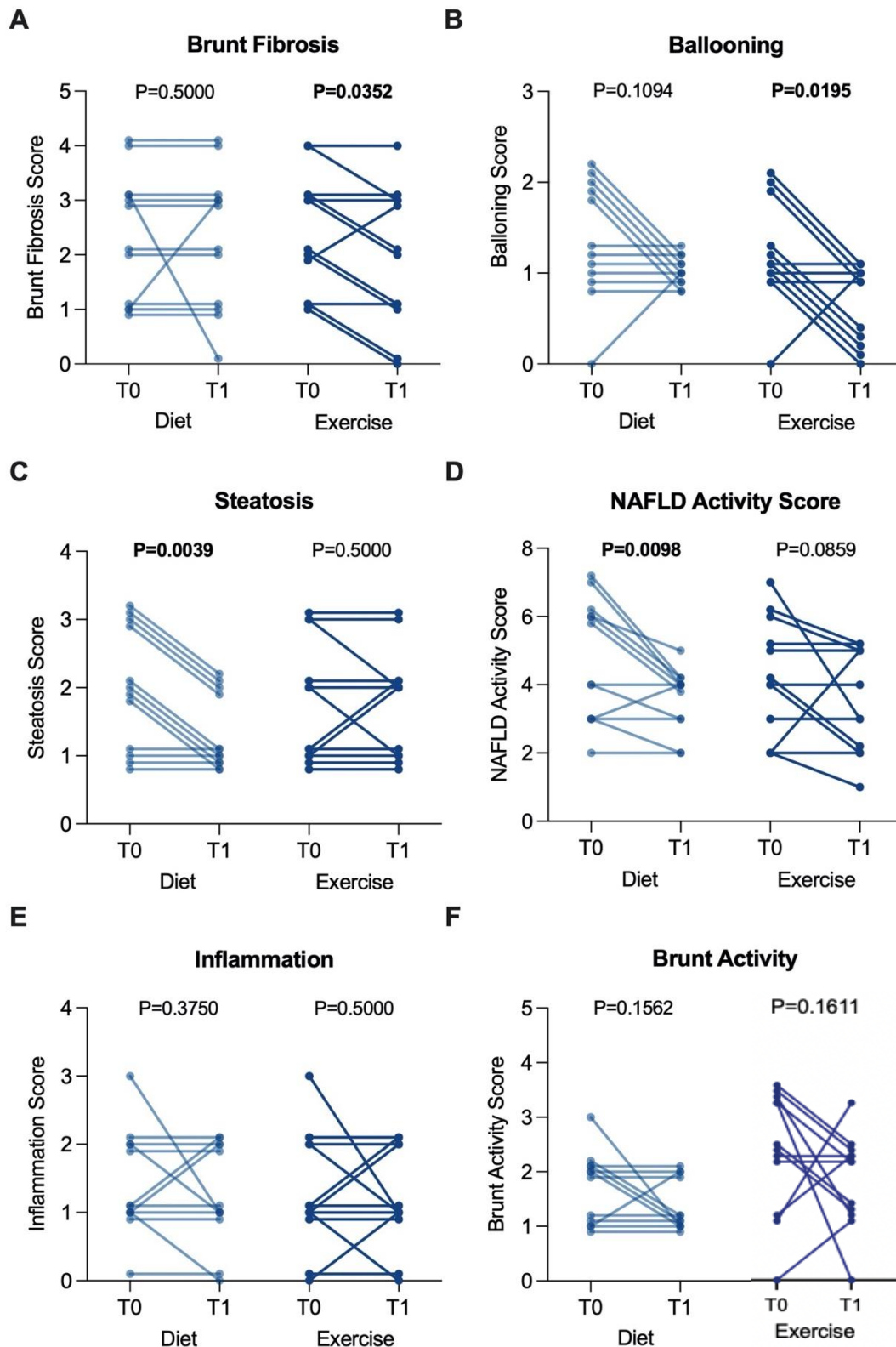


Figure 4-5 Individual histological changes in response to 12 weeks diet or exercise intervention. (A) Fibrosis, (B) Ballooning, (C) Steatosis, (D) NAFLD Activity Score, (E) Lobular inflammation, and (F) Brunt Activity Score. T0, baseline assessment; T1, post intervention assessment.

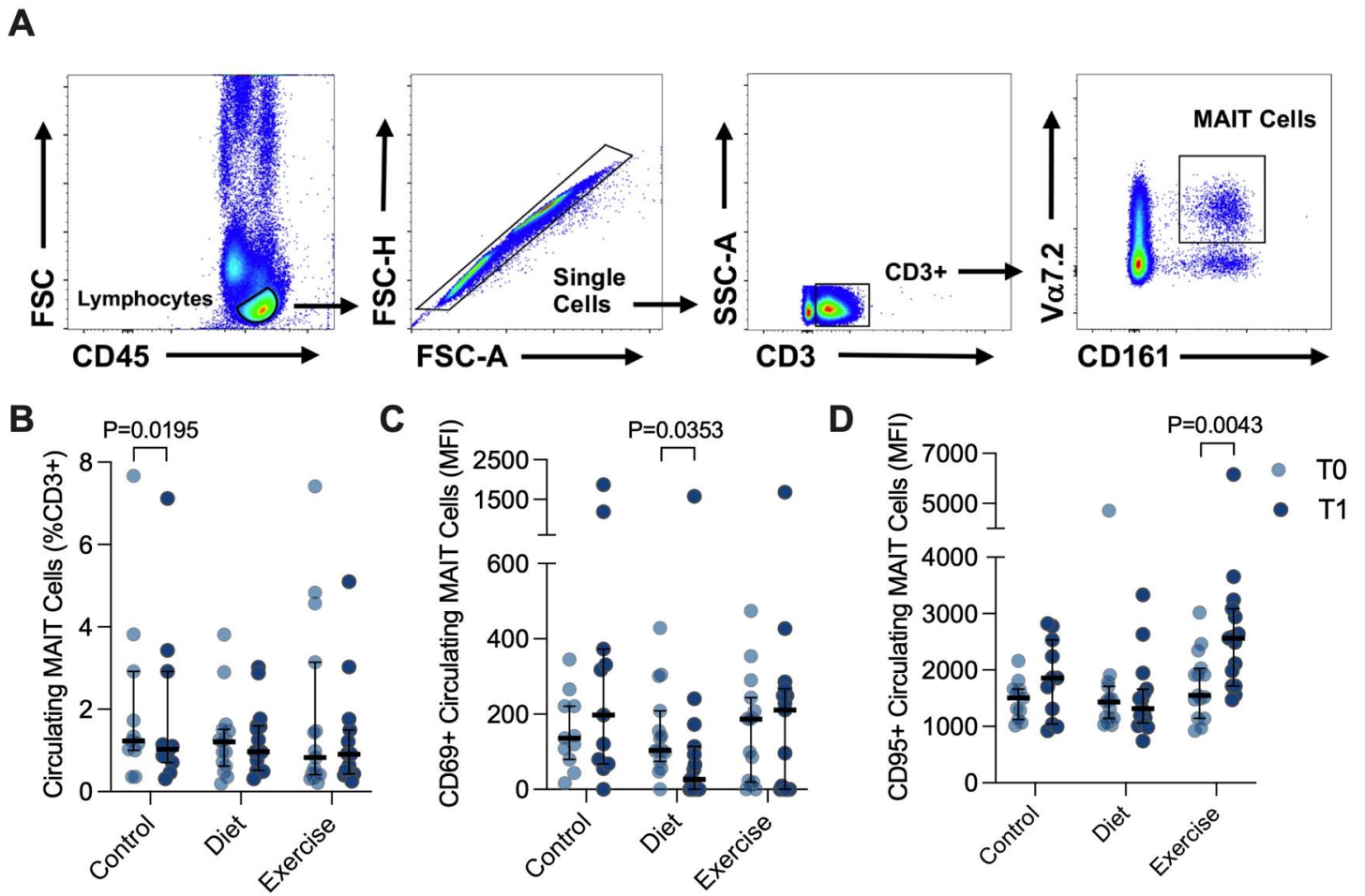


Figure 4-6 Circulating MAIT cells before (T0) and after (T1) diet or exercise intervention.

(A) Representative flow cytometry dot plots showing gating strategy for defining circulating CD3<sup>+</sup> Vα7.2<sup>hi</sup> CD161<sup>hi</sup> MAIT cells. (B) Circulating MAIT cells in control participant samples were lost during processing (n=, and participants completing either 12 weeks of dietary (n=15) or exercise (n=15) intervention. (C) CD69 and (D) CD95 expression by circulating MAIT cells before and after intervention. Data was analyzed via Wilcoxon matched-pairs signed rank test, and individual values and median  $\square$  interquartile range are depicted. MFI: median fluorescence intensity.

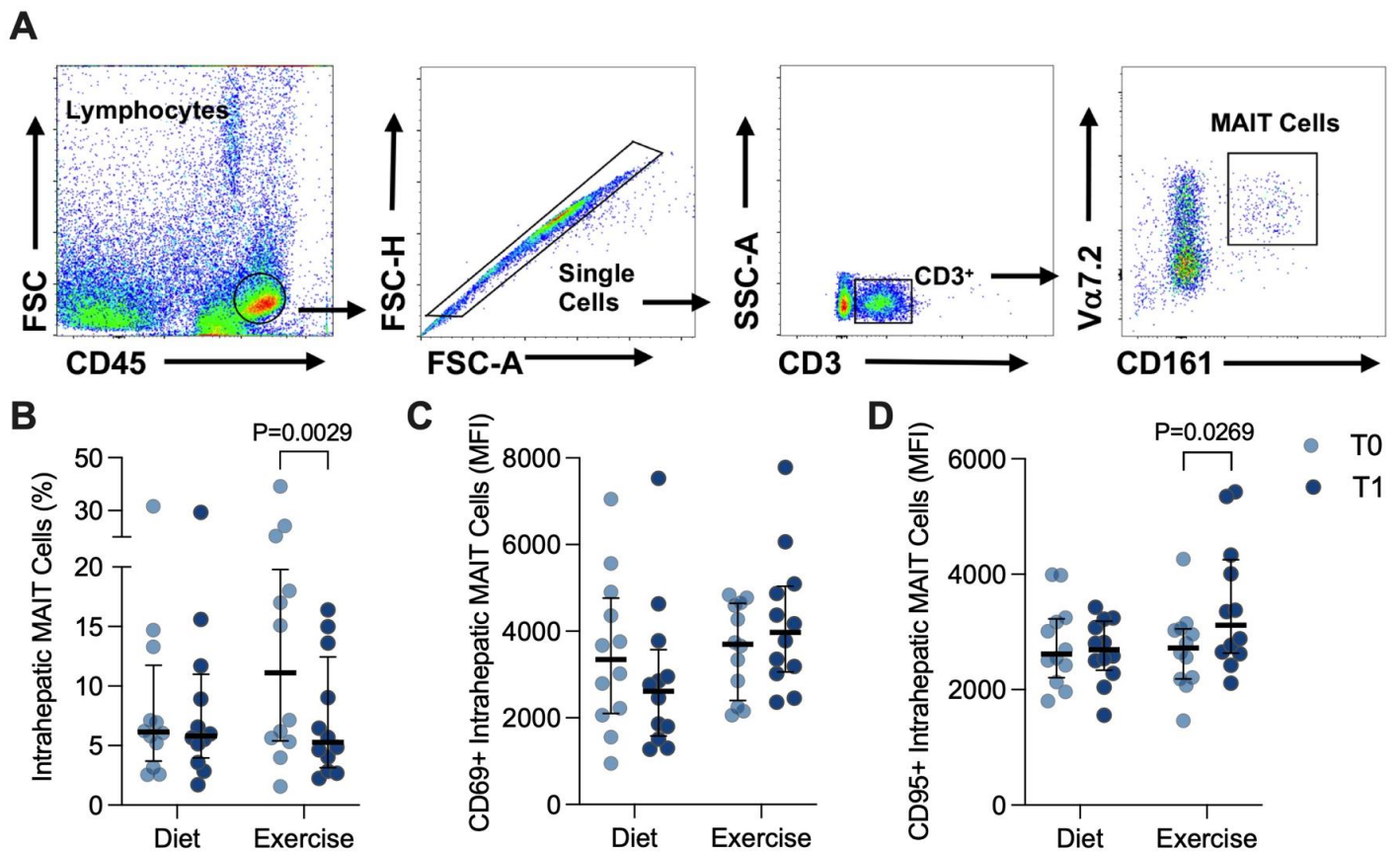


Figure 4-7 Intrahepatic MAIT cells before (T0) and after (T1) diet or exercise intervention.

(A) Representative flow cytometry dot plots showing gating strategy for defining intrahepatic CD3<sup>+</sup> V $\alpha$ 7.2<sup>hi</sup> CD161<sup>hi</sup> MAIT cells. (B) Intrahepatic MAIT cells in participants completing either 12 weeks of diet (n=12) or exercise (n=12) intervention. (C) CD69 and (D) CD95 expressed by circulating MAIT cells before and after intervention. Data were analyzed via Wilcoxon matched-pairs signed rank test and individual values, and median  $\pm$  interquartile.

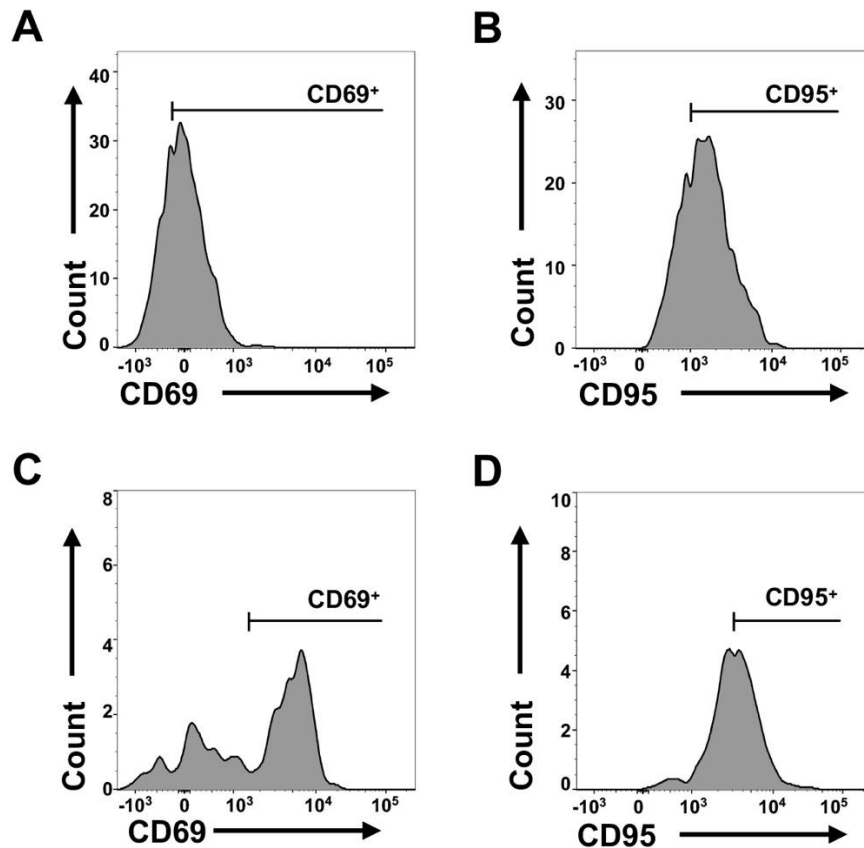


Figure 4-8 CD69 and CD95 expression in MAIT cells.

Representative histograms of distribution of surface marker expression on CD3<sup>+</sup> CD161<sup>hi</sup> V $\alpha$ 7.2<sup>+</sup> MAIT cells in peripheral blood (A) and (B); and collagenase-digested liver tissues (C) and (D). Expression calculated from median fluorescent intensity (MFI) of gated regions.

#### 4. 6 Discussion

These results demonstrate that a 12-week aerobic exercise program results in a significant reduction in the percentage of intra-hepatic MAIT cells, with increased activation marker and apoptotic marker expression in association with reductions in fibrosis and ballooning stage in patients with NAFLD. By contrast, a 12-week nutritional weight loss program results in reductions in hepatic steatosis grade alone, with no significant changes amongst intrahepatic MAIT cells. The increased metabolic demand with aerobic exercise may stimulate the activation pro-fibrogenic cell population, which may make them more vulnerable to accelerated apoptosis. Accelerated apoptosis of MAIT cells in turn may promote fibrosis regression and resolution of NASH.

To our knowledge, this is the first study to investigate circulating and intrahepatic MAIT cell populations in the context of metabolic and histological changes in patients with NAFLD who

completed either a dietary or aerobic exercise intervention. While both DI and EI participants demonstrated improvements in clinical and transient elastography parameters of NAFLD, weight reductions were more marked in DI participants, who also exhibited significant improvements in histological steatosis grade and reduced expression of the acute activation marker CD69 on circulating MAIT cells. Interestingly, in the EI participants, significant improvements were noted in fibrosis stage and hepatocyte ballooning were observed in association with increased apoptotic marker CD95 expression in association with significant reduction in the percentage of intrahepatic MAIT cells.

Circulating MAIT cells are significantly reduced in adults with obesity and/or type 2 diabetes, as well as children with obesity [81, 109, 116]. Furthermore, circulating and adipose-residing MAIT cells exhibit an activated with a pro-inflammatory (Th17) phenotype, with reduced IFN $\gamma$ , but increased TNF $\alpha$  and IL-17 production. Studies conducted amongst obese patients undergoing bariatric surgery show changes in the lymphocyte population, including MAIT cells, following weight loss from pro-inflammatory to anti-inflammatory phenotypes [272]. These shifts include decreased expression of the early activation marker CD69 [273, 274], with increases in the percentage of circulating MAIT cell post bariatric surgery [81, 116]. However, pro-inflammatory cytokine levels do not revert to those of healthy controls following weight loss [272]. In a study of bariatric surgery patients, IL-17 levels were found to be persistently elevated, despite serum levels of IL-2 and granzyme B normalising to the level of healthy controls at 6 and even 12-months following surgery [116]. However, no studies to date have examined changes in MAIT cell populations in response to weight loss from dietary or exercise interventions or have investigated for these changes in patients with NAFLD. This study is, we believe, the first to report these findings.

In our study, low levels of circulating MAIT cells as percent of all CD3<sup>+</sup> lymphocytes were observed both pre- and post- intervention, similar to data from MAIT cell analyses in participants with obesity [109, 116]. Although we did not observe changes in the total percentage of circulating MAIT cells in response to either DI or EI, there was a reduction in the expression of the early activation marker CD69 by circulating MAIT cells in participants who had completed the 12-week DI. This supports data from earlier studies suggesting weight loss reduces lymphocyte activation [273, 274]. While EI participants did decrease their

bodyweight, the reductions were not as pronounced as those in the DI group and no changes in CD69 expression in MAIT cells were observed in response to EI. It is possible that a longer, sustained period of weight loss may be needed to promote significant changes in the percentage of circulating MAIT cells.

Our results demonstrated an increase in the expression of the apoptotic marker CD95, otherwise known as the Fas death receptor, in both circulating and intrahepatic MAIT cells after EI. While stimulation of MAIT cells promotes expression of CD69, excessive chronic activation promotes CD95 expression on surface membranes, the resultant binding of the Fas ligand to the receptor in turn activates caspases and cell apoptosis [275]. Interestingly, the increased expression of CD95 in intrahepatic MAIT cells after EI was observed in conjunction with a reduction in the percentage of intrahepatic MAIT cells, with significant improvements in liver fibrosis and hepatocyte ballooning. In contrast, despite losing more weight, the DI participants had improvements in steatosis score alone; there were no measurable changes in the severity of histological inflammation or fibrosis grade.

Very few studies to date have examined circulating MAIT cells in response to exercise. Circulating MAIT cells have been shown to increase transiently in response to both acute graded exercise test to volitional exhaustion [276], and a 40-minute submaximal test,[277] with MAIT cell numbers reverting to pre-exercise levels within an hour [277], more recently, a study conducted by Bates et al reported restoration of circulating MAIT cells in overweight and obese women after a 12 week aerobic exercise intervention[278]. Additionally, exercise training in breast cancer survivors improved responsiveness of MAIT cells after 45 minutes of intermittent cycling, although not to the level observed in healthy age matched controls [279]. Both acute and chronic exercise programs have been shown to affect the immune system [280, 281]. Although more intensive exercise programs have been shown to have more pro-inflammatory effects, in contrast moderate exercise training programs have been shown to exert anti-inflammatory effects in individuals with cardiometabolic disease and cancer prevention [187].

#### *4.7 Limitations:*

This study had several limitations: (i) the small sample size (n=44) completing the study, (ii) participants were allocated to the intervention groups based on preference and were not randomised, (iii) paired liver biopsies were not performed in the control group; however, identical techniques were used to assess the changes in liver histology with the nutritional and exercise interventions which yielded significantly different results, (iv) although dietary assessments were made pre- and post-exercise intervention, changes in physical activity levels in the nutritional intervention group were not assessed (v). This study focused primarily on circulating and intra-hepatic MAIT cells and did not assess for concomitant changes in other immune cell populations which may also contribute to the histological changes observed. This descriptive study focused on MAIT cell percentages and cell surface marker expression; no measurements were made to assess for changes in MAIT cell metabolism.

#### *4.8 Conclusions*

MAIT knockout mice are protected from fibrosis in CCL4 models of NAFLD, supporting the hypothesis that the observed increase in CD95 and decreased frequency of intrahepatic MAIT cells may explain the histological improvements in fibrosis and hepatocyte ballooning that we observed in the EI participants. However, whether intrahepatic MAIT cells play a pathogenic or protective role in NAFLD remains to be determined and the results of this pilot study will need be extended to enable generalisation of the findings to a larger population. Although MAIT cells have been demonstrated to promote inflammation in both adipose and the gut of high fat fed mice [110], MAIT knock-out mice have demonstrated more severe hepatic steatosis and inflammation when fed a methionine choline deficient diet, suggesting MAIT cells could also have a protective role in NASH [130]. Recent reports have suggested a liver MAIT cell-derived tissue repair gene signature, which can be induced via TCR mediated activation [137]. In summary, further studies examining the function of MAIT cells in patients with NAFLD in response to diet and exercise alone and combined interventions are needed, to further clarify the role of MAIT cells in the pathogenesis of NAFLD [282].

#### *4.9 Study findings*

- A 12-week nutritional based intervention resulted in greater weight loss in comparison to an aerobic-exercise based intervention and led to significant reductions in the degree of hepatic steatosis on repeat biopsy with no reductions in activation marker, CD69 expression on circulating MAIT cells
- Despite reduced degree of weight loss, a 12-week aerobic exercise intervention resulted in significant improvements in histological features of NASH and fibrosis, this was associated with intrahepatic MAIT cell depletion with increased terminal marker expression

## Chapter 5: Frailty in NAFLD

## 5 Frailty in NAFLD

### *5.1 Introduction*

Frailty is a term used to define a clinical state of decline due to reduced physiological reserve across several organ systems which results in increased vulnerability to health stressors[283, 284]. The term has been used to distinguish older people who were at increased risk of poor health outcomes including falls, fractures, disability, poor quality of life, hospitalisation and mortality [284]. Although an age-associated phenomenon, the pathophysiology is distinct from ageing[143]. The phenotype is associated with features of physical frailty combined with reduced functional and cognitive capacity [284, 285]. Physical frailty assessments incorporate sarcopaenia and are centred around measures of muscle mass, strength and function [286, 287].

Whilst the term frailty encompasses a broad range of deficits, physical frailty measures have been used most frequently when assessing individuals with chronic liver disease, especially when assessing risk of adverse outcomes in the setting of liver transplantation[288-293]. Studies conducted amongst patients with advanced liver disease, demonstrate a prevalence of frailty ranging from 17% to 68%, with frailty being an independent risk factor for decompensation [288-294]. As a result, frailty has been identified as a useful predictor of clinical outcomes in patients with established cirrhosis and those with decompensated liver disease requiring liver transplantation, such that it has been recommended that frailty assessments are incorporated in clinical practice [295]. Although the development of advanced liver disease in the majority of cases results from chronic hepatic inflammation, the associated metabolic risk factors in NAFLD may confer an increased risk of frailty and mortality in this cohort [70].

The prevalence of sarcopaenia ranges from 30% to 70% in individuals with established cirrhosis with higher rates seen in individuals with more advanced, decompensated chronic liver disease [296]. Importantly, sarcopenia may be an independent risk factor for NAFLD progression and is underrecognized in patients with NAFLD [297-300].

Despite the risk factors for frailty in NAFLD, very little data is available on its prevalence amongst individuals with early stage, non-cirrhotic disease. Whilst Wang et al demonstrated an increased prevalence of pre-frailty and frailty in individuals with non-cirrhotic chronic liver disease compared to adults without liver disease [301], the number of patients with NAFLD in the study was low and they did not analyse for differences in aetiology. An added challenge when assessing frailty in NAFLD is identifying which frailty assessments are best. There are over 46 different frailty measures available, including the Fried frailty index (FFI)[143], the self-reported frailty index (SRFI)[244] and the lab-based frailty index (FI-LAB)[245]. These indices have been shown to be independently predictive of adverse clinical outcomes including mortality, hospitalisation and self-reported health scores in elderly populations. However, as the majority of these indices were developed and validated in community-dwelling older adults without known liver disease, as such, their applicability in liver diseases and NAFLD is not known.

## *5.2 Hypothesis*

Given the relationship between metabolic disease, Inflammageing and the development of sarcopaenia, the hypothesis for the study is that there is an increased prevalence of frailty amongst individuals with non-cirrhotic NAFLD.

## *5.3 Specific aims*

- To assess the prevalence of frailty across the full spectrum of NAFLD disease severity.
- To stratify frailty scores on the basis of fibrosis, using a range of frailty assessment tools and whilst assessing their suitability in identifying the presence of prefrailty and frailty in individuals with non-cirrhotic NAFLD.

## *5.4 Methods*

Study methodology inclusive of patient recruitment, frailty measures and statistical analysis is as outlined in Chapter 2, section 2.7. One hundred and nine patients with NAFLD completed the study assessments. Baseline participant characteristics for the cohort are detailed in table 5-1. The mean age of participants in the study was  $56 \pm 12$  years, with a median BMI was  $32.3 \pm 9.4$  kg/m<sup>2</sup> and 50% (n=55) of participants were women. There was a high prevalence of

hypertension (46%, n=50), diabetes (53%, n=58) and hypercholesterolaemia (61%, n=66) in the cohort. As anticipated, severity of metabolic disease was associated with the severity of liver disease, with significantly higher rates of diabetes, hypertension and higher BMIs in individuals with advanced fibrosis. Similarly, higher AST, GGT, CRP and HbA1c levels were associated with increased NAFLD fibrosis stage. LSM, CAP, F-AST scores were significantly higher in individuals with more advanced NAFLD, as was the NLR.

*Table 5-1: NAFLD participant characteristics*

| Variable                     | All<br>(n=109) | F0–F1<br>(n=41) | F2–F3<br>(n=44) | F4<br>(n=24)   | Between-group p value |
|------------------------------|----------------|-----------------|-----------------|----------------|-----------------------|
| Age, years †                 | 56 (12)        | 53 (14)         | 58 (11)         | 57<br>(11)     | 0.150 ‡               |
| Gender, n (%)                |                |                 |                 |                | 0.187 §               |
| Female                       | 55 (50)        | 18 (44)         | 21 (48)         | 16<br>(67)     |                       |
| Male                         | 54 (50)        | 23 (56)         | 23 (52)         | 8 (33)         |                       |
| Smoking status, n (%)        |                |                 |                 |                | 0.440 §               |
| Non-smoker                   | 44 (41)        | 20 (49)         | 16 (37)         | 8 (35)         |                       |
| Former-smoker                | 51 (48)        | 16 (39)         | 21 (49)         | 14<br>(61)     |                       |
| Smoker                       | 12 (11)        | 5 (12)          | 6 (14)          | 1 (4)          |                       |
| T2DM, n (%n)                 | 58 (53)        | 14 (34)         | 26 (59)         | 18<br>(75)     | <b>0.004 **§</b>      |
| Hypercholesteremia,<br>n (%) | 66 (61)        | 23 (56)         | 28 (64)         | 15<br>(63)     | 0.758 §               |
| Hypertension, n (%)          | 50 (46)        | 10 (24)         | 25 (57)         | 15<br>(63)     | <b>0.002 **§</b>      |
| BMI (kg/m <sup>2</sup> ) ¶   | 32.3<br>(9.4)  | 30.9 (4.2)      | 35.0 (8.2)      | 34.1<br>(14.3) | <b>0.002 ***††</b>    |
| AST (IU/L) ¶                 | 28 (18)        | 23 (12)         | 28 (19)         | 32<br>(14)     | <b>0.004 ***††</b>    |
| ALT (IU/L) ¶                 | 39 (30)        | 36 (29)         | 44 (33)         | 41<br>(23)     | 0.074 ††              |
| ALP (IU/L) ¶                 | 81 (43)        | 75 (39)         | 80 (43)         | 93<br>(46)     | 0.169 ††              |
| GGT (IU/L) ¶                 | 51 (63)        | 41 (54)         | 51 (64)         | 74<br>(161)    | <b>0.017 *††</b>      |
| CRP (mg/L) ¶                 | 2.4 (3.3)      | 1.6 (2.3)       | 2.6 (3.0)       | 2.9<br>(7.1)   | <b>0.047 *††</b>      |
| NLR ¶                        | 1.9 (1.1)      | 1.9 (1.4)       | 1.7 (1.0)       | 2.3<br>(1.1)   | <b>0.013 *††</b>      |
| HbA1c<br>(mmol/mol) ¶        | 43 (17)        | 38 (10)         | 44 (17)         | 50<br>(20)     | <b>0.001 ***††</b>    |
| Plasma glucose<br>(mmol/L) ¶ | 6.0 (2.9)      | 5.6 (1.4)       | 6.5 (4.6)       | 6.2<br>(3.5)   | 0.050 ††              |
| Hepatic CAP (dB/m) ¶         | 319 (64)       | 302 (62)        | 329 (61)        | 340<br>(80)    | <b>0.007 ***††</b>    |
| Hepatic stiffness<br>(kPa) ¶ | 9.4 (6.3)      | 5.9 (2.1)       | 10.0 (2.3)      | 15.9<br>(7.7)  | <b>≤0.001 ****††</b>  |
| FAST score ¶                 | 0.36<br>(0.40) | 0.16<br>(0.28)  | 0.44<br>(0.30)  | 0.62<br>(0.21) | <b>≤0.001 ****††</b>  |

Patient demographics grouped by LSM

Significant between-group difference ( $p \leq 0.05$ ), \*\*Significant between-group difference ( $p \leq 0.01$ ), \*\*\*Significant between-group difference ( $p \leq 0.001$ ).

†Normally distributed variable (mean (SD)).

‡One-way analysis of variance.

§Pearson's  $\chi^2$  test.

¶non-normally distributed variable (median (IQR)).

††Kruskal Wallis test.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transferase; AST, aspartate transferase; BMI, body mass index; CAP, controlled attenuation parameter; CRP, C reactive protein; GGT, gamma-glutamyl transferase; HbA1c, glycated haemoglobin; LSM, liver stiffness measurement; NLR, neutrophil to lymphocyte ratio.

## 5.5 Results

### 5.5.1 Prevalence of frailty and pre-frailty across the NALFD disease spectrum

Within the cohort, the median SRFI score was 0.18 (IQR=0.18; *see table 5-2*) and the median FFI score was 1 (IQR=1; *see table 2*). The FFI classified 59% ( $n=48$ ) of the study cohort as prefrail and 5% ( $n=4$ ) as frail. The frequency of prefrailty and frailty was higher when using the SRFI, with 38% ( $n=41$ ) of the cohort classified as frail and a further 39% ( $n=42$ ) classified as prefrail (*table 5-2*). Only 36% ( $n=29$ ) of patients were classified as robust using the FFI and only 23% ( $n=25$ ) of patients were classified as robust using the SRFI. The median FI-LAB score in the full cohort was 0.18 (IQR=0.12; *see table 5-2*), the median TUG score was 7.0 (IQR=1.8; *see table 2*), and the median 30STST score was 14 (IQR=7; *see table 5-2*). Defined prefrail and frail cut-off values for the FI-LAB, TUG and 30STST scores were not available.

Table 5-2: Frailty outcomes grouped by liver stiffness measurement

| Variable              | All<br>(n=109) | F0–F1<br>(n=41) | F2–F3<br>(n=44) | F4<br>(n=24)   | Between-<br>group<br>p value |
|-----------------------|----------------|-----------------|-----------------|----------------|------------------------------|
| SRFI score †          | 0.18<br>(0.18) | 0.15<br>(0.15)  | 0.20<br>(0.19)  | 0.27<br>(0.20) | <b>0.001 **‡</b>             |
| SRFI categories       |                |                 |                 |                | <b>0.007 **§</b>             |
| Robust, n (%)         | 25 (23)        | 14 (35)         | 10 (23)         | 1 (4)          |                              |
| Pre-frail, n (%)      | 42 (39)        | 18 (45)         | 16 (36)         | 8 (33)         |                              |
| Frail, n (%)          | 41 (38)        | 8 (20)          | 18 (41)         | 15 (63)        |                              |
| FFI score †           | 1 (1)          | 1 (1)           | 1 (2)           | 1 (2)          | 0.285 ‡                      |
| FFI categories        |                |                 |                 |                | 0.810 §                      |
| Robust, n (%)         | 29 (36)        | 14 (41)         | 11 (36)         | 4 (25)         |                              |
| Pre-frail, n (%)      | 48 (59)        | 18 (53)         | 19 (61)         | 11 (69)        |                              |
| Frail, n (%)          | 4 (5)          | 2 (6)           | 1 (3)           | 1 (6)          |                              |
| FI-LAB score †        | 0.18<br>(0.12) | 0.14<br>(0.11)  | 0.21<br>(0.09)  | 0.24<br>(0.15) | <b>≤0.001 ***‡</b>           |
| Timed-up and go (s) † | 7.0<br>(1.8)   | 6.7 (1.8)       | 6.8 (1.7)       | 7.5<br>(1.9)   | 0.110 ‡                      |
| 30 s sit-to-stand †   | 14 (7)         | 16 (7)          | 14 (6)          | 11 (6)         | <b>0.006 **‡</b>             |

Post hoc analysis for SRFI: F0/F1 significantly different from F4 (adjusted p=0.001). Post hoc for LBFi: F0/F1 significantly different from F2/F3 (adjusted p=0.001) and F4 (adjusted p≤0.001). Post hoc for 30 s sit-to-stand: F0/F1 significantly different from F4 (adjusted p=0.004).

\*\*Significant between-group difference (p≤0.05), \*\*Significant between-group difference (p≤0.01),

\*\*\*Significant between-group difference (p≤0.001).

††Non-normally distributed variable (median (IQR))

‡‡Kruskal-Wallis test

§§Pearson's  $\chi^2$  test

Abbreviations: FFI, Fried frailty index; FI-LAB, lab-based frailty index; SRFI, self-reported frailty index.

### 5.5.2 Frailty is increased in patients with NAFLD with advanced liver fibrosis and cirrhosis

To determine if higher frailty scores were associated with severity of liver fibrosis and or cirrhosis, the cohort was stratified on the basis of liver stiffness measurement (LSM) using established cut-off values for NAFLD [302]. These LSM groupings included 41 patients with minimal or no fibrosis (F0/F1, <8.2 kPa); 44 patients with moderate to advanced fibrosis (F2/F3, 8.2–13.5 kPa) and 24 patients with established cirrhosis (F4, ≥13.6 kPa) (see table 5-1). There were no significant differences in age or gender between these groups, however increased frequencies of diabetes and hypertension were noted with significantly higher BMI,

HbA1c and liver enzymes in participants with more advanced liver disease (table 1), highlighting the known risk factors for disease progression.

When comparing the frailty assessments between F0/F1, F2/F3 and F4 patients, a significant difference was observed in the SRFI scores ( $p=0.001$ ; see *figure 5-1A and table 5-2*), FI-LAB scores ( $p\leq 0.001$ ; see *figure 5-1B and table 2*) and 30STST scores ( $p=0.006$ ; see *figure 5-1C and table 5-2*). There was no statistical difference between the LSM groupings for either the FFI scores ( $p=0.285$ ; see *figure 5-1D and table 2*) or the TUG scores ( $p=0.110$ ; see *figure 5-1E and table 5-2*). Post hoc analysis identified a significant increase in SRFI scores between F0/F1 patients and F4 patients (median 0.15 vs 0.27; adjusted  $p=0.001$ ).

There was a significant decrease in the 30STST scores between F0/F1 patients and F4 patients (median 16 vs 11; adjusted  $p=0.004$ ). The post hoc analysis also identified a significant increase in FI-LAB scores between both F0/F1 patients and F4 patients (median 0.14 vs 0.24; adjusted  $p<0.001$ ) as well as F0/F1 patients and F2/F3 patients (median 0.14 vs 0.21; adjusted  $p=0.001$ ).

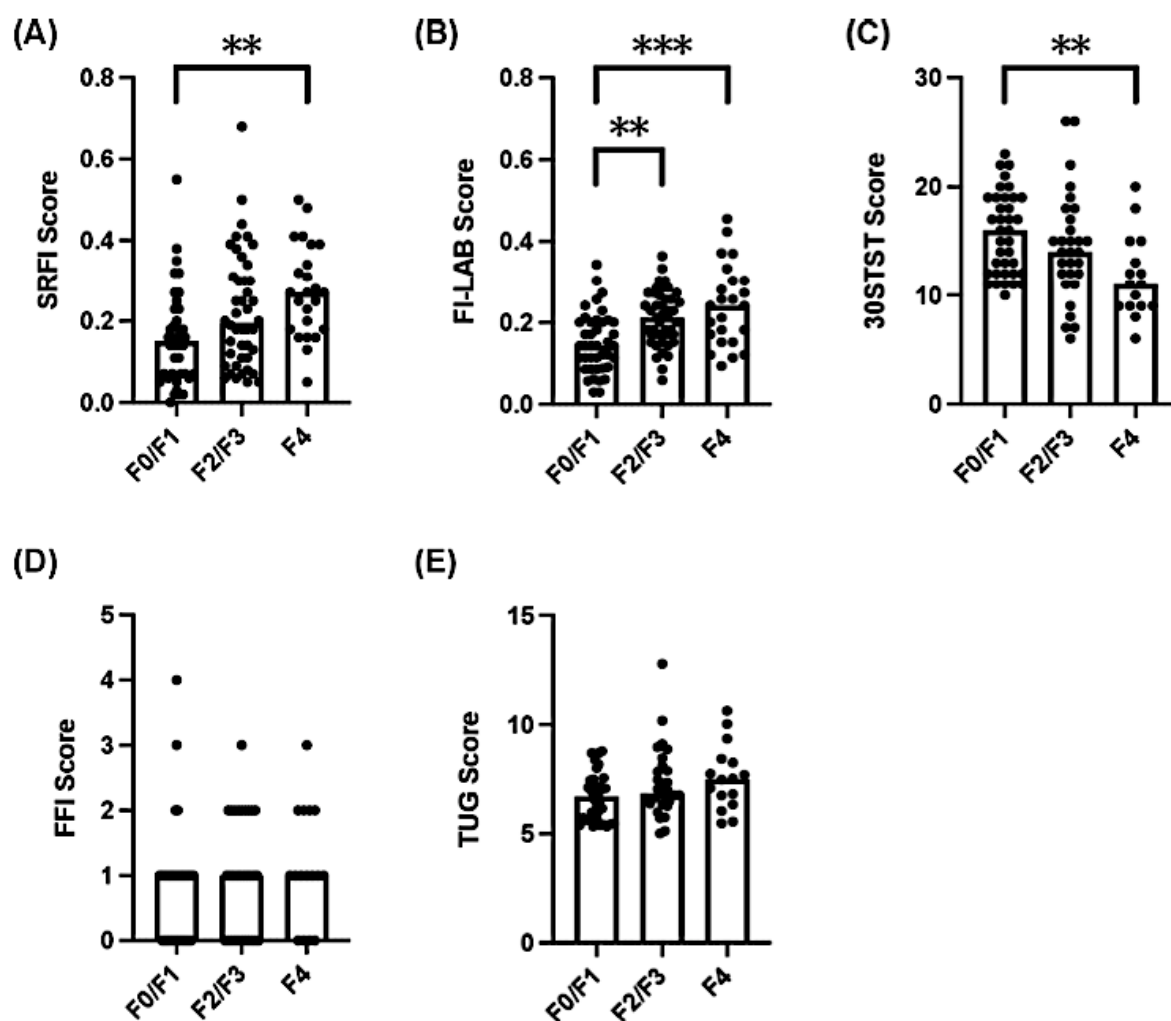


Figure 5-1 The frequency of frailty increases in patients with non-alcoholic fatty liver disease (NAFLD) with higher stages of liver fibrosis.

Participants were categorised into three groups based on liver stiffness measurement (LSM) cut-off values for NAFLD31: no/minimal fibrosis (F0/F1, <8.2 kPa); moderate/advanced fibrosis (F2/F3, 8.2–13.5 kPa) and cirrhosis (F4, ≥13.6 kPa). (A) Self-reported frailty index (SRFI) scores in patients classified as F0/F1, F2/F3 and F4. (B) Lab-based frailty index (FI-LAB) scores in patients classified as F0/F1, F2/F3 and F4. (C) 30 s sit-to-stand (30STST) scores in patients classified as F0/F1, F2/F3 and F4. (D) Fried frailty index (FFI) scores in patients classified as F0/F1, F2/F3 and F4. (E) Timed up and go (TUG) scores in patients classified as F0/F1, F2/F3 and F4. Between-group differences were assessed using a Kruskal-Wallis test and Mann-Whitney post-hoc analysis with Bonferroni correction. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

### 5.5.3 Female gender is associated with increased frailty scores in patients with NAFLD

In order to assess which clinical parameters were associated with elevated frailty scores within our cohort, we performed multivariate linear regression for the three instruments that demonstrated significant differences between F0/F1 and F4 groups (SRFI scores, FI-LAB scores and 30STST scores). Variables were selected on the basis of clinical relevance to disease progression and included CAP scores, LSM values, gender, smoking history, diabetes,

hypercholesterolaemia, hypertension, age and BMI. CAP score, gender and hypercholesterolaemia were each identified as statistically significant independent predictors of SRFI score (see table 5-3). Gender was the only statistically significant independent predictor of the FI-LAB score (see table 5-4), whilst gender and age were statistically significant independent predictors of the 30STST score (see table 5-4).

*Table 5-3: Multivariate linear regression predicting SRFI score*

| Variable                 | B (Standard Error) | T value | P value        |
|--------------------------|--------------------|---------|----------------|
| Constant                 | -0.26 (0.09)       | -2.877  | <b>0.005**</b> |
| CAP (dB/m)               | 0.001 (0)          | 2.969   | <b>0.004**</b> |
| LSM (kPa)                | 0 (0.002)          | 0.1     | 0.921          |
| Gender                   | 0.064 (0.02)       | 3.106   | <b>0.003**</b> |
| Smoking history          | -0.003 (0.016)     | -0.171  | 0.864          |
| Diabetes                 | 0.039 (0.021)      | 1.875   | 0.064          |
| Hypercholesterolaemia    | 0.051 (0.021)      | 2.372   | <b>0.02*</b>   |
| Hypertension             | 0.034 (0.022)      | 1.581   | 0.117          |
| Age (years)              | 0.002 (0.001)      | 1.98    | 0.051          |
| BMI (Kg/m <sup>2</sup> ) | 0.001 (0.002)      | 0.716   | 0.476          |

Dependent variable is SRFI score. Abbreviations: SRFI, self-reported frailty index, CAP, controlled attenuation parameter, LSM, liver stiffness measurement, BMI, body mass index, \*  $p < 0.05$ , \*\*  $p < 0.01$

Table 5-4: Multivariate linear regression predicting FI-LAB score

| Variable                 | B (Standard Error) | T value | P value        |
|--------------------------|--------------------|---------|----------------|
| Constant                 | -0.048 (0.072)     | 0.67    | 0.504          |
| CAP (dB/m)               | 0 (0)              | 0.721   | 0.472          |
| LSM (kPa)                | 0.002 (0.001)      | 1.662   | 0.1            |
| Gender                   | 0.046 (0.016)      | 2.835   | <b>0.006**</b> |
| Smoking history          | 0.005 (0.012)      | 0.436   | 0.664          |
| Diabetes                 | 0.03 (0.016)       | 1.791   | 0.077          |
| Hypercholesterolaemia    | 0.024 (0.017)      | 1.434   | 0.155          |
| Hypertension             | 0.019 (0.017)      | 1.119   | 0.266          |
| Age (years)              | 0 (0.001)          | 0.434   | 0.665          |
| BMI (Kg/m <sup>2</sup> ) | 0 (0.001)          | -0.129  | 0.897          |

Dependent variable is FI-LAB score. Abbreviations: FI-LAB, lab-based frailty index, CAP, controlled attenuation parameter, LSM, liver stiffness measurement, BMI, body mass index, \*\*  $p < 0.01$

Table 5-5: Multivariate linear regression predicting 30STST score

| Variable                 | B (Standard Error) | T value | P value         |
|--------------------------|--------------------|---------|-----------------|
| Constant                 | 26.353 (3.944)     | 6.681   | <b>0.000***</b> |
| CAP (dB/m)               | 0.009 (0.010)      | 0.916   | 0.363           |
| LSM (kPa)                | -0.080 (0.101)     | -0.788  | 0.433           |
| Gender                   | -2.578 (0.929)     | -2.777  | <b>0.007**</b>  |
| Smoking history          | -1.458 (0.732)     | -1.991  | 0.050           |
| Diabetes                 | -1.564 (0.991)     | -1.578  | 0.119           |
| Hypercholesterolaemia    | 0.182 (1.023)      | 0.177   | 0.860           |
| Hypertension             | 1.162 (0.971)      | 1.196   | 0.236           |
| Age (years)              | -0.111 (0.041)     | -2.708  | <b>0.008**</b>  |
| BMI (Kg/m <sup>2</sup> ) | -0.161 (0.086)     | -1.885  | 0.063           |

Dependent variable is 30STST score. Abbreviations: 30STS, 30 second sit to stand, CAP, controlled attenuation parameter, LSM, liver stiffness measurement, BMI, body mass index, \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

To further characterise this association with gender for the SRFI, the FI-LAB and the 30STST, we divided the full cohort on the basis of gender. There were significant differences in SRFI scores ( $p<0.01$ ; see figure 5-2A), FI-LAB scores ( $p<0.01$ ; see figure 5-2B) and 30STST scores ( $p<0.01$ ; see figure 5-2C) when comparing male and female patients. There were no differences in CAP scores, LSM values or age between the male and female patients (see figure 2D–F). The SRFI scores and FI-LAB scores were both higher in women (see figure 5-2A, B), whilst the 30STST score was lower in women (see figure 5-2C), indicating a significantly higher prevalence of frailty in female patients with NAFLD.

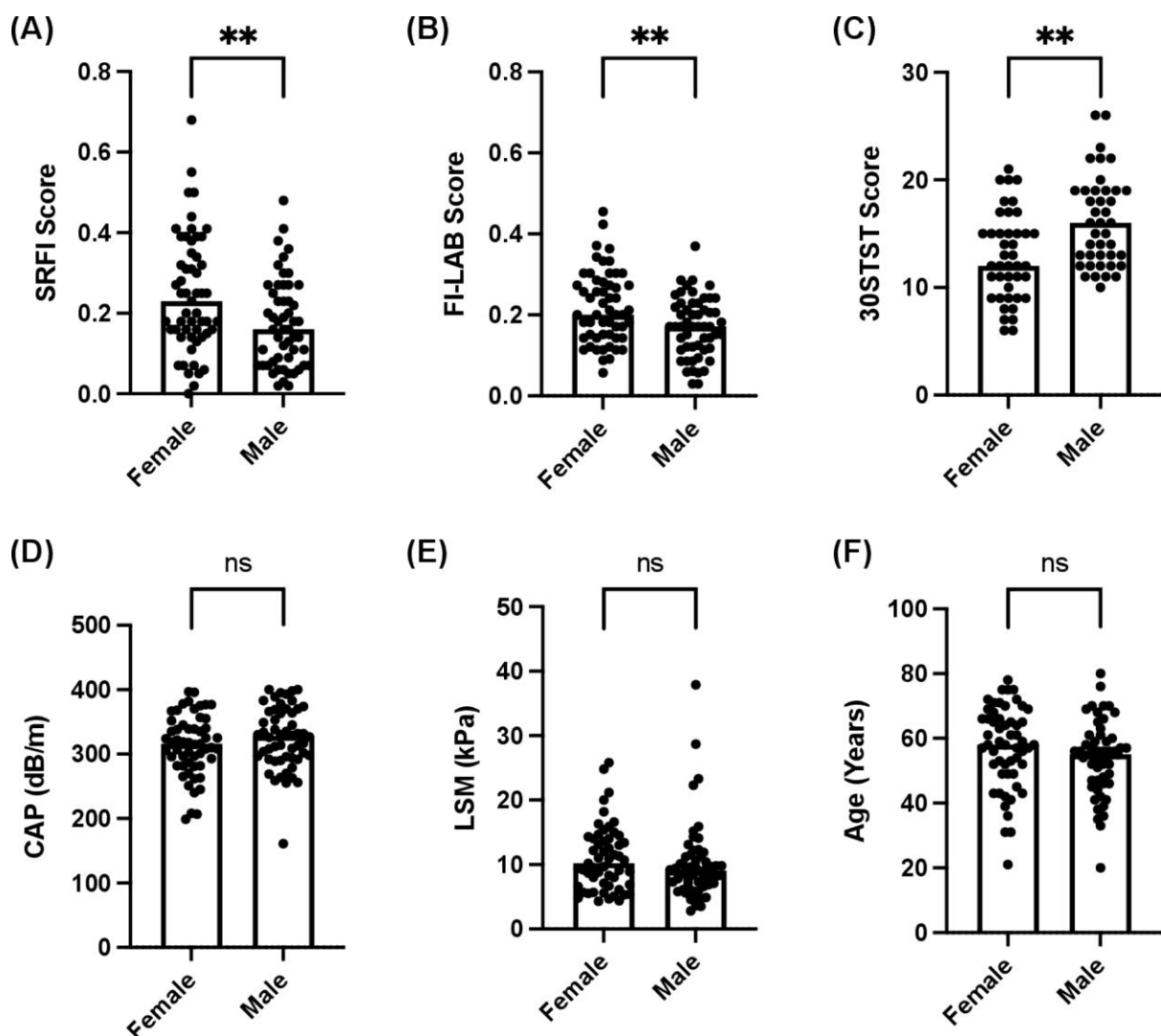


Figure 5-2 Female patients with non-alcoholic fatty liver disease (NAFLD) have higher frailty scores, despite no difference in liver fibrosis staging or age, compared with males.

All participants were categorized into on the basis of gender, irrespective of the degree of liver disease. (A) Self-reported frailty index (SRFI) scores in female and male patients. (B) Lab-based frailty index (FI-LAB) scores in female and male patients. (C) 30 s sit-to-stand (30STST) scores in female and male patients. (D) Controlled attenuation parameter (CAP) measurements (dB/m) in female and male patients. (E) Liver stiffness measurement (LSM; in kPa) in female and male patients. (F) Age (in years) in female and male patients. Between-

group differences were assessed using either an unpaired t test (F) or a Mann-Whitney test (A–E). ns, not significant; \*\*p<0.01.

### *5.6 Discussion*

This study highlights the high prevalence of prefrailty and frailty amongst patients with NAFLD. Interestingly, side by side comparison of different frailty measures demonstrated a direct relationship between increased frailty using the SRFI, FI-LAB and 30STST scores with increasing liver stiffness. In contrast, there was no significant relationship between frailty and fibrosis score when TUG or FFI instruments were used. Furthermore, our results support an association between frailty and female gender.

These results highlight the high prevalence of prefrailty and frailty among patients with NAFLD at non-cirrhotic disease stages. 20% of patients with F0/F1, 41% of patients with F2/F3 compared with 63% of patients with F4 disease were classified as frail using the SRFI with median SRFI scores of 0.15, 0.20 and 0.27 in the F0/F1, F2/F3 and F4 groups, respectively. In contrast, the mean SRFI score amongst healthy, community-dwelling Irish adults has been shown to be approximately 0.10 [244]. In the Irish longitudinal study on ageing (TILDA), the overall frequency of frailty among 4961 individuals aged over 50 using the SRFI was 11%[244, 303]. Similar frequencies have been reported amongst healthy community-dwelling adults in Japan[304]. Using the FFI, the prevalence of frailty amongst healthy adults has been shown to be approximately 5.6% compared to 17.9% in non-cirrhotic patients with chronic liver disease and 37.0% in patients with established cirrhosis[305]. This would support the hypothesis that individuals with non-cirrhotic liver disease are at increased susceptibility to frailty than the general population.

Our results identify female gender as a strong independent predictor of increased frailty in NAFLD, as assessed by the SRFI, FI-LAB and 30STST scores. These results are supportive of data from a review of 4611 NHANES subjects, assessing the association between NAFLD and sarcopenia, which distinguished older age, female gender, non-Hispanic white ethnicity and lower baseline physical activity levels as independent predictors for sarcopaenia [306]. Similar gender disparities have been reported in a number of population studies of frailty amongst individuals without chronic liver disease. It is likely that multiple factors, including reduced physiological reserve with lower baseline muscle mass, account for this difference [307-309].

Furthermore, hormonal influences may also be contributory, with a recent metanalysis reporting an association between menopause and frailty, with increased risk of frailty in women who undergo early menopause or hysterectomy [310].

Our results indicate that the CAP score, age and hypercholesterolaemia were also independently associated with increased frailty when assessed with the different frailty measures. Due to our small sample size, we could not distinguish between the relative contributions of different clinical parameters on the risk of frailty. It is possible that the presence of comorbidities such as type 2 diabetes, elevated CRP and hypertension, which were all more common in patients with greater degrees of fibrosis, may also have influenced the risk of frailty among patients with NAFLD, independent of the presence of liver disease.

The SRFI, the FI-LAB and the 30STST were significantly associated with the hepatic fibrosis stage as measured by liver stiffness; however, this was not seen with the FFI instrument and TUG test. One of the components of the FFI includes unintentional weight loss, this is not frequently seen in individuals with metabolic disease and NAFLD. In obese patients, BMI and weight do not correspond with functional muscle mass, as a result the FFI may under-represent frailty [311]. Furthermore, the cut-off parameters for assessing grip strength based on BMI may not be sensitive for patients with non-cirrhotic NAFLD. Each component of the frailty index assesses different aspects of frailty and it is likely that some components of the frailty assessment may be more predictive of specific clinical outcomes compared with others. Our results highlight that FFI scores do not significantly increase in patients with NAFLD during the early stages of disease progression.

To address the demand for a frailty assessment tool in patients with chronic liver disease, Lai et al developed the Liver Frailty Index (LFI)[291]. This consists of 3 measurements, grip strength, chair stands and balance testing. According to results, the LFI performed better than MELD-Na in predicting waiting list mortality[291, 294]. However, at the time we began enrolling patients in our study, the LFI had only been validated for use in cirrhotic patients and consequently was not included in our study. More recent data has demonstrated the validity of the LFI in non-cirrhotic populations [301]. Future studies should assess the LFI in non-cirrhotic patients with NAFLD alongside the FFI, 30STS and SRFI measures to assess which

frailty assessment instruments are best suited for this patient population and are more closely associated with fibrosis stage.

The presence of sarcopenia was not formally examined in our cohort. The European Working Group on Sarcopenia in Older People guideline recommends measurement of muscle mass, strength and function and requires documentation of low muscle mass in addition to one of two further criteria: low muscle strength or low physical performance[287]. Although this study was not designed to formally quantify muscle mass by means of imaging, we did assess muscle strength as part of the FFI score and physical performance using the 30STST and TUG tests. Physical performance as assessed by 30STST varied significantly across the spectrum of liver fibrosis, however, the TUG scores did not. This may be due to differences in the sensitivity of these measures amongst patients with NAFLD.

Frailty is a potentially modifiable condition; consequently, recognising frailty in non-cirrhotic patients with NAFLD is an essential first step in identifying at risk individuals and implementing therapeutic interventions to improve outcomes. Lifestyle interventions inclusive of dietary supplementation and exercise-based rehabilitation programs have shown improvements in physical performance, muscle strength and mobility [312, 313]. Exercise-based interventions in NAFLD have shown to result in significant improvements in hepatic inflammation and fibrosis which is independent of the degree of weight loss [314, 315] although patient adherence is a significant challenge which must be addressed. It will be important to assess the impact of exercise on frailty in non-cirrhotic NAFLD populations in future interventional studies.

This study had several limitations: (i) small sample size (n=109), this was due in part to the detailed assessment required for each patient and future prospective studies will need to prioritise the assessments of frailty, including the LFI as well as measurements of sarcopenia; (ii) our cohort was selected from patients attending a tertiary referral service which may select for individuals with more severe metabolic disease compared with patients with NAFLD in the community; (iii) our study lacked a direct age-matched controls without NAFLD and while Irish data on the use of SRFI is available from community-based well-described cohorts from different age groups, these data are not directly matched for BMI or gender; (iv) as a

cross-sectional study, we were unable to measure the impact of frailty on clinical outcomes in non-cirrhotic NAFLD patients, or to report if changes in frailty measurements correlate with liver disease progression or regression. It is likely that frailty assessment tools will need to be assessed in longitudinal studies to establish the optimal frailty assessment tool for this patient population, (v) our cohort did not record data on menopause or use of hormone replacement therapy and consequently it was not possible to subcategorize frailty findings in female patients with reproductive stage of life.

Our results highlight the complex and multifaceted impact of metabolic disease on overall health in patients with NAFLD, even in patients with early fibrosis stage. Frailty indices are important tools that aid early identification of patients with NAFLD who may be more vulnerable to adverse health outcomes. Incorporation of frailty assessment tools in clinical practice can facilitate more accurate prognostication, facilitate rehabilitation strategies to address physical frailty deficits and consequently improve patient care. Further validation studies of frailty assessment tools with long-term follow-up in patients with non-cirrhotic liver disease are required.

#### *5.7 Study findings:*

- One third of individuals with non-cirrhotic NAFLD were identified as frail and a further one third were prefrail
- Frailty was more common in females with a diagnosis of NAFLD

## Chapter 6: Discussion

## 6 Discussion

With rising rates globally, NAFLD increasingly poses a risk to public health. It is estimated to affect approximately 52 million individuals in Europe, with a prevalence of 20-40% and is becoming the fastest growing indication for liver transplantation with a greater than seven-fold increase in the rates of NAFLD associated hepatocellular carcinoma over the last 15 years. [316] Of the 20-40% of individuals with NAFLD, approximately 10-20% go onto develop NASH of which a further 24% progress to cirrhosis, with an estimated overall annual progression rate of 0.03 stages [317]. Not only is it associated with significant liver related morbidity and mortality, but NAFLD also confers an increased risk of cardiovascular disease, renal disease and extra-hepatic malignancies, with an exponential rise in liver related and all-cause mortality with each stepwise increase in fibrosis stage [318-321]. Individuals with NAFLD undergoing liver transplantation are considered higher operative risk candidates, with increased risk of post-transplant related complications [322]. In addition, NAFLD has a notable impact on an individual's perception of health and quality of life [318]. Unsurprisingly, given its widespread effects, NAFLD places a significant economic burden, costing healthcare systems in Europe approximately 35 billion euros [318]. Interestingly, these statistics may even be an underrepresentation of the true extent of NAFLD, as guidelines for screening at risk populations have only recently been developed. Despite its clinical importance, NAFLD still poses a challenge both diagnostically, in terms of assessing disease severity and optimal therapeutic management.

Whilst prognosis is dependent on fibrosis stage, the first step towards NAFLD progression and hepatic fibrosis is the development of steatohepatitis. This transition, is perpetuated by immune dysregulation. Excess free fatty acid accumulation in the liver disrupts organelle function, particularly that of mitochondria, resulting in the release of reactive oxygen species, endoplasmic reticulum stress and hepatocyte apoptosis [323]. This local hepatocellular injury, leads to the release of DAMPs which in conjunction with dysbiosis mediated translocation of pro-inflammatory gut bacteria to the portal circulation leads to the activation of Kupffer cells, causing the release of pro-inflammatory cytokines and chemokines, promoting hepatic migration of leukocytes [323]. Although it is challenging to ascertain the full sequence of events and much of the information to date has been obtained from experimental animal

studies. Immunohistochemical analysis of liver biopsies in patients with increased hepatic steatosis has shown early portal accumulation of macrophages and monocytes, in conjunction with increased MCP-1 expression [324]. This suggests that they act as first responders to hepatocellular injury in NAFLD. In vitro studies have shown that activated macrophages and monocytes release copious quantities of pro-inflammatory cytokines,  $\text{TNF}\alpha$ , IL  $1\beta$  and IL 12, directly activating stellate cells, promoting inflammation and further hepatic leukocyte chemotaxis [324]. As a result, monocytes and macrophages are believed to be instrumental in the progression of NAFLD to NASH and the development of fibrosis. By comparison, liver biopsies performed in individuals with more advanced disease including established NASH show portal and lobular inflammation with infiltration predominantly by T, B lymphocytes, NK T cells and neutrophils in conjunction with increased IL 6, IL 8 and Th17 related cytokine production, IL 17, IL 21 and IL 23 [323-325], with plasma cell and IL 17 positive neutrophil infiltration showing the strongest correlation with hepatic fibrosis grade [325]. The challenge that remains in clinical practice is identifying the patients with NASH who are at risk of progressive liver disease and advanced fibrosis. This is increasingly difficult with the move away from liver biopsies to non-invasive methods for the assessment of liver disease severity.

Given the critical role of the immune system in the evolution of NASH, it is likely that detailed analysis and characterization of immunological changes can not only inform the development of novel therapeutic strategies, but also aid in the diagnosis of NASH, through the formulation of an immune signature. Our findings support this hypothesis, demonstrating that pooled analysis of monocytes, MAIT cells,  $\text{CD8}^+$  Lymphocytes and the NLR is strongly associated with and predictive of both NASH and advanced NAFLD fibrosis with an AUC of 0.813 and 0.947, respectively, exceeding the AUC of the non-invasive scoring systems currently validated for use in clinical practice. The combined predictive capacity of the immune cells outperforms each cell population in isolation, likely reflecting their concentric and synergistic activity in NAFLD. Thus, it is likely that any immune signature for NASH and advanced NAFLD would consist of a composite 'immunoscore,' although further studies are required amongst larger populations to assess the reproducibility and validate our results. From our pilot study, most circulating immune cells are depleted with advancing fibrosis, although, the effect is greatest amongst the  $\text{CD8}^+$  lymphocyte population, including  $\text{CD8}^+$  MAIT cells. Whilst within the liver, only the  $\text{CD8}^+$  T lymphocytes appears to be diminished. This observed reduction is associated

with increased acute and terminal activation marker expression, suggesting that excess stimulation in advanced NAFLD may play a factor in the accelerated apoptosis of these cells. These findings are novel as there has not been much exploration of T lymphocyte and MAIT cell subpopulations in NAFLD. Interestingly, both MAIT cells and CD8<sup>+</sup> T lymphocytes have been shown to rely on the mTOR mediated glycolytic pathway when stimulated [81, 267]. This metabolic pathway has been shown to be significantly impaired in obesity and it is possible that the same pathophysiology is involved in NAFLD, where reduced energy supply despite increasing demand may have a deleterious effect on cellular function and result in increased vulnerability to apoptosis [81]. Functional studies and assessments of circulating and intrahepatic CD8<sup>+</sup> T lymphocytes with MAIT cells are needed to further develop our understanding of the role and behaviour of these cells in NAFLD. Of all the cell populations analysed, total circulating monocytes exhibited the strongest relationship with histological inflammation. This would support existing literature suggesting that they act as ‘first-responders,’ involved in the early stages of NASH development [324]. What remains to be determined is how dynamic these immunological changes are, is there a potential for serial analyses to indicate disease progression and if there is potential for restoration of populations with histological improvement.

Leading on from our initial study, we then proceeded to focus on MAIT cells and assess for changes in response to weight loss interventions in patients with NAFLD. Given the global impact and burden of NAFLD, there has been much interest in the development of pharmacotherapeutic strategies, however weight loss still remains the cornerstone of management. Current data on lifestyle measures in NAFLD have reported a direct correlation between the degree of weight loss achieved and histological improvement, with resolution of NASH in 58% of patients who achieved  $\geq 5\%$  weight loss with no worsening of fibrosis and 100% in those who achieved  $\geq 10\%$  weight loss after one year, with regression of fibrosis seen in up to 45% of participants [178]. Data from NAFLD patients post bariatric surgery has also reported improvements in lobular inflammation in 50% of participants, reduction in ballooning in 76% and reduction in fibrosis stage in 40%, one year post-operatively [190]. Although 53% of our nutritional intervention cohort lost  $\geq 5\%$  of their weight with an additional 27% losing  $\geq 10\%$ , only significant reductions in steatosis grade were observed. This

may be due to the length of the interval between paired biopsies. Most studies evaluating histological response post weight loss repeated histological assessment after one year.

Weight loss has been shown to alter circulating chemokine profile. Increased adiponectin and reduced leptin levels improve insulin sensitivity with increased fatty acid oxidation, reduced gluconeogenesis and increased mitochondrial biogenesis. This leads to reduced oxidative stress within tissue, reducing pro-inflammatory cytokine expression and immune cell infiltration of adipose tissue [326]. Adiponectin also directly promotes hepatic stellate cell apoptosis reducing intra-hepatic pro-inflammatory cytokine expression and extra-cellular matrix deposition [327].

Small population studies have investigated the impact of weight loss on circulating immune cell populations with the most significant findings being seen post-bariatric surgery. Changes in circulating lymphocyte populations have been observed, with reductions in the Th1/Th2 ratio in association with improved insulin sensitivity and reductions in CD4<sup>+</sup> lymphocytes correlating directly with improvement in BMI [272]. Shifts in B lymphocyte phenotype have also been observed from the effector (IL 6) to a regulatory type (IL 10) post Roux-en Y bypass, suppressing pro-inflammatory T lymphocyte cytokine secretion [272]. In addition, some conflicting data has been documented on T cell activation marker expression post Bariatric surgery, with one study of 13 patients finding reduced T cell CD69 and CD25 expression in patients who achieved a mean total weight loss of 13.5% and another study of 25 patients showing significant reductions T lymphocyte CD62L and Fas ligand (CD95) expression without any significant changes in T lymphocyte CD69 expression [273].

Current published data on MAIT cells in obesity has shown lower circulating levels with an altered, predominantly Th17 phenotype [81, 109]. In this pathological setting, MAIT cells demonstrate mitochondrial dysfunction and impaired glycolytic activity with reduced production of IFN $\gamma$ , higher levels of TNF $\alpha$  and IL 17 [81]. Following weight loss 3 months post bariatric surgery, the percentage of circulating MAIT cells has been shown to increase, with lower levels of Granzyme B, IL 2, but persistently high IL 17 production [115]. No studies to date have assessed for these changes in patients with NAFLD.

By comparison, our results did not show any increase in circulating MAIT cells post nutritional intervention, however they did express lower levels of CD69. Improved metabolic status and reduced systemic inflammation with weight loss could explain the decrease observed in circulating MAIT cell activation. However, a greater degree of weight loss may be needed to promote significant increases in the percentage of circulating MAIT cells.

It is well established that exercise leads to reduction in visceral adipose tissue mass and promotes effective weight loss in conjunction with dietary changes. However, more limited data is available on the impact of exercise alone interventions in NAFLD, with small population studies showing no change in liver histology following six months of either aerobic or combined aerobic and resistance training [328]. Our previously published paper highlighted the histological improvements in association with increased fitness levels [315].

Both acute and chronic exercise programs have been shown to influence the immune system. Stimulation of skeletal muscle leads to release of myokines, muscle derived IL-6, decreased Toll-like receptor expression with increased expression of IL-1 receptor agonist on circulating mononuclear cells which suppress the release of pro-inflammatory cytokines by T lymphocytes and inhibit the infiltration of adipose tissue by monocytes and macrophages [329].

The release of catecholamines and cortisol in the immediate phase of exercise leads to the mobilisation of CD4<sup>+</sup>, CD8<sup>+</sup> lymphocytes and Natural Killer cells to the peripheral circulation [330-332]. The degree of catecholamine and cortisol release with exercise is proportionate to the nature and duration of exercise, with greatest levels with aerobic exercise lasting greater than one and a half hours [330-332]. Larger circulating concentrations of these mediators have greater immunosuppressive effects, reducing circulating TNF $\alpha$ , which in turn inhibits lymphocyte proliferation and makes activated lymphocytes more vulnerable to apoptosis. [330-332] In addition to this, cortisol directly stimulates Fas and TNF receptors, further promoting lymphocyte apoptosis [331].

Skeletal muscle is the primary site of glutamine synthesis, activated lymphocytes and macrophages are heavily reliant on skeletal muscle derived glutamine for nucleotide

biosynthesis and proliferation as they lack glutamine synthetase [333, 334]. Prolonged exercise has been shown to reduce glutamine availability and reduction in supply of this key mediator with exercise may contribute to increased metabolic stress experienced by lymphocytes, contributing further to their increased vulnerability to apoptosis [334].

In the resting state, Th1, Th2, Th17 cells rely on oxidative phosphorylation and ATP production. When activated, they switch to aerobic glycolysis, a more efficient method for more rapid energy and macromolecule synthesis [334]. This is facilitated with mTORC1 mediated glucose metabolism in Th1 and Th17 cells and mTORC2 mediated glucose metabolism in Th2 cells [335]. Dysregulation of this metabolic pathway with persistent chronic activation of the glycolytic pathway has been documented within the lymphocytes of individuals with obesity, diabetes and reduced baseline fitness levels [336]. Lymphocytes exhibit greater levels of mitochondrial dysfunction, lower levels of mTOR and Bcl2 leading to impaired autophagy and increased rates of apoptosis in response to increased metabolic demand and oxidative stress [337]. Exercise has been shown to modulate this metabolic dysfunction, with a short course of particularly high intensity exercise training through reduced IL-4, TNF $\alpha$  and increased circulating IFN $\gamma$  which promotes autophagy, the sequestration of dysfunctional mitochondria, improving the anti-oxidative capacity of lymphocytes and reducing the rate of apoptosis [337].

There is very little data examining the impact of exercise interventions on MAIT cell numbers and activation marker expression. One small study conducted among 20 participants found an acute increase in circulating MAIT cells in association with increased TNF $\alpha$  production during exercise with normalisation post exercise, reflecting similar patterns observed with CD4<sup>+</sup>, CD8<sup>+</sup> lymphocytes and NK cells [276, 277]. Increased circulating MAIT cells following a 16-week aerobic exercise program in patients with breast cancer [338]. This was contrary to our finding, in which no significant difference in the percentage of circulating MAIT cells was observed. Additionally, animal studies have shown that MAIT cell blockade with Acetyl-6-formylpterin promoted fibrosis regression among mice injected with CCL4 [339].

We hypothesize that dysfunctional MAIT cell metabolism in NAFLD could pre-dispose these cells to accelerated apoptosis within the liver, particularly when excessively stimulated and

exposed to increased oxidative stress during exercise. The accelerated apoptosis of this pro-inflammatory and pro-fibrogenic cell population within the liver may explain the histological improvements observed.

Our study is the first to evaluate the impact of exercise on MAIT cells within the liver. In vitro studies focusing on MAIT cell metabolism and cytokine production have noted that circulating MAITs exhibit features of metabolic dysfunction, particularly with impaired glycolysis due to reduced mTOR activation and reduced IFN $\gamma$  production similar to that documented in lymphocytes [81]. While an exercise regimen has been shown to modulate metabolic dysfunction in circulating lymphocytes, the same may not be true for intrahepatic MAIT cells in patients with NAFLD. Impaired glycolysis and energy output despite increased activation may render the cells unable to meet the rising metabolic demand, promoting Fas ligand expression and apoptosis.

Finally, we wanted to assess the impact of NAFLD on clinical measures of frailty. Frailty represents a state of physiological decline and increased vulnerability to adverse health events. Although it has classically been considered an age associated phenomenon, with the majority of studies focusing on community dwelling older adults, more recently there has been increased understanding of its distinction from physiological ageing and its link with pathophysiological mechanisms underlying different disease states, such as obesity, that increase an individual's risk of accelerated ageing. The term frailty encompasses a wide spectrum of pathology including features of physical frailty, centred around measures of muscle mass and function, with deficit accumulation, a cumulative index of an individual's co-morbidities. Methods of assessing and measuring frailty have become very useful in clinical practice as they are highly predictive of risk of disability, hospitalization and mortality. NAFLD is the hepatic manifestation of obesity, a multisystem inflammatory condition [323, 340]. As such with the associated lipotoxicity, mitochondrial dysfunction and inflammasome activation it leads to the generation of reactive oxygen species which damage cellular DNA, promoting senescence whilst also impairing autophagy. This leads to the accumulation of senescent cells within muscle tissue impairing its function. In addition, inflammation directly stimulates myocyte apoptosis and inhibits myogenesis through reduction of irisin production, reducing muscle mass. Furthermore, NAFLD is linked with many co-morbidities that have

been associated with increased risk of frailty including diabetes with a 58% prevalence, morbid obesity with a 30% prevalence, hypertension and sleep apnoea. Much of the current literature on frailty in NAFLD focuses on individuals with decompensated chronic liver disease where it has been a helpful predictive marker of peri-operative risk and adverse outcomes post liver transplantation. However, very little data has been published on frailty in patients with pre-cirrhotic NAFLD. An additional challenge is identifying the most suitable measure for individuals with NAFLD, as there are up to 67 different frailty instruments available, the majority of which have been developed and validated in individuals who are aged over 65 without a history of known liver disease [341]. We performed a side-by-side analysis of five different measures of frailty, including the FFI, SRFI, FI-LAB, 30STS and TUG in a cohort of patients with different stages of NAFLD. Our results demonstrated a high prevalence of pre-frailty and frailty in patients with non-cirrhotic NAFLD using the SRFI, FI-LAB and 30STS tests. Whilst there was no significant intergroup variability with the FFI and TUG tests. The FFI in particular may not be optimal for NAFLD patients in particular as one of the components of the index includes unintentional weight loss of  $\geq 10$  lbs (4.5 Kg), with the majority of NAFLD patients would not experience unintentional weight loss, by contrast high BMI and weight gain can mask lean weight loss or sarcopenia.[342] Interestingly with the SRFI, FI-LAB and 30STS, there was a stepwise increase in the prevalence of frailty with each fibrosis stage. Of note, a considerable proportion, 20%, of patients with early stage, F0/F1 NAFLD, were frail according to the SRFI. We also demonstrated a correlation between steatohepatitis and frailty, with patients with higher FAST scores, scoring higher on both the SRFI and FI-LAB measurements. These findings are novel and support the hypothesis that NAFLD encompasses a spectrum of metabolic dysfunction, immune cell activation and inflammation resulting in measurable features of accelerated ageing even in the early stages. The liver frailty index, is one measure that has been developed and validated in patients with chronic liver disease with its use primarily as risk stratification for liver transplantation, more recently it has been validated in patients with non-cirrhotic chronic liver disease, although there is still very little data available on its use in individuals with non-cirrhotic NAFLD [301].

Female gender was independently associated with frailty in our cohort, this gender difference in the absence of chronic liver disease, has been shown in population studies on frailty [143]. This is likely due to comparatively reduced biological reserve in women, with significantly

lower baseline lean body mass leaving them at higher risk of developing sarcopaenia with increasing age.

The findings of this study are important as frailty is tied to clinical outcomes, these results signify the underrecognized increased risk of adverse health events in NAFLD patients, even in individuals with early-stage disease.

### *6.1 Conclusion*

NAFLD is a state of chronic inflammation, resulting from persistent systemic immune cell activation. Our results from this thesis further support this, showing both circulating and intrahepatic immune cells are measurably reduced, with features of terminal activation, in correlation with histological severity. These changes are most marked amongst CD8<sup>+</sup> T lymphocytes and MAIT cells. These results shed further light on their potential involvement in NAFLD progression and also diagnostic utility. Composite analysis of circulating MAIT cells, CD8<sup>+</sup> T lymphocytes, monocytes and the NLR is predictive of both NASH and advanced hepatic fibrosis, suggesting these changes could aid in the non-invasive staging of NAFLD. Whilst weight loss remains the cornerstone of NAFLD management, side by side comparison of dietary and exercise-based weight loss interventions shows that despite resulting in more modest weight loss, exercise may have unique immunomodulating effects, leading to improvements in histological inflammation and fibrosis in association with intrahepatic MAIT cell depletion. Finally, our results support the link between chronic inflammation and accelerated ageing, with significantly increased rates of pre-frailty and frailty in individuals with NAFLD, even in early F0/F1 stages, directly correlating with histological severity.

### *6.2 Future directions*

Collectively the work in this thesis has identified further questions and areas for future work. Notably our analysis of immune cells was predominantly descriptive in nature, assessing phenotypic changes and looking for correlates with the severity of hepatic inflammation and fibrosis. Whilst the identified changes suggest the possible involvement of monocytes, CD8<sup>+</sup> T lymphocytes and MAIT cells in NAFLD progression, a causal relationship cannot be

confirmed. Functional studies are required, looking at changes in cytokine production and co-culture with stellate cells and hepatic myofibroblasts to elucidate the role of these different immune cell populations in the evolution and progression of NAFLD. Of particular interest would be determining if there are features of metabolic dysfunction on a cellular level in particular in MAIT cells and CD8<sup>+</sup> T lymphocytes and if there is a direct relationship between cellular metabolism, cytokine dysfunction and terminal activation. Data from functional and metabolic analyses could potentially help determine if potentially impaired cellular metabolism drives a switch, to a pro-inflammatory, persistently activated phenotype. This could inform the development of novel therapeutic approaches. Additionally, it would be informative to evaluate the CD4<sup>+</sup> T lymphocyte with the CD4<sup>+</sup> and double negative MAIT cell populations, to clarify if the observed reduction in the CD8<sup>+</sup> lymphocytes was due to their selective depletion or if it could be explained by the expansion of the CD4<sup>+</sup> and double negative populations. Our results demonstrated reductions in the total circulating monocyte population and increased NLR with worsening NAFLD, however, concurrent analysis of intrahepatic monocyte and neutrophil populations in conjunction with chemokine receptor expression would help to determine if these reductions could be explained by increased hepatic migration. The data from this thesis have been obtained by small population, pilot studies. An additional limitation was that control data in Chapter 3 was obtained from different lab with no accompanying demographic information, thus, it would be important to repeat these analyses amongst larger populations with a fully characterized control populations to validate these results confirm the relationship with histological stage and determine optimal diagnostic cut offs.

With increasing research on the importance of the intestinal microbiome in the evolution of disease, it would be very interesting to assess the microbial influences behind these immunological changes through evaluation of the microbiome and co-cultures. What also remains to be determined is how dynamic these immunological changes are? Can MAIT and CD8<sup>+</sup> lymphocyte populations be repleted post therapeutic intervention? Surprisingly, we found significant reductions in intrahepatic MAIT cells with increased terminal activation marker expression post exercise intervention, suggesting that accelerated apoptosis may be behind exercise mediated histological improvements. However, it would be of interest to repeat these measures at a longer interval post intervention and to compare with results

following other interventions namely bariatric surgery and treatment with GLP-1 agonists. To complete our understanding of the immunological changes with weight loss interventions it would be informative to perform side by side comparisons of changes in the monocyte, neutrophil and T lymphocyte populations pre and post exercise intervention. Another question raised by this body of work is what exercise intervention is superior for the management of NAFLD, we focused on a 12- week aerobic intervention, however it would be of interest to compare these results with the outcomes of a resistance-based exercise intervention. Are immunological changes and histological improvements in NAFLD influenced by improved cardiovascular fitness or increased muscle mass?

Finally, our frailty assessments were limited by measure of muscle function, but not direct measures of muscle mass. What would be of particular interest would be combining the SRFI, FI LAB and 30STS results with measures of muscle mass and assessing for immunological correlates of frailty and sarcopaenia in NAFLD.

## Appendix:

### Abstract Presentations

Irish Endocrinology Society Annual Meeting 2017

Irish Society of Gastroenterology Winter Meeting 2017

EASL NAFLD summit Sept 2018

AASLD- Nov 2018

EASL ILC 2019

Irish Society of Gastroenterology Winter Meeting 2019

Irish Society of Gastroenterology Winter Meeting 2021

### Publications

#### **Prevalence of frailty in patients with non-cirrhotic non-alcoholic fatty liver disease**

**Sara Naimimohasses\***, Philip O'Gorman\*, Emma McCormick, Damien Ferguson, Ann Monaghan, Marie McGrath, Mark W Robinson, John Gormley, Suzanne Norris

*BMJ Open Gastroenterol*, 2022 May;9(1)

*Department of Hepatology, St James's Hospital, Dublin, Ireland.*

**\*Both authors contributed equally**

#### **Differential Effects of Dietary versus Exercise Intervention on Intrahepatic MAIT Cells and Histological Features of NAFLD**

**Sara Naimimohasses**, Philip O'Gorman, Ciara Wright, Deirdre Ni Fhloinn, Dean Holden, Niall Conlon, Ann Monaghan, Megan Kennedy, John Gormley, Peter Beddy, Stephen Patrick Finn, Joanne Lysaght, Jacintha O'Sullivan, Margaret R. Dunne, Suzanne Norris and J. Bernadette Moore

*Nutrients*, 2022, 14(11), 2198

*Department of Hepatology, St James's Hospital, Dublin, Ireland.*

#### **Letter: Proving the benefit of exercise intervention in metabolic associated fatty liver disease-authors' reply.**

Philip O'Gorman, **Sara Naimimohasses**, Ann Monaghan, Megan Kennedy, Stephen P Finn, J Bernadette Moore, John Gormley, Suzanne Norris

*Aliment Pharmacol Ther* 2020 10;52(8):1426-1427

*Department of Hepatology, St James's Hospital, Dublin, Ireland.*

#### **Determinants of Physical Activity Engagement in Patients with Nonalcoholic Fatty Liver Disease: The Need for an Individualized Approach to Lifestyle Interventions.**

Philip O'Gorman, Ann Monaghan, Marie McGrath, **Sara Naimimohasses**, John Gormley, Suzanne Norris

*Phys Ther* 2021 02;101(2)

*Department of Hepatology, St James's Hospital, Dublin, Ireland.*

**Improvement in histological endpoints of MAFLD following a 12-week aerobic exercise intervention.**

*Philip O'Gorman\*, Sara Naimimohasses\*, Ann Monaghan, Megan Kennedy, Stephen P Finn, J Bernadette Moore, John Gormley, Suzanne Norris*

*Aliment Pharmacol Ther 2020 10 27;52(8):1387-1398. Epub 2020 Jul 27.*

*Department of Hepatology, St James's Hospital, Dublin, Ireland.*

***\*Both authors contributed equally***

**Development and relative validation of a short food frequency questionnaire for assessing dietary intakes of non-alcoholic fatty liver disease patients.**

*Carla Bredin, Sara Naimimohasses, Suzanne Norris, Ciara Wright, Neil Hancock, Kathryn Hart, J Bernadette Moore*

*Eur J Nutr 2020 Mar 25;59(2):571-580. Epub 2019 Feb 25.*

*Department of Nutritional Sciences, University of Surrey, Guildford, GU2 7XH, Surrey, UK.*

## Grants and Awards

|                                                        |           |
|--------------------------------------------------------|-----------|
| ISG Winter Meeting Presentation Prize, Dublin          | Nov 2019  |
| EASL NAFLD Summit Young Investigator Bursary, Zurich   | Sept 2018 |
| ISG Winter Meeting Presentation Prize, Dublin          | Nov 2018  |
| Trinity Translational Medicine Institute Grant, Dublin | Feb 2017  |
| Trinity College Dublin Dean's Initiative Grant, Dublin | Feb 2017  |

SJH/TUH Research Ethics Committee Secretariat  
email: [researchethics@tuh.ie](mailto:researchethics@tuh.ie)

Dr Sara Naiminohasses,  
Hepatology Research Fellow,  
St James's Hospital,  
James' Street,  
Dublin 8

21<sup>st</sup> December 2018

**RE.: Inflammaging in patients with NAFLD: A study of cognition, sarcopenia and fragility in chronic liver disease**

**REC REF 3: 2018-12 List 47 (6)**

**REC REF 1: 2018-09 (01)**

*(Please quote reference on all correspondence)*

**Date of Valid Submission to REC: 03.12.2018**

**Date of Ethical Review: 20.11.2018**

Dear Dr Naimimohasses,

Thank you for your correspondence in which you sent in a response to the Committee's letter which detailed the Committee's queries and concerns in relation to the submission of the above referenced research study.

The Chairman has reviewed your response on behalf of the Committee, and gives approval for this study to proceed.

The following document was reviewed:

- Response to comments, dated 03.12.2018
- PIL, version 1, dated 1 May 2018 (tracked changes)
- PIL, version 2, dated November 2018 (clean)
- Consent form (tracked & clean) [Please add version and date]

*Applicants must submit an annual report for ongoing projects and an end of project report upon completion of the study. It is the responsibility of the researcher/research team to ensure all aspects of the study are executed in compliance with the General Data Protection regulation (GDPR), Health Research Regulations and the Data Protection Act 2018.*

Yours sincerely,



---

REC Officer – Dr Sadhbh O'Neill - SJH/TUH Research Ethics Committee

The SJH/TUH Joint Research and Ethics Committee operates in compliance with and is constituted in accordance with the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004 & ICH GCP guidelines.

SJH/AMNCH Research Ethics Committee Secretariat  
[researchethics@sjh.ie](mailto:researchethics@sjh.ie)



Tallaght  
University  
Hospital

An Academic Partner of Trinity College Dublin

Ospidéal  
Ollscoile  
Thamhlachta

Prof Suzanne Norris,  
St James's Hospital,  
James' Street,  
Dublin 8

11<sup>th</sup> May 2019

**Re: Inflammaging in Patients with NAFLD: A Study of Cognition, Sarcopenia and Fragility  
in Chronic Liver Disease**

**REC Reference: 2019-06 List 21 (05)**  
**Previous REC Reference: 2018-12 List 47 (6)**  
*(Please quote reference on all correspondence)*

**EudraCT Number: N/A**

**Date of Valid Submission to REC: 25.05.2019**  
**Date of Ethical Review: 05.06.2019**  
**R&I application Number: N/A**

Dear Prof Norris,

Thank you for your correspondence in which you submitted an amendment for the above named study.

The Chairman has reviewed the documentation you submitted and noted the addition of Dr Mark Robinson to the study. This amendment is approved.

The following documents were reviewed:

- Non-clinical amendment request form, dated 24.05.2019

*Applicants must submit an annual report for ongoing projects and an end of project report upon completion of the study. It is the responsibility of the researcher/research team to ensure all aspects of the study are executed in compliance with the General Data Protection regulation (GDPR), Health Research Regulations and the Data Protection Act 2018. **Additionally, please note for documents submitted for GDPR purposes that the REC and the Chair are not confirming that you're documents are GDPR compliant, they are approving the document from an ethical perspective.***

Yours sincerely,

REC Officer – Dr Sadhbh O'Neill - SJH/TUH Research Ethics Committee

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THIS NOTEPAPER MUST NOT BE USED FOR  
PRESCRIPTIONS OR INVOICING PURPOSES

SJH/AMNCH Research Ethics Committee Secretariat  
Claire Hartin Ph: 4142199  
email: [claire.hartin@amnch.ie](mailto:claire.hartin@amnch.ie)



**THE ADELAIDE & MEATH  
HOSPITAL, DUBLIN**  
INCORPORATING  
THE NATIONAL CHILDREN'S HOSPITAL

TALLAGHT, DUBLIN 24, IRELAND  
TELEPHONE: +353 1 4342000

Dr. Sara Naimimohasses  
Hepatology Department  
St. James's Hospital  
James's Street  
Dublin 8

31<sup>st</sup> May 2017

**Re: A Pilot study to develop a non-invasive bio-clinical tool for the identification of obesity related fatty liver disease**

**REC Reference: 2017-05 List 17 (12)**  
*(Please quote reference on all correspondence)*

Dear Dr. Naimimohasses,

Thank you for your recent correspondence to SJH/AMNCH Research Ethics Committee in which you requested an amendment in relation to the above referenced study.

The Chairman, Dr. Peter Lavin, on behalf of the Research Ethics Committee, has reviewed this request and grants permission for this amendment.

Yours sincerely,

Claire Hartin  
Secretary  
SJH/AMNCH Research Ethics Committee

The SJH/AMNCH Joint Research and Ethics Committee operates in compliance with and is constituted in accordance with the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004 & ICH GCP guidelines.



# OSPIDÉAL NAOMH SÉAMAS ST. JAMES'S HOSPITAL



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## **A pilot study to develop a non-invasive bio-clinical tool for the identification of obesity-related fatty liver disease**

DEPARTMENT: Hepatology

**Dear Mr., Ms., Dr.,** You are being invited to participate in a research study. Thank you for taking time to read this.

Who is carrying out this research?: Researchers from the Hepatology Department in St James' Hospital are carrying out this study to investigate the effects of exercise and dietary weight loss interventions on non-alcoholic fatty liver disease.

### Introduction:

Non-alcoholic fatty liver disease (NAFLD) has now become the most common cause of liver disease in the developed world and is believed to affect more than 1 in 5 people globally.

It is caused by too much fat on the liver in the absence of excessive alcohol intake and in 20% of people it can lead to fat-related inflammation of the liver. This is called non-alcoholic steatohepatitis (NASH). NASH is the more aggressive form of fatty liver disease or NAFLD, and NASH can lead to liver damage and scarring (fibrosis). If untreated over a period, of time this can lead to cirrhosis of the liver. Cirrhosis is defined by severe, irreversible scarring (fibrosis) of the liver. It can increase the risk of liver cancer and can lead to liver failure.

There are many factors that can contribute to inflammation in the liver. Excess fat tissue in the body can cause inflammation and may encourage more severe inflammation and scarring in the liver. Recent studies have shown specific bacteria in the gut, identified by analysis of stool samples, can promote weight gain, increase the risk of obesity, diabetes and NAFLD. Genetics also plays an important role in NAFLD, with some genes increasing the chance of more severe liver scarring. To date, no studies have yet been performed to see how common these genes are in Ireland.



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At present, blood tests and FibroScan® (liver scan) can assess the risk of advanced fibrosis, but are not helpful for the diagnosis of NASH. The only way to confirm a diagnosis NASH is by performing a liver biopsy.

## WHY HAVE I BEEN CHOSEN TO PARTAKE IN THIS STUDY?

You have been chosen to partake in this study because your liver blood tests have been significantly elevated and/ or your Fibroscan results have indicated that you may have established scarring (fibrosis) of the Liver.

## THE AIM OF THIS STUDY IS AS FOLLOWS;

- 1) Develop a clinical tool based on blood tests to detect patients with NASH without the need of a liver biopsy.
- 2) Look at the effect of weight loss on the clinical tool.

## WHAT DOES THE STUDY INVOLVE?

This will involve taking additional research blood tests, stool sample, a body scan and a superficial fat tissue biopsy at the beginning of the study.

**Blood tests:** You will need to be fasting for 12 hours when you attend the Hepatology unit for your review. Blood tests will be taken from a vein in your arm by a trained phlebotomist. The blood tests will check for diabetes, pre-diabetes, cholesterol, liver function and will include experimental blood tests to measure inflammation and scarring of the liver.

**Body scan:** The body scan involved is a dual-energy x-ray absorptiometry (DEXA) scan. It enables accurate measurement of bone density, body fat and muscle content.

**Liver biopsy:** This study will involve a liver biopsy. A liver biopsy is currently the standard of care to evaluate for the presence of NASH and to confirm the degree of scarring (fibrosis) of the liver. It is recommended for patients who



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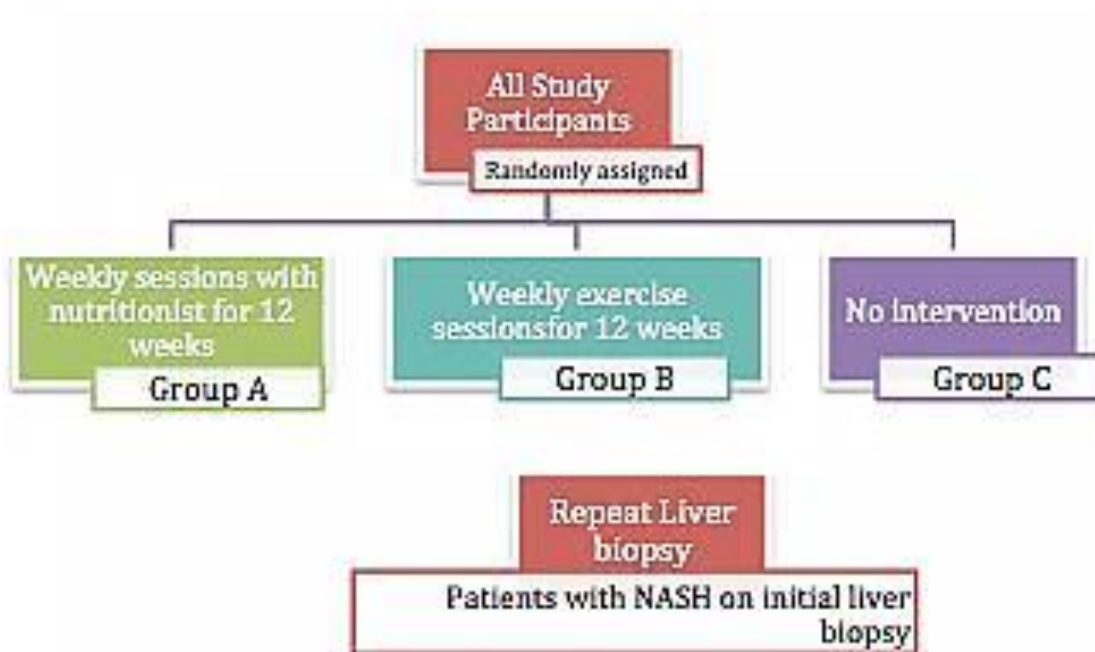
have persistently high liver enzymes or for whom blood tests and Fibroscan results suggest a high risk of scarring (fibrosis).

**Superficial fat tissue biopsy:** This study will also involve a superficial fat samples. The procedure will be performed as a day procedure, in the radiology department at the same time as the liver biopsy. This is to assess if the severity of fat tissue inflammation is related to the severity of inflammation in the liver.

After the initial assessment, you will be assigned into one of 3 groups to look at the effect of weight loss.

**Group A:** will have a weekly reviews with a dietician over a 12-week period, cooking classes and recipe guides will also be provided to help effective weight loss.

**Group B:** will involve a 12 week exercise-based weight-loss program supervised by a physiotherapist and will focus on exercise to improve participants cardiovascular health.





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**Group C:** will act as a control group with no planned weight-loss program.

Subjects in group A and B will receive additional information leaflets outlining the dietary and exercise programs.

Allocation to these groups is completely random and the research team is not permitted to allocate participants to a preferred group.

All participants will be reviewed after 12 weeks at the end of both weight-loss programs with repeat blood tests, stool sample, a repeat body scan and superficial fat biopsy. Participants will have another follow up in clinic at 6 months for repeat blood tests only.

If you have evidence of fat related inflammation on your first liver biopsy you will be asked to have a repeat liver biopsy after a 12 weeks. This is not part of standard care but we do this routinely to assess if there has been any improvement in inflammation.

## WHAT WILL HAPPEN IF I VOLUNTEER TO PARTICIPATE?

If you volunteer for the study, you will be called in for an initial assessment.

Following your assessment, you will be randomly assigned to one of the 3 groups.

## ARE THERE ANY RISKS INVOLVED IN PARTICIPATING?

**DEXA scan:** The radiation dose involved in the DEXA scan is less than the dose of

**Superficial fat biopsy:** Local anaesthetic will be used to numb the area and to reduce pain experienced during the procedure. Superficial fat samples will be taken using a sterile needle from an area overlying the right lower ribcage. There is a small risk of bleeding during the procedure. A sterile technique will be used to reduce the risk of infection. A sterile dressing will be applied to the area. You will be examined immediately and a week after the procedure to review the site.





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NASH. The procedure will be performed as a day procedure by an experienced interventional radiologist. Local anaesthetic will be used to reduce discomfort experienced during the procedure. Pain relief will be provided as required following the procedure. There is a risk of bleeding. Subjects who are on Aspirin or Clopidogrel will be asked to remain off Aspirin/Clopidogrel for 5-7 days prior to the biopsy to reduce the risk of bleeding. A sterile technique will be used to reduce the risk of infection. The biopsy will be performed under ultrasound guidance to increase the accuracy of sampling and reduce the risk of damaging nearby structures. If any complications occur during the procedure, you may have to be admitted to the hospital for closer observation.

## ARE THERE ANY BENEFITS INVOLVED IN PARTICIPATING?

The benefits of this study include :

- 1) Developing a tool to be able to identify the presence of NASH before the development of significant liver scarring without the need for further liver biopsies.
- 2) Aiding the development of a bioclinical tool to monitor people's response to treatment.
- 3) Assess the effectiveness of dietary and exercise interventions for the resolution of NASH and improvement of NAFLD.

## WHAT HAPPENS IF I DO NOT AGREE TO PARTICIPATE?

If you do not wish to participate you will continue your regular outpatient follow up with the Hepatology Department

## WILL MY PARTICIPATION OR WITHDRAWAL HAVE ANY IMPACT ON MY ROUTINE CARE?

You can withdraw at any stage of the study and your participation will not have any impact on your routine care.

## WILL MY PARTICIPATION BE CONFIDENTIAL?

Your participation in this study will be completely confidential.



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The research team are covered by standard medical malpractice insurance. Nothing in this document restricts or curtails your rights.

## WHO IS ORGANISING AND FUNDING THIS RESEARCH?

This study has been organised by the Hepatology department in St. James' Hospital and it is funded by Trinity College Dublin

## HAS THIS STUDY REVIEWED BY AN ETHICS COMMITTEE?

Yes

## CONTACT DETAILS

Name: Sara Naimimohasses

Email: [SNaimimohasses@stjames.ie](mailto:SNaimimohasses@stjames.ie)

Address: Hepatology Department, St. James' Hospital, James' St., Dublin, Dublin 8

Phone: (01) 416 2688

This study and this consent form have been explained to me. A member of the research team has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study. I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement.

**PARTICIPANT'S NAME:** \_\_\_\_\_

**PARTICIPANT'S SIGNATURE:** \_\_\_\_\_ **Date:** \_\_\_\_\_

**Date on which the participant was first furnished with this form:** \_\_\_\_\_

Where the participant is capable of comprehending the nature, significance and scope of the consent required, but is physically unable to sign written consent, signatures of two witnesses present when consent was given by the participant to a registered medical practitioner treating him or her for the illness.

NAME \_\_\_\_\_ OF \_\_\_\_\_ 1st \_\_\_\_\_ WITNESS:  
SIGNATURE: \_\_\_\_\_

NAME \_\_\_\_\_ OF \_\_\_\_\_ 2ND \_\_\_\_\_ WITNESS: \_\_\_\_\_ SIGNATURE:  
\_\_\_\_\_

**Statement of investigator's responsibility:** I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

**Investigator signature:** \_\_\_\_\_ **Date:** \_\_\_\_\_





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## PARTICIPANT INFORMATION AND CONSENT FORM

**STUDY TITLE:** Evaluating the Prognostic Benefit Fibroscan scores in obese patients with NAFLD undergoing Bariatric Surgery

NAME OF PRINCIPAL INVESTIGATOR: Professor Donal O'Shea

## INTRODUCTION:

Studies have shown that individuals who have diabetes, are overweight or obese are at increased risk of developing Non-alcoholic fatty liver disease (NAFLD).

It is caused by too much fat on the liver in the absence of excessive alcohol intake and in 20% of people it can lead to fat-related inflammation of the liver. This is called non-alcoholic steato-hepatitis (NASH). NASH is the more aggressive form of fatty liver disease or NAFLD, and NASH can lead to liver damage and scarring (fibrosis).

At present routine blood tests and a liver scan (Fibroscan) can help identify patients who are at high risk of advanced liver scarring (fibrosis). Current international guidelines recommend that individuals who are identified as having a high risk of advanced liver scarring have a liver biopsy to confirm the severity of liver disease and continue their follow up in a specialist Hepatology clinic.

## WHAT IS THE PURPOSE OF THIS STUDY?

The aim of the study is to:

- 1) Assess the severity of NAFLD in patients attending the National Weight Management clinic in St. Columcille's Hospital
- 2) Refer patients who have significant liver scarring (fibrosis) to a Hepatology unit for continued care
- 3)

## WHY HAVE I BEEN CHOSEN?

You have been chosen to participate in this study because you have identifiable risk factors for developing NAFLD.

## WHAT WILL HAPPEN IF I VOLUNTEER?

Your participation is entirely voluntary. If you initially decide to take part you can subsequently change your mind without difficulty. This will not affect your future treatment in any way.

If you agree to participate, you will be requested to have a liver scan (Fibroscan) on the same day as your clinic review. This scan is recommended as part of routine clinical practice for estimating the severity of liver disease.

A Fibroscan is similar to an ultrasound that measures the stiffness of your liver. You will be required to fast for four hours before the scan.

During the procedure, the Fibroscan uses an ultrasound probe which emits a mechanical pulse at the surface of the skin, measuring the condition of the liver through sound waves. The data is analysed by a computer which displays a two-dimensional picture of the liver. It is used to measure the level of scarring (fibrosis) in the liver.

During the procedure you will be asked to lie on your back with your right arm raised behind your head. The procedure does not normally take more than fifteen minutes. There is no need for an anaesthetic and there are no side-effects. Please see the image below for an example of what a Fibroscan procedure looks like.



No additional blood samples will be taken.

You will be informed of the result of the scan on the same day.

If your test results are suggestive of advanced scarring (fibrosis) a liver biopsy will be discussed with you as recommended by current international guidelines. This test is recommended by current international guidelines and can be scheduled at the same time as your Bariatric surgery. In addition, you will be referred to the Hepatology Department in St. James' Hospital, for continued specialist care.

ARE THERE ANY BENEFITS FROM MY PARTICIPATION?

You may or may not benefit from your participation in this study.

ARE THERE ANY RISKS INVOLVED IN PARTICIPATING?

There are no risks associated with participation in this study.

#### WHAT HAPPENS IF I DO NOT AGREE TO PARTICIPATE?

If you decide not to participate in this study your treatment will not be affected in any way.

#### CONFIDENTIALITY

Your identity will remain confidential. A study number will identify you. Your name will not be published or disclosed to anyone.

#### COMPENSATION

Your doctors are adequately insured by virtue of their participation in the clinical indemnity scheme.

#### WHO IS ORGANISING AND FUNDING THIS RESEARCH?

This study is organised by St. Columcille's and St. James' Hospital

#### **WILL I BE PAID FOR TAKING PART IN THIS STUDY?**

There will be no payment for participation in this study

#### **WILL MY EXPENSES BE COVERED FOR TAKING PART IN THIS STUDY?**

There will be no additional expenses for taking part in this study

#### HAS THIS STUDY BEEN REVIEWED BY AN ETHICS COMMITTEE?

The St. Vincent's Healthcare Group, Ethics and Medical Research Committee have reviewed and approved this study.

#### CONTACT DETAILS

Name: Dr Johan Meurling

Email: [imran.meurling@hse.ie](mailto:imran.meurling@hse.ie)

Address: National Weight Management Clinic  
St. Columcille's  
Phone: (01) 211 5040



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**RESEARCH PARTICIPANT'S RIGHTS**

If you have any questions about your rights as a research participant, then you may contact the Hospital's Quality & Patient Safety Department 01 2214013

PLEASE TICK YOUR RESPONSE IN THE APPROPRIATE BOX

- I have read and understood the Participant Information YES ☐ NO ☐
- I have had the opportunity to ask questions and discuss the study YES ☐ NO ☐
- I have received satisfactory answers to all my questions YES ☐ NO ☐
- I have received enough information about this study YES ☐ NO ☐
- I understand that I am free to withdraw from the study at any time without giving a reason and without this affecting my future medical care YES ☐ NO ☐
- I agree to take part in the study YES ☐ NO ☐

Participant's Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Participant's Name in print: \_\_\_\_\_

Investigator's Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Investigator's Name in print: \_\_\_\_\_

# Inflammaging in Patients with NAFLD: A study of Cognition, Sarcopaenia and Frailty in Chronic Liver Disease

DEPARTMENT: Hepatology

**Dear Mr., Ms., Dr.,** You are being invited to participate in a research study. Thank you for taking time to read this.

Who is carrying out this research?: Researchers from the Hepatology Department in St James' Hospital are carrying out this study to investigate the effects of Non-alcoholic fatty liver disease on memory, concentration, blood pressure changes and muscle strength.

Introduction:

Non-alcoholic fatty liver disease (NAFLD) has now become the most common cause of liver disease in the developed world and is believed to affect more than 1 in 5 people globally.

It is caused by too much fat on the liver in the absence of excessive alcohol intake and in 20% of people it can lead to fat-related inflammation of the liver. This is called non-alcoholic steatohepatitis (NASH). NASH can lead to liver damage and scarring (fibrosis).

Studies have identified links between diabetes, obesity and reduced memory, concentration, low mood and reduced muscle strength, however this has yet to be explored in patients with NAFLD.

## WHY HAVE I BEEN CHOSEN TO PARTAKE IN THIS STUDY?

You have been chosen to partake in this study because your liver blood tests may have been elevated and your Fibroscan results have indicated that you have NAFLD.

### THE AIM OF THIS STUDY IS AS FOLLOWS;

- 1) To investigate features of accelerated ageing, including assessments of attention, cognition, memory, blood pressure variability and frailty amongst patients with NAFLD.

## WHAT DOES THE STUDY INVOLVE?

This study will involve taking additional research blood tests, memory, attention, cognitive tests, mobility assessments, blood pressure measurements and grip strength. The visit will be approximately 30 minutes in duration.

### **At your attendance to the clinic following measurements will be taken:**

Blood tests will be performed to check your liver function, diabetes, cholesterol and experimental bloods will also be taken to assess for inflammation.(This will consist of 5 additional vials of blood)



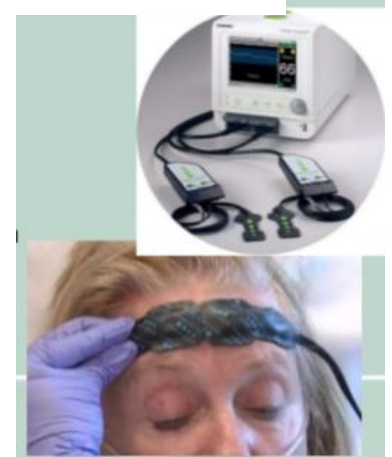


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undergo tests to assess variation in your blood pressure and blood flow to the brain that may occur when you stand up and sit down. This will involve the use of a Near-Infra-red spectrometer or NIRS device. This is a small device which fits around your forehead and measures the oxygen level in the blood using infrared light. We use this to estimate blood flow to the brain. The device will be attached by one of our engineers and you will also have a device to enable continuous measurement of your blood pressure. You will be asked to lie down on your back initially for a few minutes and then be asked to stand. The machine will record any changes that occur in the measurements when you stand.



This visit will take approximately 30 minutes.

Benefits: Full analysis of fitness levels, activity levels and body composition measurements, along with exercise progression and tolerance will be provided to volunteers on completion of the testing procedures, if requested, which may be of potential benefit.

## **There are no significant risks involved in participating in this study**

Exclusion from participation:

You cannot be in this study if you are pregnant or:

- You are unable to give informed consent.
- You are co-infected with HIV or Hep B.
- You are currently undergoing drug treatment for HIV or Hep B
- Anyone for whom exercise is not allowed as per results of the PAR-Q or anyone who is experiencing any of the following:
  1. Heart attack or any cardiac event within 2 days
  2. Unstable chest pains
  3. Uncontrolled irregular heart rhythms causing symptoms
  4. Heart failure causing symptoms
  5. Narrowing of the aorta
  6. Weakness or bulging of artery walls
  7. Current infection of the heart
  8. Current blockage or clot in a lung blood vessel
  9. Current infection accompanied by fever, body aches or swollen lymph glands.

Please ask your doctor or inform the research team if you feel you may fall under any of the above items.



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## WHAT HAPPENS IF I AGREE TO PARTICIPATE IN THE STUDY?

If you volunteer for the study, you will be called in for an in depth assessment on the day you attend the Hepatology clinic.

## ARE THERE ANY RISKS INVOLVED IN PARTICIPATING?

There are no risks associated with participating in this study.

## ARE THERE ANY BENEFITS INVOLVED IN PARTICIPATING?

The benefits of this study include:

- 4) To characterise the degree, if any, of impairment in cognition, blood pressure control and muscle strength in patients with NAFLD.

## WHAT HAPPENS IF I DO NOT AGREE TO PARTICIPATE?

If you do not wish to participate you will continue your regular outpatient follow up with the Hepatology Department

## WILL MY PARTICIPATION OR WITHDRAWAL HAVE ANY IMPACT ON MY ROUTINE CARE?

You can withdraw at any stage of the study and your participation will not have any impact on your routine care.

## WILL MY PARTICIPATION BE CONFIDENTIAL?

Your participation in this study will be completely confidential.

## WHO IS ORGANISING AND FUNDING THIS RESEARCH?

This study has been organised by the Hepatology department in St. James' Hospital and it is funded by Trinity College Dublin





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HAS THIS STUDY REVIEWED BY AN ETHICS COMMITTEE?

Yes

CONTACT DETAILS

Name: Dr Sara Naimimohasses

Position: Hepatology Research Fellow

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|                                                                                                                                                                                                                                                          | Yes | No |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|----|
| I have read and understood the Participant Information and have had the opportunity to ask questions and discuss this study.                                                                                                                             |     |    |
| I have received enough information about this study and I have received satisfactory answers to all my questions.                                                                                                                                        |     |    |
| I understand that I am free to withdraw from the study at any time without giving a reason and without this affecting my future medical care.                                                                                                            |     |    |
| I consent to giving blood samples for the purposes of this study                                                                                                                                                                                         |     |    |
| I understand that anonymised data collected for this study will be kept for a 10 years period on a secure password protected server. These data may be used for further research and audit purposes if approved by the research ethics committee.        |     |    |
| I understand that samples collected during this study may be retained in a biobank. They may be stored for a period of 10 years and may be utilized in future research projects where approval from the institutional research ethics board is obtained. |     |    |
| I agree that my anonymised samples, collected during this study may be transferred to other institutions and laboratories both within and outside the Republic of Ireland for testing relate to this study.                                              |     |    |
| I agree to take part in the study                                                                                                                                                                                                                        |     |    |

**PARTICIPANT'S NAME:** \_\_\_\_\_

**PARTICIPANT'S SIGNATURE:** \_\_\_\_\_ **Date:** \_\_\_\_\_

**INVESTIGATOR'S SIGNATURE:** \_\_\_\_\_ **Date:** \_\_\_\_\_



Ospidéal Ollscoile Choláiste na Tríonóide, Baile Átha Cliath.

University Hospital of Trinity College Dublin.

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