To the soul of my beloved mother Sarah

To the souls of my dear sister Hussah and my little nephew Faisal

To you who left this life while I was away from you, I present this work…

UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Human Development & Health Academic Unit

Investigation of the relation between red blood cell omega-3 fatty acids and cardiovascular risk factors in patients with non-alcoholic fatty liver disease

by

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Thesis for the degree of Doctor of Philosophy

in Clinical Nutrition

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ABSTRACT

FACULTY OF MEDICINE

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Thesis for the degree of Doctor of Philosophy

INVESTIGATION OF THE RELATION BETWEEN RED BLOOD CELL OMEGA-3 FATTY ACIDS AND CARDIOVASCULAR RISK FACTORS IN PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE

Albandri Saleh Alshathry

Background: Non-alcoholic fatty liver disease (NAFLD) is a spectrum of conditions ranging from simple fatty liver to non-alcoholic steatohepatitis, fibrosis and cirrhosis. Up to 30% of the global adult population is affected by NAFLD and there is no licensed treatment for this condition. NAFLD has a strong link with cardiovascular disease (CVD), suggesting that NAFLD itself promotes atherogenesis. Benefits are reported for omega-3 fatty acids (mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) in diseases of the cardiovascular system and some guidelines recommend their use. No prior study has related omega-3 fatty acid status to CVD risk in NAFLD patients without intervention.

Aim: The aim of this research was to determine the relation between red blood cell omega-3 fatty acids, as a recognised marker of status of these fatty acids, and cardiovascular risk factors in patients with NAFLD.

Methods: Data and blood samples were collected from 63 patients with NAFLD who were participants in the INSYTE study. Status of omega-3 fatty acids was determined by the level of EPA, DHA, and docosapentaenoic acid (DPA) in red blood cells. Cardiovascular risk was determined using history report, anthropometric measurements, blood pressure, carotid intima-media thickness measurements, and blood biomarkers. The latter included blood lipids and markers of insulin resistance and vascular inflammation. Data from 105 patients with NAFLD from the WELCOME cohort were used to confirm the findings.

Results: The NAFLD patients studied had elevation of a number of cardiovascular risk factors. Most had a body mass index (BMI) indicating obesity, a waist circumference indicating excess abdominal fat, and high systolic blood pressure (SBP). Many had high blood cholesterol, triglycerides and glucose and high cholesterol to high density lipoprotein (HDL)-cholesterol ratio. More than two-thirds of the NAFLD patients had moderate or severe insulin resistance, while more than one third had low grade inflammation according to C-reactive protein concentration. Moreover, more than two-thirds of the patients were being treated for diabetes, hypertension, or hyperlipidaemia. However, diastolic blood pressure was not raised and carotid intima-media thickness was almost normal. The NAFLD patients had low omega-3 status (mean = 5.5) which is indicative of medium risk of CVD. Increasing age was a determinant of increasing omega-3 fatty acid status in these patients. A higher red blood cell DHA status was associated with lower BMI and waist circumference. Higher red blood cell DPA and DHA (and in some cases EPA and omega-3 index) was associated with lower concentrations of some inflammatory markers.

Conclusions: The NAFLD patients studied here have a normal omega-3 fatty acid status, but this indicates a medium risk of CVD. Among these patients, a higher omega-3 status is associated with a better cardiovascular risk factor profile. This suggests that treating patients with NAFLD with omega-3 fatty acids might lower their cardiovascular risk.

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DECLARATION OF AUTHORSHIP

I, Albandri Saleh Alshathry declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Investigation of the relation between red blood cell omega-3 fatty acids and cardiovascular risk factors in patients with non-alcoholic fatty liver disease

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. (26.09.2016) none of this work has been published before submission.

Signed: .....

Date: 11.09.2017 .....

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Definitions and Abbreviations

AA	Arachidonic Acid
ADP	Adenosine Diphosphate
ANOVA	Analysis Of Variance
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
BMI	Body Mass Index
BRC	Biomedical Research Centre
CAD	Coronary Artery Disease
CAM	Cellular Adhesion Molecule
CCA	Common Carotid Artery
CE	Cholesteryl Ester
CHD	Coronary Heart Disease
CHE	Cholesteryl Esterase
CIM	Carotid Intima-Media
CIMT	Carotid Intima-Media Thickness
COX	Cyclooxygenase
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
CoV	Coefficients Of Variation
DBP	Diastolic blood pressure
DHA	Docosahexaenoic Acid
DNA	Desoxyribonucleic Acid
DPA	Docosapentaenoic Acid
DRI	Dietary Reference Intake
ECA	External Carotid Artery
EDTA	Ethylenediaminetetraacetic Acid
EFA	Essential Fatty Acid
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Eicosapentaenoic Acid
ER	Endoplasmic Reticulum
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FFA	Free Fatty Acid
GC	Gas Chromatography
GLP-1	Glucagon-Like Peptide
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulphuric Acid
HDL	High Density Lipoprotein
HOMA	Homeostasis Model Assessment
ICAM-1	Intercellular Adhesion Molecule-1
ICM	Internal Carotid Artery
IDF	International Diabetes Federation

IFN-γ	Interferon Gamma
IHN	Institute Of Human Nutrition
IL	Interleukin
IL-10	Interleukin 10
IL-6	Interleukin 6
IL-8	Interleukin 8
INR	International Normalized Ratio
IQR	Interquartile Range
IR	Insulin Resistance
JNK	C-Jun N-terminal Kinase
LA	Linoleic Acid
LDL	Low Density Lipoprotein
LOX	Lipoxygenase
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
LT	Leukotriene
LYVE-1	Lymphatic Vessel Endothelial Hyaluronan Receptor
MAC-1	Macrophage Antigen-1
MCP-1	Monocyte Chemoattractant Protein 1
MMP-9	Metalloproteinase-9
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
MS	Metabolic Syndrome
MUFA	Monounsaturated Fatty Acid
NAFLD	Non Alcoholic Fatty Liver Disease
NASH	Non Alcoholic Steatohepatitis
NEFA	Non Esterified Fatty Acid
NF-KB	Nuclear Factor Kappa B
NO	Nitric Oxide
P	P-value
PAI-1	Plasminogen Activator Inhibitor-1
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PG	Prostaglandin
PNPLA3	Phospholipase Domain-Containing Protein-3
POD	Presence Of Peroxidase
PPAR-α	Peroxisome Proliferator- Activated Receptor Alpha
PPAR-γ	Peroxisome Proliferator-Activated Receptor Gamma
PUFA	Polyunsaturated Fatty Acid
RBC	Red Blood Cell
SBP	Systolic blood pressure
SD	Standard Deviation
SEM	Standard Error of the Mean
SFA	Saturated Fatty Acid
SPM	Specialized Pro-resolving Lipid Mediator

SPSS	Statistical Package for Social Sciences
SST	Serum Separator Tube
TBS	Tris-Buffered Saline
TC	Total Cholesterol
TG	Triglyceride
TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor Alpha
UPR	Unfolded Protein Response
VAT	Visceral Adipose Tissue
VCAM	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
VOI	Volumes Of Interest
WHO	World Health Organization
Z-score	Standardized Values
α-LNA	α-Linolenic Acid

Chapter 1: **General introduction**

1.1 Context of the study

Non-alcoholic fatty liver disease (NAFLD) has a strong link with cardiovascular disease (CVD) risk. Adiposity, dyslipidaemia, high blood pressure, insulin resistance and inflammation are cardiovascular risk factors related to NAFLD (Bhatia et al., 2012, Samuel and Shulman, 2012, Tarantino and Caputi, 2011, Targher et al., 2010). Omega-3 fatty acids have been shown to play an important role in preventing and treating both CVD and NAFLD (Saravanan et al., 2010, Masterton et al., 2010, Marsman et al., 2011). Substantial benefits are reported for omega-3 fatty acids in relation to diseases of the cardiovascular system and some guidelines recommend use of these agents in some cardiac disorders (Saravanan et al., Harris and von Schacky, 2004, Siscovick et al., 1995, Yokoyama et al., 2007, Albert et al., 2002). Knowing the actual status of omega-3 fatty acids in patients with NAFLD and the relation of these fatty acids with CVD risk is crucial to designing interventions to lower cardiovascular risk in those patients.

The purpose of this research was to investigate the relationships between omega-3 fatty acid status and CVD risk in patients with NAFLD. The measures of omega-3 fatty acid status include eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA) and the omega-3 index. The CVD factors that were investigated include blood lipids, carotid intima-media thickness, blood pressure, insulin resistance, body fatness, and inflammatory markers.

1.2 Non-alcoholic fatty liver disease

1.2.1 Liver structure and function

The liver is located in the right upper quadrant of the abdominal cavity, beneath the diaphragm, and on top of the stomach, right kidney, and intestines. An adult liver weighs about 1.5 kg and consists of two main lobes. Each of these is made up

of eight segments, each of which is a complete functional unit with a single portal pedicle, that supplies oxygenated blood flowing in from the hepatic artery, and a hepatic vein, that supplies nutrient-rich blood flowing in from the hepatic portal vein. Each segment consists of 1,000 lobules (small lobes). These lobules are connected to small ducts (tubes) that in turn connect with larger ducts to form the common hepatic duct. The common hepatic duct transports the bile made by the hepatocytes to the gallbladder and duodenum via the common bile duct. Liver tissue contains two main cell types: Kupffer cells and hepatocytes. Kupffer cells are a type of macrophage that capture and degrade old red blood cells. Hepatocytes are cuboidal epithelial cells that make up the majority of cells in the liver. Hepatocytes perform most of the liver's functions – metabolism, storage, digestion, and bile production. The bile aids digestion by emulsifying lipids. The liver has a central role in metabolism of bilirubin (biosynthesis from from haem), bile salts (the principal mechanism for clearance of cholesterol), carbohydrates (e.g. maintenance of blood glucose within a narrow range and storage of glycogen), amino acids and ammonia (e.g. control of the plasma concentration of amino acids, clearing of portal venous ammonia generated within the gut lumen, conversion of ammonia to urea), proteins (most circulating plasma proteins being produced by hepatocytes), lipids and lipoproteins (synthesis and export of cholesterol, fatty acids, triglycerides and ketone bodies; synthesis, export and clearance of lipoproteins) and toxins (the liver is themajor site of detoxification of xenobiotics and drugs). Interruption to these processes results in the major metabolic consequences of acute and chronic liver disease (Sherlock and Dooley, 2008, Saladin and Miller, 1998).

1.2.2 Definition of non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease is a pathologic state that arises from the deposition of triglyceride (TG) in the liver to more than 5% of liver weight with consumption of alcohol being zero or no more than 10 g/day (Brea and Puzo, 2013, Marchesini et al., 2003, Bhatia et al., 2012a). The term NAFLD covers a series of

liver lesions similar to those induced by alcohol, but not caused by alcohol use (Brea and Puzo, 2013).

In fact, NAFLD consists of a range of liver disease. The progress of the disease starts from simple fat accumulation (steatosis) to non-alcoholic steatohepatitis (NASH) then NASH can develop to cirrhosis; NASH or cirrhotic liver in some cases can develop to hepatocellular carcinoma (Bhatia et al., 2012a) as depicted in Figure 1-1.

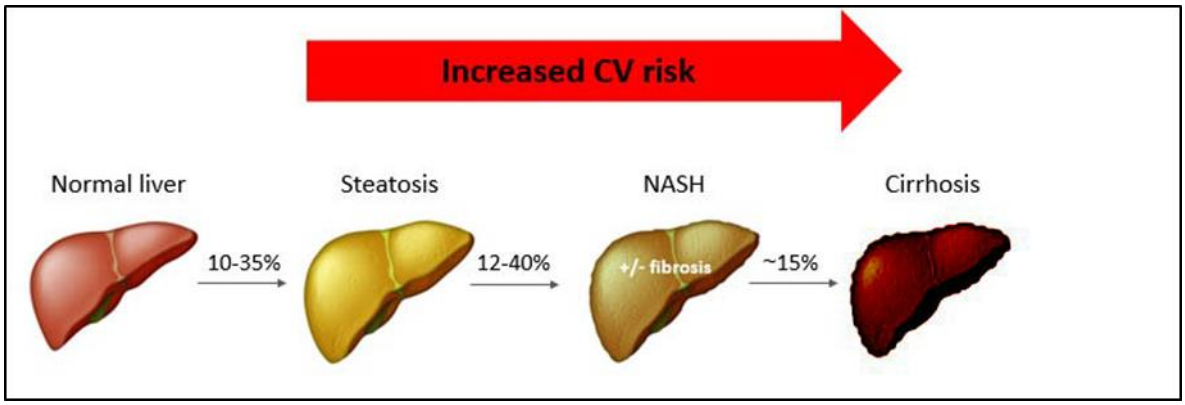


Figure 1-1 Spectrum and progression of NAFLD.
Spectrum and progression of NAFLD (usually over several years) with different grades of severity in each stage developed from simple steatosis and non-alcoholic steatohepatitis. These stages are generally reversible, apart from more severe forms of non alcoholic steatohepatitis + fibrosis. CVD risk is increased as NAFLD becomes more severs. Figure redraw from (Bhatia et al., 2012a).

1.2.3 Steatosis

The steatosis is the first stage of NAFLD. It is defined as an accumulation of fat in the liver. As such, steatosis is related to abnormalities of the synthesis and oxidation of lipid (Parnell et al., 2012). Figure 1-2 shows the appearance of normal and steatotic liver.

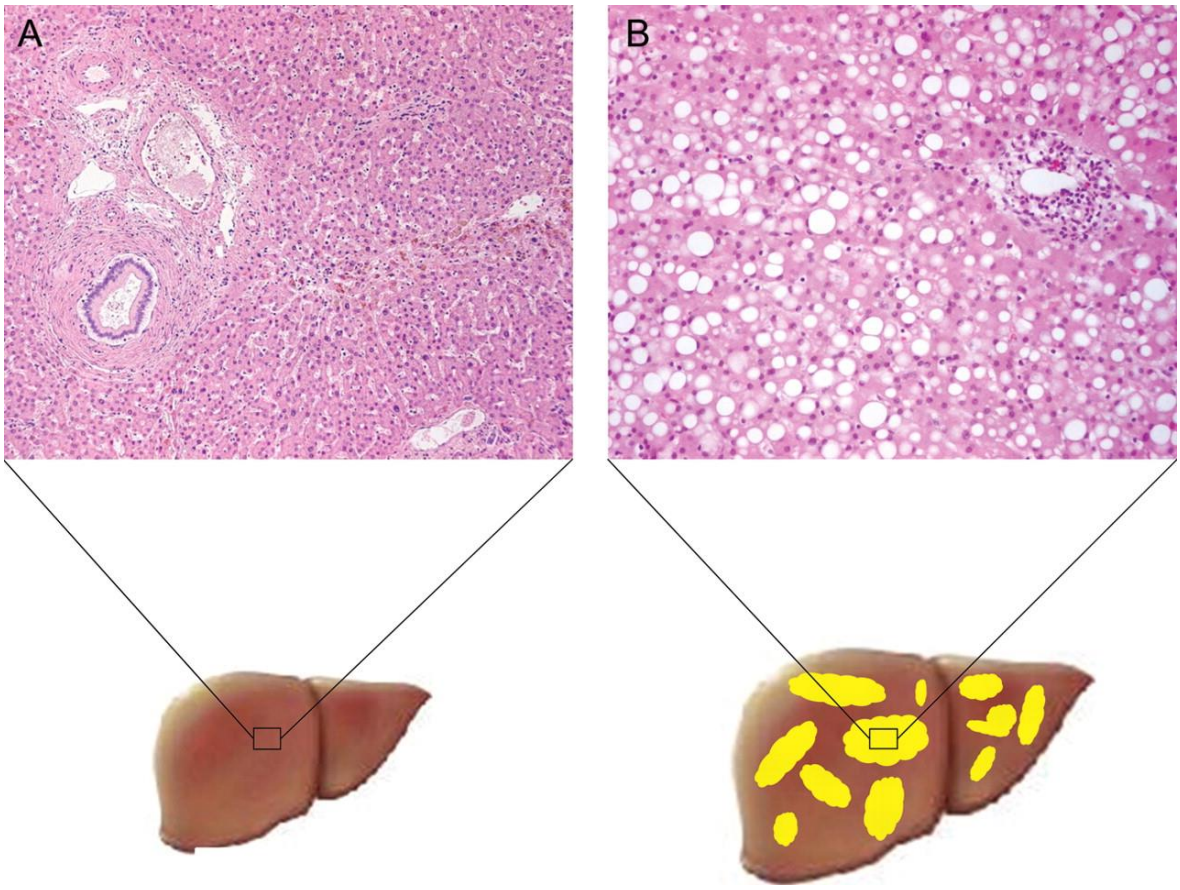


Figure 1-2 Histological section of steatosis.
(A) showing normal liver tissue, (B) showing fat accumulation in hepatocytes.
figure taken from (Bhatia et al., 2012a)

1.2.4 Non-alcoholic steatohepatitis (NASH)

NASH is the second stage of NAFLD, defined as liver inflammation and hepatocyte injury caused by a build-up of fat in the liver. Inflammatory markers like C-reactive protein (CRP), interleukin 6 (IL-6), and tumour necrosis factor alpha (TNF–α) are elevated in the blood of patients with NASH. The NASH stage of NAFLD acts to accelerate disease progression as depicted in Figure 1-1. Furthermore, the presence of inflammation along with steatosis in NASH greatly increases risk of CVD and of mortality compared with simple steatosis (Bhatia et al., 2012a).

1.2.5 Prevalence of NAFLD

The prevalence of NAFLD is about 20-30% in Western Countries (Brea and Puzo, 2013, Marchesini et al., 2003, Bhatia et al., 2012a). That prevalence increases along with increasing type 2 diabetes, obesity and dyslipidaemia. In children, the prevalence ranges from 2.6% in Japan to 9.6% in the United States. In obese children, the incidence of NAFLD increases to 38 % (Parnell et al., 2012). The reported figure for Saudi Arabia is 7-10% of the general adult population (Al-hamoudi et al., 2012); however the prevalence in people with type 2 diabetes increased to 48% (Elmakki et al., 2014). NAFLD is currently the greatest cause of chronic hepatic disease (Bhatia et al., 2012a).

NAFLD can be regarded as the liver manifestation of the metabolic syndrome (MS), which is a condition defined as combinations of visceral obesity with hypertension, type 2 diabetes, , insulin resistance (IR) and dyslipidaemia (Brea and Puzo, 2013, Marchesini et al., 2003, Bhatia et al., 2012a). Many of these features co-exist. 30% of NAFLD patients have MS (Bhatia et al., 2012b), while four-times more people with MS have NAFLD than not (Bhatia et al., 2012a). NAFLD is found in 67% of individuals who are overweight, and in 94% of those who are obese (Argo and Caldwell, 2009).

1.2.6 Morbidity and mortality of NAFLD

Although the mortality risk of CVD doubled in patients with MS, NAFLD itself has a strong link with CVD, suggesting that NAFLD itself promotes atherogenesis. Moreover, NAFLD involves pathological dysfunction of fat tissue, which is correlated to CVD risk (Bhatia et al., 2012a). Although the primary liver pathology in NAFLD affects hepatic structure and function to cause morbidity and mortality from cirrhosis, liver failure and hepatocellular carcinoma, the majority of deaths among NAFLD patients are attributable to CVD (Byrne and Targher, 2015). The

mortality rate for NAFLD is 26% over 5 years, most deaths are from cardiovascular causes but a substantial number result from liver-related diseases (Argo and Caldwell, 2009). According to the Office for National Statistics in the United Kingdom (UK national statistics, 2006), liver disease is now the fifth most common cause of death after heart disease, stroke, chest disease and cancer. However, unlike other major causes of mortality, liver disease rates are increasing rather than declining. A recent UK study showed that mortality rates from 1950 to 2002 in Scotland for subjects with cirrhosis have more than doubled for males and have increased by almost half for females (Leon and McCambridge, 2006, Wang et al., 2012). According to European Association for the Study of the Liver, results from a large population-based cohort of 929465 individuals in the UK found that the chances of dying from NASH, over a 14-year period from 2000 to 2013; were 15% for patients diagnosed with NAFLD and over 20% with NASH and 50% with related-cirrhosis. Congestive cardiac failure was less prevalent in NAFLD than NASH and cirrhosis (Mann et al., 2015). Thus, the severity and mortality increase with the progression of NAFLD.

1.2.7 Pathogenesis of NAFLD

The liver plays a central role in lipid metabolism. It is a site for biosynthesis of cholesterol, fatty acids and triglycerides (TG) and for the assembly and export of TG-rich lipoproteins called very low density lipoproteins. It is also a site for fatty acid beta-oxidation and synthesis of ketone bodies. The liver can also take up free fatty acids (FFAs) from the plasma. If these are not oxidised as a source of energy, they are exported or stored as TG. A series of abnormalities in local and systemic factors which control the balance between flow, oxidation and export of lipids leads to the accumulation of triglycerides in the liver (Brea and Puzo, 2013). In the pathogenesis of NAFLD, insulin resistance (IR) and obesity are important factors because they increase the flow of FFAs to the liver from visceral and subcutaneous fat (Brea and Puzo, 2013). The influx of FFAs may exceed the capacity to oxidise

them, promoting triglyceride synthesis and storage. Hence, control of the synthesis of triglycerides in the liver may be the key to reduce NAFLD (Ahmed and Byrne, 2009). The data support the hypothesis that liver steatosis leads to liver IR through activating gluconeogenesis and stimulating the c-Jun N-terminal kinase (JNK) pathway. Accumulation of liver fat promotes IR per se through defective intracellular insulin signalling as shown in Figure 1-3. In fact, IR can enhance the development of hepatic steatosis and vice versa (Wei et al., 2009). Thus, whether IR is a cause or effect of NAFLD is a matter of on-going debate (Tarantino and Caputi, 2011).

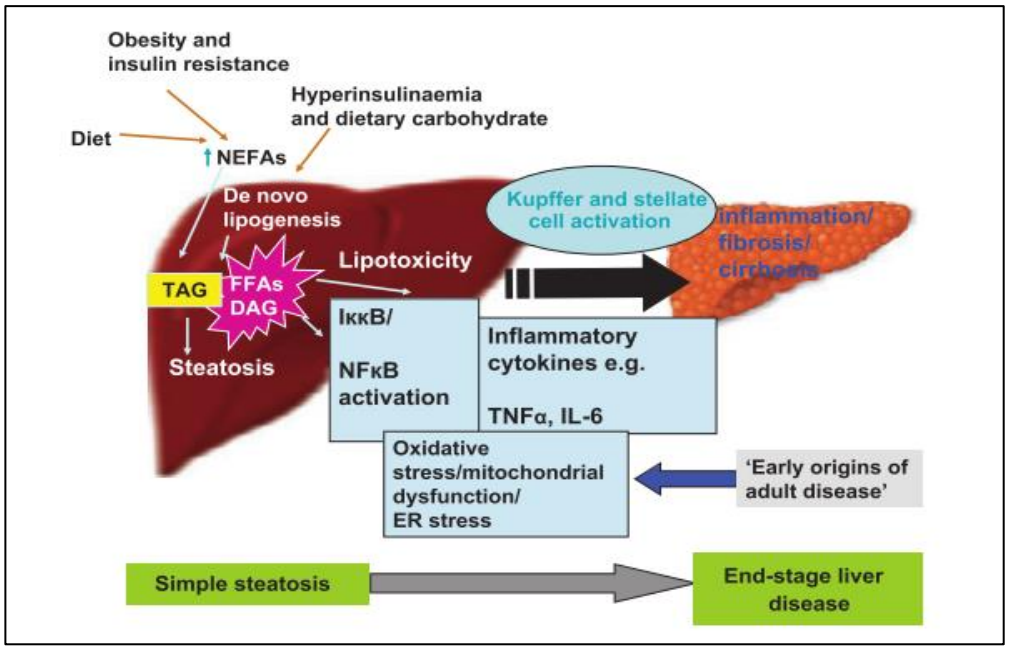


Figure 1-3 Interaction of factors involved in NAFLD.
Showing the importance of insulin resistance Initial insulin resistance and fatty acid release from adipocytes promotes hepatic steatosis. Increased fatty acid fluxes through the liver promote hepatic gluconeogenesis, worsening hepatic insulin resistance and potentially worsening of whole-body insulin resistance with adverse changes in cardio-metabolic risk factors. ER, endoplasmic reticulum; FFAs, free fatty acids; IκkB, inhibitor of nuclear factor kappa-B kinase subunit beta; IL-6, interleukin 6; NEFAs, non-esterified fatty acids; NFκB, nuclear factor kappa B; TAG, triacylglycerol; TNF–α, tumour necrosis factor α. Figure taken from (Byrne, 2012).

However, hepatic steatosis and even NASH can develop in some patients with no evidence of IR and obesity, even though few NAFLD patients are non-obese and non-insulin resistant, and direct evidence for a causal relationship between hepatic steatosis and insulin resistance is incomplete, although a correlation between fatty liver and IR is well established (Wei et al., 2009).

1.3 Cardiovascular diseases

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels, including heart disease, peripheral vascular disease and cerebrovascular disease. These diseases are caused by atherosclerosis which is the build-up of fatty deposits in the blood vessel walls (i.e. in the sub-endothelial space) (Targher et al., 2010). Manifestations of CVD include angina, myocardial infarction and stroke.

CVD is one of the main causes of death worldwide (Abubakar et al., 2015). CVD causes over four million deaths every year in Europe and is responsible for 52% of total mortality in women and 42% in men. In the UK, CVD is one of the main causes of death. CVD was the most common cause of death for women (28% of all female deaths), but not for men, where cancer is now the most common cause of death (32% of all male deaths) (Bhatnagar et al., 2015).

1.3.1 Mechanisms linking NAFLD with CVD

Although the contribution of NAFLD to the progress of CVD is still the subject of on-going research, and the mechanisms linking NAFLD with CVD are not yet fully understood, the potential underlying mechanisms are likely to involve insulin resistance, visceral adipose tissue, inflammation, oxidative stress, procoagulant factors, and lipid profile (Munteanu and Mircea, 2014,Wang et al., 2012,Targher et al., 2007). Figure 1-4 describes the possible mechanisms leading to CVD in patients with NAFLD (Targher et al., 2010).

The first potential underlying mechanism is IR. According to Munteanu and Mircea (2014) IR seems to explain the accumulation of triglycerides in the liver, being the main pathogenic link involved in the onset and progression of NAFLD. IR has consequences for the metabolism of fatty acids and carbohydrates. Hyperinsulinemia induces disturbances in the transport of FFAs, leading to the accumulation of fat in key metabolic organs, such as the liver and the skeletal muscles. IR is also associated with increased risk for CVD and atherosclerosis.

The second potential underlying mechanism is accumulation of visceral adipose tissue (VAT). There is a strong connection between the VAT and liver fat. Plasma FFAs are the main source of fat for the liver, because of the increased lipolysis due to the IR. VAT is associated with glucose intolerance, IR, and dyslipidaemia, thus conferring increased risk for CVD. Also, adipose tissue is a metabolically active tissue, and it can release proinflammatory adipocytokines, which in turn increase the risk of CVD and atherosclerosis (Munteanu and Mircea, 2014, Bhatia et al., 2012a).

The third potential underlying mechanism is inflammation. The liver is a key organ in the systemic inflammatory response. In NAFLD, the liver is both a source of inflammatory factors and a target for them. Accumulation of fat in the liver increases the production of proinflammatory cytokines by hepatocytes and by, Kupffer cells and stellate cells. Inflammatory markers like CRP, IL-6, TNF-α are elevated in patients with NASH and correlate with increased cardiovascular risk, with a progressive increased level of these markers with an increasing severity of the liver disease. These inflammatory changes occur through activation of the transcription factor nuclear factor kappa-B (NF-kB), which, when activated, leads to increased hepatic production of IL-6, interleukin 1 beta (IL-1β), TNF-α. These atherogenic inflammatory cytokines secreted by the liver have an important role in the pathogenesis of systemic inflammation and atherosclerosis (Munteanu and Mircea, 2014, Bhatia et al., 2012a).

The fourth potential underlying mechanism is oxidative stress. In the progression of NAFLD from simple steatosis to NASH, oxidative stress appears to play an important role. The oxidative stress is characterized by increased reactive oxygen species and by increasing lipid peroxidation. In addition, the oxidative stress leads to mitochondrial dysfunction and to an inadequate response of the endoplasmic reticulum. Mitochondrial dysfunction and mitochondrial destruction are associated with both IR and atherosclerosis.

The fifth potential underlying mechanism is an increased level of procoagulant factors. In the presence of NASH, the levels of procoagulant factors like plasminogen activator inhibitor-1 and fibrinogen were observed to be elevated. These factors are closely related to the development of ischemic heart disease and diabetes.

The sixth potential underlying mechanism is the lipid profile. The lipid profile in NAFLD patients includes increased levels of TG, low density lipoprotein (LDL) cholesterol, and very low density lipoprotein (VLDL) cholesterol, and decreased level of high density lipoprotein (HDL) cholesterol. This kind of lipid profile is associated with atherosclerosis and CVD. To reduce hepatic lipid content, it begins to produce excessive levels of TG rich VLDL, That lead to reduction in the quality and quantity of HDL, which is the lipid fraction that is considered protective against CVD and atherosclerosis (Munteanu and Mircea, 2014).

The end result of the increase in risk factors is atherosclerosis. This involves increased vascular wall intimal thickness. As such carotid intima-media thickness is a marker of subclinical and asymptomatic atherosclerotic vascular diseases (Targher et al., 2010, Del Ben et al., 2012).

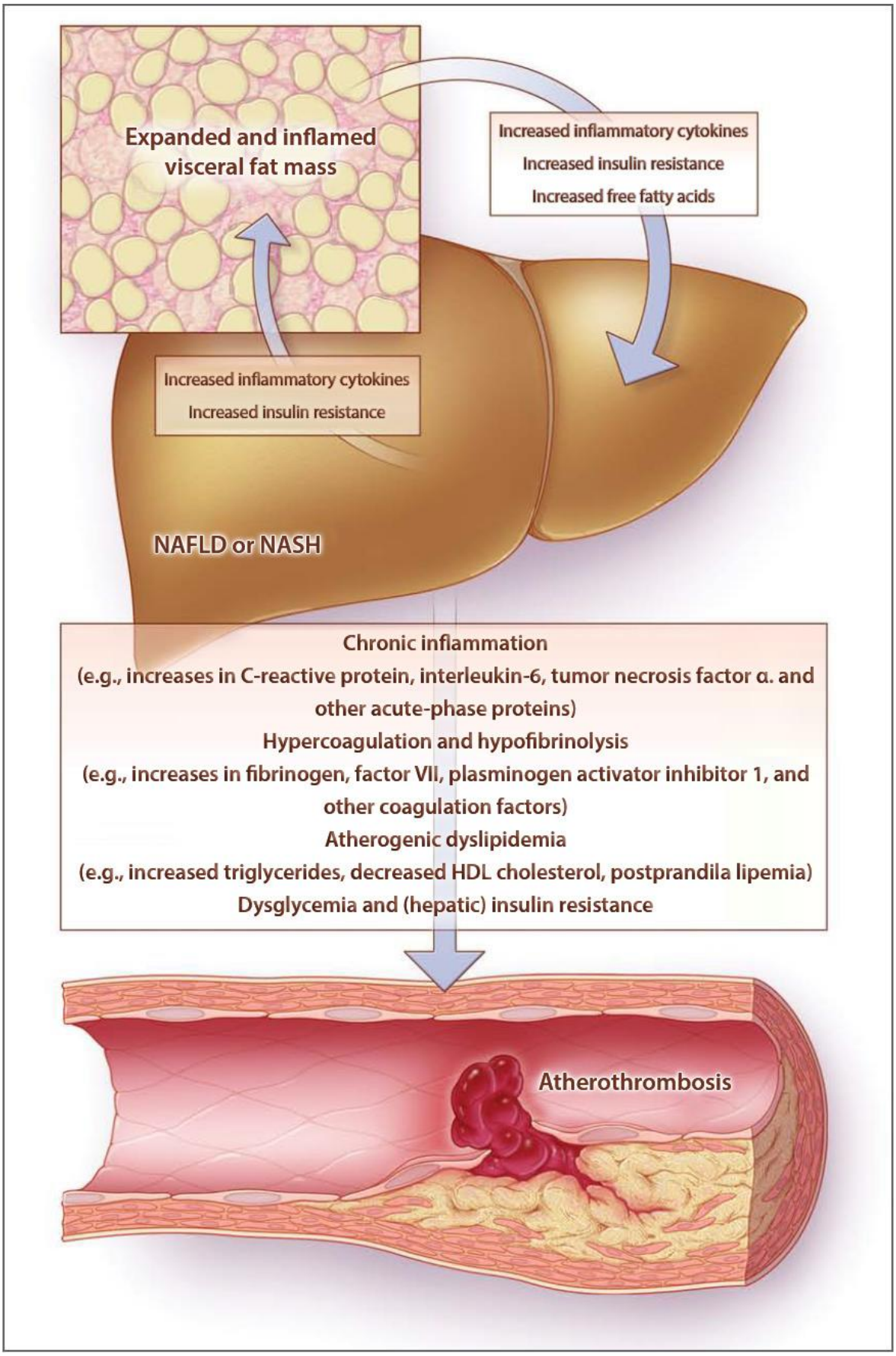


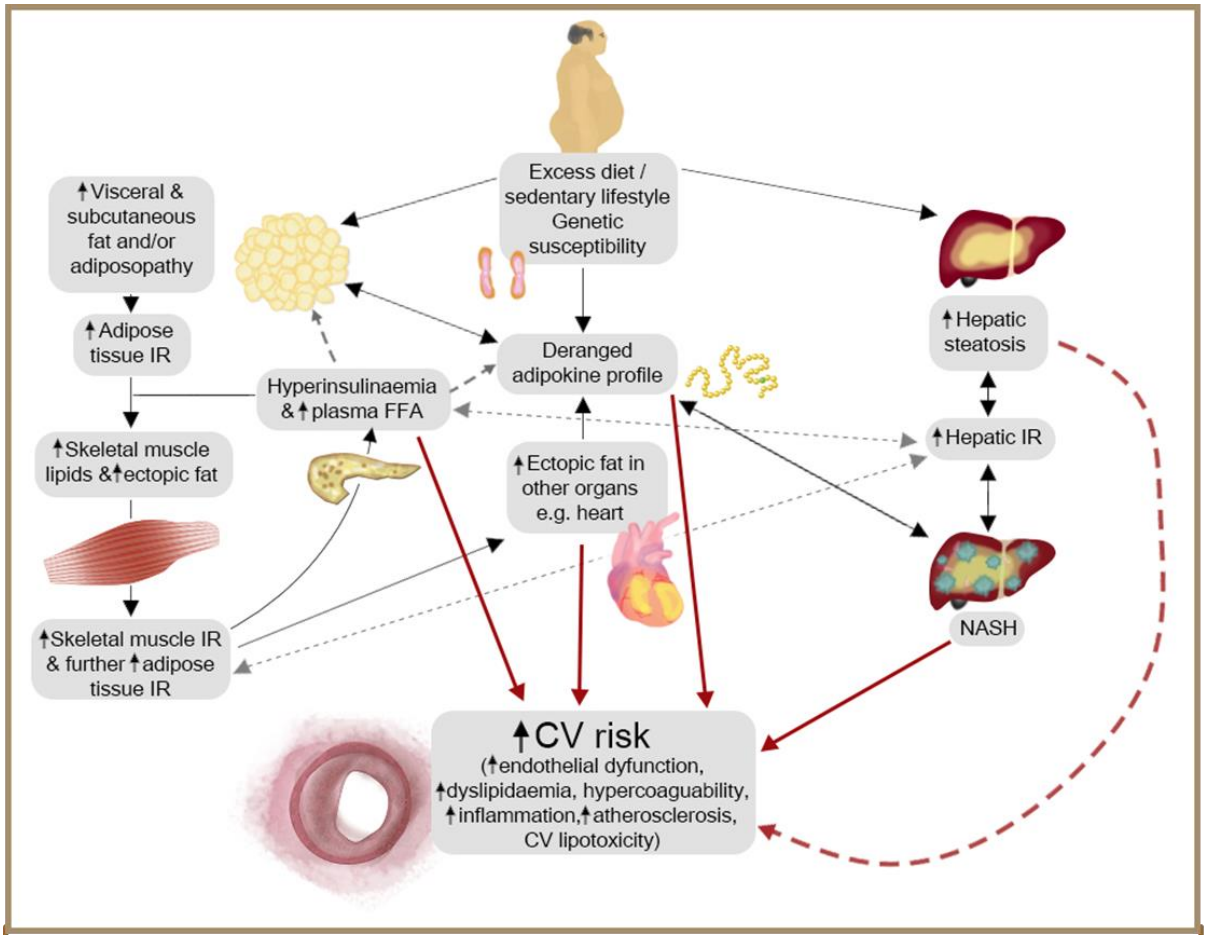
Figure 1-4 Possible mechanisms linking NAFLD with CVD.
Figure taken from (Targher et al., 2010).

1.4 Cardiovascular risk factors in NAFLD

A risk factor is a characteristic that is related to a subsequent occurrence of a disease. Adiposity, dyslipidaemia, high blood pressure, insulin resistance and inflammation are CV risk factors. These also form the link between NAFLD and CVD.

1.4.1 Adiposity

Visceral adipose tissue (VAT) is positively and independently associated with liver fat (Diaz et al., 2015). Plasma FFAs are the main source of liver TGs in NAFLD, arising in part by increasing lipolysis from insulin resistant adipose tissue. VAT itself could explain the raised CV risk seen with NAFLD instead of hepatic fat content per se. VAT, in addition to being a fat store, is also metabolically active, secreting different adipokines, cytokines, and hormones that serve to regulate hepatic fat, inflammation, IR, and affect CV disease outcome as shown in Figure 1-5 (Bhatia et al., 2012a).



Schematic diagram of the pathophysiological processes involved in non-alcoholic fatty liver disease leading to increased CV risk, highlighting the complex inter-relationships between visceral adipose tissue, adipocytokines, insulin resistance, ectopic fat accumulation and non-alcoholic fatty liver disease. FFA, free fatty acids

Figure 1-5 Pathophysiological processes involved in NAFLD leading to increased CV risk. Figure redrawn from (Bhatia et al., 2012a).

In addition to the adverse effect of visceral fat, abnormal ectopic fat storage like increased myocardial and epicardial fat could be amongst the cumulative effects of IR and NAFLD with consequent adverse CV outcome (Bhatia et al., 2012a). Furthermore, obesity strictly linked to low grade chronic inflammation causes insulin resistance that interacts with other complex mechanisms like hypertension, hypercholesterolemia, and hyperglycaemia to elevate the risk of coronary heart disease (Tarantino and Caputi, 2011). Accumulation of ectopic lipid metabolites, activation of innate immune pathways and activation of the unfolded protein

response pathway are all believed to contribute to the pathogenesis of insulin resistance. (Samuel and Shulman, 2012).

1.4.2 Dyslipidaemia

NAFLD is characterised by an atherogenic lipid profile, which includes low HDL cholesterol, high TG levels, an increase in LDL and VLDL cholesterol levels. This kind of atherogenic dyslipidaemia is highly linked to adverse CV outcome (Bhatia et al., 2012a).

Insulin inhibits adipose tissue lipolysis. However, in the case of insulin resistance, lipolysis is overstimulated leading to an increase of the flux of FFAs from adipose tissue. Increasing plasma FFAs from the lipolysis of white adipose tissue is the main contributor to the accumulation of triglyceride in NAFLD (Tarantino and Caputi, 2011). Insulin resistance also decreases the activity of lipoprotein lipase following the elevation of the serum triglyceride level (Wei et al., 2009), thus reducing clearance from the bloodstream.

Additionally, an increasingly insulin resistant individual must secrete more and more insulin in order to prevent the consequences of insulin resistance. However, the combination of insulin resistance with compensatory hyperinsulinaemia increases the possibility that an individual will be hypertensive, and have a dyslipidaemia characterized by a low HDL cholesterol and high plasma TG concentration. These changes have been shown to be associated with increased CVD risk (Tarantino and Caputi, 2011, Reaven, 2011).

CRP can also accelerate atherosclerosis by increasing the expression of PAI-1 and adhesion molecules in endothelial cells, inhibiting nitric oxide formation and increasing LDL uptake into macrophages as shown in Figure 1-6 (Tarantino and Caputi, 2011).

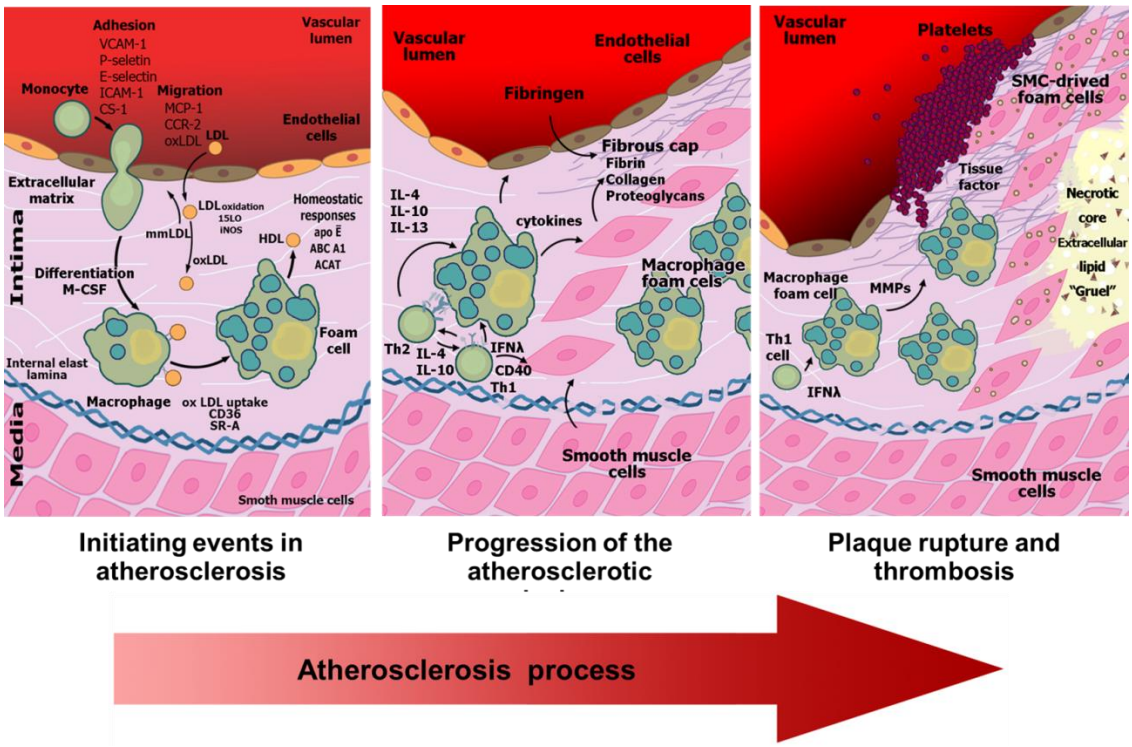


Figure 1-6 The process of atherosclerosis.
The initial steps of atherosclerosis include adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes recruited into macrophages, and their uptake of lipid, to form foam cells. Then progression of the atherosclerosis involves the migration of smooth muscle cells from the media to the intima, and the heightened synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans; plaque macrophages and smooth muscle cells died in advancing lesions; extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque. Plaque rupture and thrombosis: shown is a fracture of the plaque's fibrous cap, which has enabled blood coagulation components to come into contact with tissue factors in the plaque's interior, triggering the thrombus that extends into the vessel lumen, where it can impede blood flow. Figure adapted from (Glass and Witztum, 2001).

Carotid intima-media thickness

Carotid intima-media thickness (CIMT) is defined as an echogenic thickening of tissue starting at the luminal-intimal interface and the media-adventitia interface of the common carotid artery (CCA) as shown in Figure 1-7 (Kasliwal et al., 2014). In atherosclerosis, arteries become clogged with fatty substances called plaques or

atheroma. Measurments of carotid intima-media thickness (CIMT) can be used to detect the early stages of atherosclerosis. The test measures the thickness of the inner two layers of the carotid artery (intima and media). Intima-media thickness values of more than 0.9 mm should be considered abnormal. Increasing carotid wall intimal thickness is a marker of subclinical and asymptomatic atherosclerotic vascular diseases (Targher et al., 2010, Del Ben et al., 2012). The amount of lesion in the common carotid artery has been reported to correlate to the extent of atherosclerotic lesions elsewhere in the body (Kasliwal et al., 2014). Consequently, an increase in CIMT is related with both NAFLD and atherosclerosis according to recent studies (Ahmed et al., 2010).

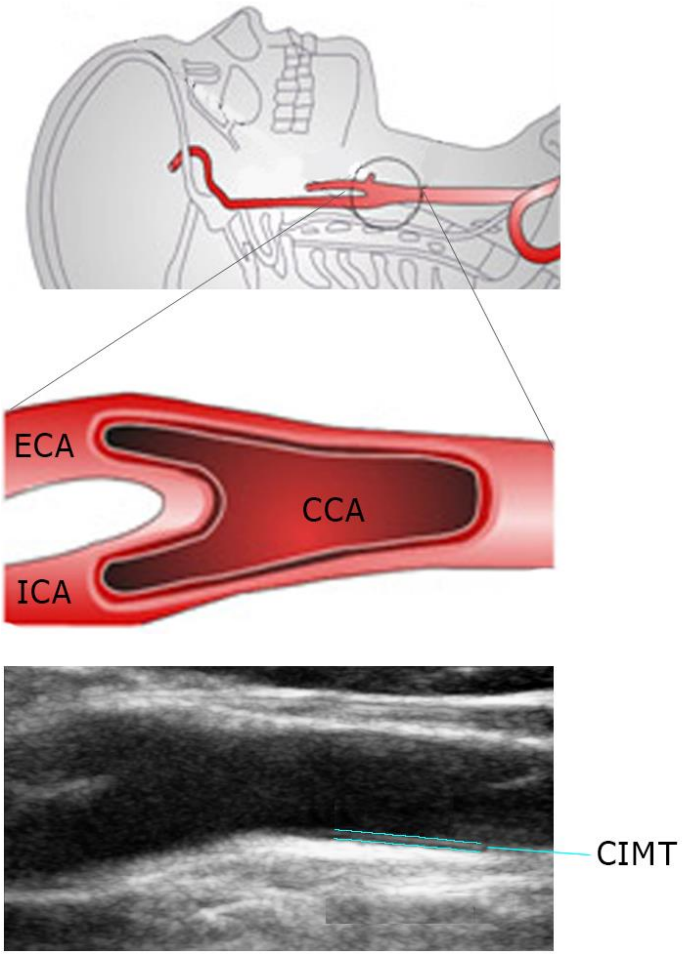


Figure 1-7 The procedure to detect the early stages of atherosclerosis.
Top: Cartoon image of the proper location of the carotid artery located on each side of the neck. Middle: Cartoon image for the common carotid artery dividing into an internal and an external branch. Bottom: An ultrasonographic examination of carotid intima-media thickness taken by the researcher. Abbreviations: common carotid artery (CCA), external carotid artery (ECA); internal carotid artery (ICA).

1.4.3 Blood pressure

The blood pressure level, even in people without hypertension, is significantly associated with NAFLD (López-Suárez et al., 2011). Both systolic and diastolic blood pressure levels have a close relationship with metabolic syndrome, being linked with increasing fasting blood glucose, TC, TG, and LDL (Chen et al., 2007) and hypertension is one of the major factors increasing risk of CVD (Wilson et al., 1998).

1.4.4 Insulin Resistance

Insulin resistance (IR) as a simple definition is a condition in which normal amounts of insulin are insufficient to produce a normal response from the cells of liver, muscle and fat as a result of defects in intracellular signaling pathways. However, the IR state is a multifactorial condition in which overnutrition triggers increased inflammation, changes in lipid metabolism, and changes in the gastrointestinal microbiota, all of which are co-dependent to different degrees (Samuel and Shulman, 2012). It has been shown that in patients with NAFLD the prevalence of insulin resistance is increased (Reaven, 2005)**.** Even though there is general consensus that insulin resistance is a result of defects in intracellular insulin signaling, the development of hepatic insulin resistance has been proposed to describe how these insulin signaling defects appear in NAFLD. Inflammation, activation of endoplasmic reticulum (ER) stress, and accumulation of hepatocellular lipids have all been suggested to cause hepatic insulin resistance in animal models of NAFLD (Wei et al., 2009, Kumashiro et al., 2011). IR in the liver leads to impaired suppression of glucose production by insulin in liver cells leading to hyperglycaemia. One important and initial complication of insulin resistance in the liver is the induction of hepatic VLDL production, through de novo lipogenesis, or through increased FFA flux from adipocytes into the liver. In addition, IR encourages the production of C-reactive protein (CRP) and plasminogen activator inhibitor-1 (PAI-1), which are markers of the inflammatory state. All these

abnormalities are subsequently metabolically related to hepatic IR and have been shown to stimulate atherosclerosis directly or indirectly (Tarantino and Caputi, 2011).

Thus, in IR there are multiple interacting mechanisms (Samuel and Shulman, 2012), that cannot be explained by one etiological pathway. Accumulation of ectopic lipid metabolites, activation of innate immune pathways and activation of the unfolded protein response (UPR) pathway are all believed to contribute to the pathogenesis of insulin resistance. However, these pathways are also closely related to changes in lipogenesis, fatty acid uptake, and energy expenditure that can affect ectopic lipid deposition. Thereafter, these cellular changes may converge to promote the accumulation of specific lipid metabolites (ceramides and/or diacylglycerols) in skeletal muscle and liver, a common final pathway leading to impaired insulin signaling and insulin insensitivity (Samuel and Shulman, 2012).

1.4.5 Inflammation

Inflammation is now recognised to play an important role in atherosclerosis, which results from interaction between modified lipoproteins, monocyte-derived macrophages, T cells and the normal cellular elements of the blood vessel wall as shown in Figure 1-6. The earliest lesions, fatty streaks, are pure inflammatory lesions consisting only of monocyte-derived macrophages and T lymphocytes. Endothelial injury increases the adhesiveness of the endothelium to leukocytes (and platelets) by upregulating expression of adhesion molecules as well as its permeability (Calder, 2012)

Poorer cardiometabolic outcomes with worsening inflammatory and IR states are the result of increasing severity of NAFLD. Hepatic steatosis can activate liver inflammation over lipotoxicity and endoplasmic reticulum oxidative stress responses, along with mitochondrial dysfunction through oxidation of excess fatty acids. In several studies there are associations of mitochondrial dysfunction with atherosclerosis and IR, showing a probable link between rising CV risk and NAFLD

(Bhatia et al., 2012a). In the case of insulin resistance, lipolysis is overstimulated leading to an increase of the flux of free fatty acids (FFAs). Increasing plasma FFAs from the lipolysis of white adipose tissue is the main contributor to the accumulation of triglyceride in NAFLD (Tarantino and Caputi, 2011). Circulating FFAs may be cytotoxic by encouraging hepatocyte apoptosis and lipid peroxidation. Often insulin resistance is associated with chronic low-grade inflammation and the release of numerous mediators from immune cells and adipocytes could contribute to liver disease progression and liver damage (Johnson and Olefsky, 2013, Byrne, 2012).

In addition, IR encourages the production of C-reactive protein (CRP) and plasminogen activator inhibitor-1 (PAI-1), which are markers of the inflammatory state. All these abnormalities are subsequently metabolically related to hepatic IR and have been shown to stimulate atherosclerosis directly or indirectly (Tarantino and Caputi, 2011). It is now commonly accepted that low-grade chronic inflammation associated with obesity induces IR in the liver. Low-grade chronic inflammation is characterized by the production of certain cytokines and adipokines such as IL-6, TNF-α, interleukin-1 (IL-1), leptin and resistin. These factors inhibit insulin signaling in hepatocytes (Tarantino and Caputi, 2011), thus providing a direct link between inflammation and hepatic IR.

In dysfunctional or activated endothelium, an early stage in atherosclerosis, the endothelial cells express various adhesion molecules (CAMs) like intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, responsible for the adherence of leukocytes. These molecules may be looked upon as markers of endothelial dysfunction as they are related to the development of atherosclerosis (Yli-Jama et al., 2002). The injured endothelium also produces vasoactive molecules, cytokines and growth factors. Some of these molecules such as monocyte chemoattractant protein 1 (MCP-1), act as chemoattractants attracting monocytes and T cells to the vessel wall. In accord with the proposed central role of inflammation in the development of the

atherosclerotic plaque, several inflammatory markers present in the serum or plasma are associated with CVD risk. Soluble P-selectin was identified as an independent predictor of future CVD risk in the Women's Health Study. ICAM-1 concentration was shown to be an independent predictor of coronary heart disease. Serum IL-6 concentrations may also be predictive. However, of the inflammatory markers, CRP has been most investigated, and has been found to be predictive of future myocardial infarction, stroke, peripheral vascular disease and CVD mortality (Zimmermann et al., 1999). CRP has been compared with other inflammatory and lipid risk factors and found to be a strong predictor of risk. CRP has been considered simply a marker of inflammation. However, more recent studies have indicated that CRP may play an active role in inflammation by stimulating monocytes to release inflammatory cytokines and by increasing upregulation of adhesion molecule expression and MCP-1 synthesis by endothelial cells (Calder, 2012).

Thus, NAFLD has a strong link with CV risk. Cardiovascular risk factors: adiposity, dyslipidaemia, high blood pressure, insulin resistance and inflammation are related to NAFLD and involved in the pathogenesis of CVD. As will be discussed in the next section, omega-3 fatty acids have been shown to play an important role in preventing and improving NAFLD and CVD risk.

1.5 Omega-3 fatty acids

Omega-3 fatty acids have been shown to play an important role in preventing and treating both CVD and NAFLD. Omega-3 fatty acids definition and metabolism, dietary sources and intakes, red blood cell omega-3 fatty acids and omega-3 index, effect of omega-3 fatty acids status on health, and effect of omega-3 fatty acids on NAFLD and cardiovascular risk are discussed in this section.

1.5.1 Definition and metabolism

Omega-3 fatty acids are a component of foods and of body lipids. Normally fatty acids are found in esterified forms such as a triglycerides, phospholipids and cholesteryl esters in adipose tissue, in tissues such as liver, in cell membranes, and in blood as components of lipoproteins (Gurr et al., 2002). Each lipid pool (e.g. phospholipids) at each location (e.g. a cell or tissue) has a specific fatty acid composition.

Omega-3 fatty acids are a family of long chain (18 carbons) and very long chain (20 and 22 carbons) highly unsaturated fatty acids. They are defined by the position of the methyl terminal double bond. Omega-3 fatty acids include α-linolenic acid (αLNA; 18:3n-3), stearidonic acid (18:4n-3), eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Humans cannot synthesis α-linolenic acid so it must come from the diet; however, other omega-3 fatty acids can come from the diet or from endogenous biosynthesis from α-LNA as shown in Figure 1-8. However, EPA, DPA and especially DHA are poorly synthesised in humans (Calder, 2014). Therefore, preformed sources of these omega-3 fatty acids are likely to be important.

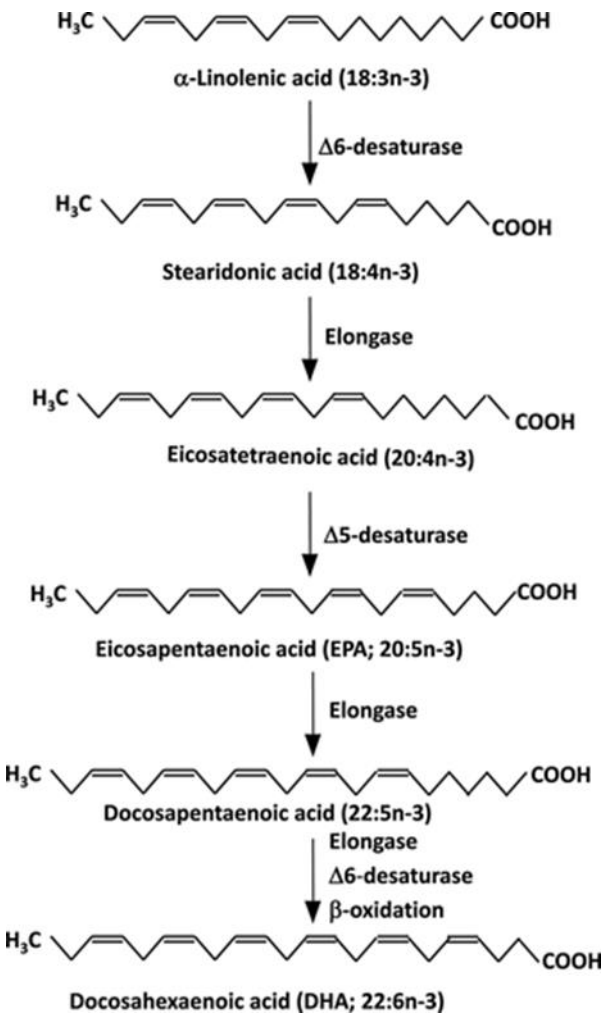


Figure 1-8 A general pathway for the conversion of α-linolenic acid. (18:3n-3) to polyunsaturated fatty acids: eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Taken from (Cunnane et al., 1994, Calder, 2014b).

1.5.2 Dietary sources and intakes

Omega-3 fatty acids have different dietary sources. Plants are a source of α-linolenic acid and it is found in green leaves, some plant oils, some nuts and some seeds. EPA, DPA and DHA are found in sea food, especially oily fish (mackerel, canned pilchards, canned sardines, salmon and trout), and in fish oil supplements (Calder, 2004).

Typical adult intake of α-LA is about 1 g/day Table 1-1. Typical intakes of EPA and DHA are low. Mean UK adult EPA plus DHA intake is about 0.2 g/day, but this

reflects a bimodal distribution since only 25% of the population consumes oily fish, the only dietary constituent that is very rich in these fatty acids (Burdge and Calder, 2005). Mean daily intakes of EPA, DPA and DHA in Australian adults were 0.056, 0.026, and 0.106 g/d respectively (Total = 0.188 g/d); however, median daily intakes of EPA, DPA and DHA were 0.008, 0.006, and 0.015 g/d respectively (Total = 0.029 g/d) see in Table 1-2 (Meyer et al., 2003).

Table 1-1 Consumption of α-linolenic acid among adults in some European countries, and in Australia and North America.
Table adapted from (Burdge and Calder, 2005)

	α-Linolenic acid intake (g·/day)	
	Men	Women
Belgium	1.7	1.4
Denmark	2.2	2.1
France	0.6	0.5
France c	0.9	0.7
Germany	0.9	0.7
Netherlands	1.7	1.2
Italy (a)	0.8	
Spain (a)	0.8	
UK (a)	1.4	
Australia a	1.2	
USA	2.0	1.0
Canada		1.6 (b)

(a) Separate data are not available for men and women
(b) Pregnant women

Table 1-2 Mean and median intakes of omaga-3 fatty acids.
EPA 20:5, DPA 22:5 and DHA 22:6 by adult Australians.
Adapted from (Meyer et al., 2003)

Fatty acid	Mean intakes	Median intakes	Median/mean (%)
n	10,851	10,851	
n-6 PUFA			
18:2	10.8	9.0	83
20:2	0.006	0.001	10
20:3	0.039	0.008	20
20:4	0.052	0.024	46
22:4	0.001	0.000	0
Σ n-6 PUFA	10.9	9.01	83
n-3 PUFA			
18:3	1.17	0.95	81
20:5	0.056	0.008	14
22:5	0.026	0.006	23
22:6	0.106	0.015	14
Σ LC n-3PUFA	0.189	0.029	15
Σ n-3 PUFA	1.36	1.08	74
Total PUFA	12.24	10.28	84

Increased intakes of omega-3 fatty acids are reflected in increased proportions of these fatty acids in blood lipid, blood cell, and many tissue pools (Table 1-3); However, increasing the omega-3 status (i.e. content) in the body by increasing the intake of omega-3 fatty acids is influenced by many factors including age, sex, genotype, dose and duration. In general, women have higher omega-3 status than men; a systemic review of human studies identified that women appear to differ from men in the ability to synthesise EPA and DHA from α-linolenic acid, with also associated differences in omega-3 status (Table 1-3). It is suggested that sex hormones play important roles in men and women to affect omega-3 status (Childs et al., 2008, Lohner et al., 2013). On other hand, some studies report that older people have higher omega-3 status than young. The study of Rees et al (2006) found that older men incorporate EPA more readily than do younger men. In addition, common gene variants are likely to be an important determinant of EPA and DHA status and associated physiological impacts (Minihane, 2016). Researchers tested the effect of genetic variation on the enrichment of EPA and

DHA in red blood cells in NAFLD patients in the WELCOME study, which was a double blind placebo controlled trial investigating the effect of high dose, 4 g/day purified long chain omega-3 fatty acids (Omacor), for 15-18 months. In this study, genetic variation in both patatin-like phospholipase domain-containing protein-3 (PNPLA3) (I148M) and the transmembrane 6 superfamily member 2 protein (TM6SF2) (E167K) were assessed; PNPLA3 148M/M variant was independently associated with percentage of DHA enrichment in red blood cells ($p = 0.036$) but not percentage of EPA enrichment ($p = 0.56$) (Scorletti et al., 2015).

Table 1-3 EPA and DHA content of lipid pools in selected cells and tissues before and after increased intake of EPA and DHA. Abbreviations: EPA, Eicosapentaenoic acid; DHA, docosahexaenoic acid. Table adapted from (Calder, 2015 a).

		Without omega-3 fatty acids intervention			After omega-3 fatty acids intervention		
Tissue/Cell Type	**Lipid fraction**	**EPA**	**DHA**	**Details of EPA +DHA intervention**	**EPA**	**DHA**	**References**
Liver	Total	0.4	6.8	—			Araya et al. (2004)
	Phospholipid	4.8	15.1	—			Elizato et al. (2007)
	Total	1.4	6.3	—			Stephenson et al. (2013)
Adipose tissue	Total	0.2	0.2	13 g/week EPA + DHA for 1 year	0.3	0.4	Browning et al. (2012)
Skeletal muscle	Phospholipid	0.7	1.9	3.4 g/day EPA + DHA for 8 weeks	2.6	4.1	Smith et al. (2011)
	Total	0,6	1.5	4.4 g/day EPA + DHA for 4 weeks	2.4	2.1	McGlory et al. (2014)
Cardiac muscle	Total	0.2	1.5	1 g/day EPA + DHA for 6 months	0.6	2.3	Harris et al. (2004)
	Phospholipid	0.5	4.8	6 g/day EPA + DHA for 33 days	3.0	8.5	Metcalf et al. (2007)
	Phospholipid	0.7	5.7	—			Metcalf et al. (2010)
	Phosphatidylcholine	0.7	3.3	1.7 g/day EPA + DHA for 17 days	1.2	4.5	Saravanan et al. (2010)
Mononuclear cells	Total	0.3	2.8	3.2 g/day EPA + DHA for 12 weeks	3.6	4.9	Yaqoob et al. (2000)
	Total	0.8	1.9	3.3 g/week EPA + DHA for 12 months	0.9	2.3	Browning et al. (2012)
	Total	0.7	1.9	6.6 g/week EPA + DHA for 12 months	2.0	2.6	Browning et al. (2012)
	Total	0.7	1.9	13 g/week EPA + DHA for 1 year	2.3	3.3	Browning et al. (2012)

1.5.3 Red blood cell omega-3 fatty acids and omega-3 index

Red blood cell (RBC) omega-3 fatty acids are a strong long-term marker (averaged over the red blood cell lifespan ≥3 months) of omega-3 fatty acid intake (Harris and von Schacky, 2004), and therefore RBCs are a potentially useful marker of omega-3 status. In contrast, plasma concentrations reflect intake over only the last few days (Arab, 2003). In addition, RBC membrane omega-3 fatty acid composition is more biologically stable than plasma concentrations (Harris and Thomas, 2010), and has been shown to be highly correlated with omega-3 fatty acid concentrations in tissues such as the heart tissues (Harris et al., 2004a).

Harris et al. established the correlation between omega-3 fatty acid levels in heart tissue and in the red blood cells. They defined the omega-3 index as the sum of EPA and DHA in red blood cell membranes expressed as a percentage of total fatty acids. Omega-3 index reflects best the average myocardial tissue content of omega-3 fatty acids. They found the omega-3 index of RBCs the most suitable replacement for cardiac omega-3 content. For that reason, DPA as another omaga-3 fatty acid was not included in the calculation of the omega-3 index. The omega-3 index has been proposed as both a risk marker and risk factor for CHD death. (Harris et al., 2004b). The RBC omega-3 fatty acid response to supplementation was similar to that of the heart; RBCs are easily collected and analysed and they have a less variable fatty acids composition than plasma. Therefore, RBC omega-3 index may be the preferred surrogate for cardiac (and other tissue) omega-3 fatty acid status (Harris et al., 2004a).

1.5.4 Effect of omega-3 fatty acids status on health

Omega-3 fatty acids are bioactive. Therefore omega-3 fatty acids influence cell and tissue metabolism, function and responsiveness; therefore they affect physiology, health status, well-being and disease risk and any change in the omega-3 status will influence health. EPA and DHA are functionally the most important omega-3 fatty acids; however, roles for DPA are now emerging (Calder, 2014, Calder, 2015b). Omega-3 fatty acids are important for the function of cell membranes. They contribute importantly to the biophysical properties of the phospholipid bilayer by providing an appropriate environment for the activities of membrane-associated proteins. In addition, some of them are substrates for the biosynthesis of messengers in signalling pathways. Concentration level of the omega-3 fatty acids in cell membranes produces significant changes in cell function. Optimal tissue function probably requires an optimal concentration of omega-3 fatty acids in cell membranes (Bakewell et al., 2006a).

Omega-3 fatty acids act directly on membrane organization and affect signalling pathways leading to gene expression. An example is within the inflammatory cascade where omega-3 fatty acids act to inhibit NF-κB activation so reducing expression of a range of pro-inflammatory genes. This may be the result of switching the production of inflammatory mediators into more regulatory molecules like resolvins (Serhan et al., 2008). According to Serhan and Recchiuti (2012) chemical mediators derived from omega-3 fatty acids were identified that control the acute inflammatory response by activating local resolution programs. Among these are the specialized pro-resolving lipid mediators (SPMs) that include lipoxins, resolvins, protectins, and maresins. These are enzymatically biosynthesized during resolution of self-limited inflammation. The SPMs are biosynthesized fromboth EPA and DHA (Serhan and Recchiuti, 2012).

1.5.5 Effect of omega-3 fatty acids on NAFLD and cardiovascular risk

The potential for beneficial effects of omaga-3 fatty acids in NAFLD may be related to their effect in regulating hepatic lipid metabolism, adipose tissue function, and inflammation (Pacifico et al., 2015). To date, there have been a few randomized trials investigating the effects of omega-3 fatty acids in adults and children with NAFLD (Pacifico et al., 2015). Omega-3 fatty acids may play an important role in preventing and improving NAFLD. For example, omega-3 fatty acids may reduce hepatic fatty acid content through decreasing hepatic de novo lipogenesis by activation of peroxisome proliferator-activated receptor alpha (PPAR-α). Also, omega-3 fatty acids can decrease insulin resistance, reduce inflammation by reducing activation of NF-kB and lower plasma TG and alanine aminotransferase (Saravanan et al., 2010, Masterton et al., 2010, Marsman et al., 2011). A dose of omega-3 fatty acids of 2–3 g/day decreases plasma levels of TG by 30%, and there is some evidence of benefit in treating atherosclerosis and a theoretical benefit for the use of omega-3 fatty acids in NAFLD (Saravanan et al., 2010). A meta-analysis of omega-3 fatty acid supplementation in adults with NAFLD that included several studies (total of 355 subjects) demonstrated a significant benefit in reducing liver fat but, the optimal dose is currently not known (Parker et al., 2012). The effect of 4 g/day EPA+DHA on 103 NAFLD participants for 15-18 months was to decrease liver fat percentage (Scorletti et al., 2014a, Scorletti et al., 2014b).

Over the last 50 years, a large amount of research has been conducted on the cardiovascular benefits of the omega-3 fatty acids (mainly EPA and DHA) (Minihane, 2013). An omega-3 index (EPA+DHA) of 4% or less has been reported to be associated with the least cardio protection (Harris and von Schacky, 2004), whereas an index of 8% or higher gives the greatest cardio protection (Figure 1-9). Risk of coronary heart disease mortality was inversely associated with the omega-3 index (Harris and von Schacky, 2004).

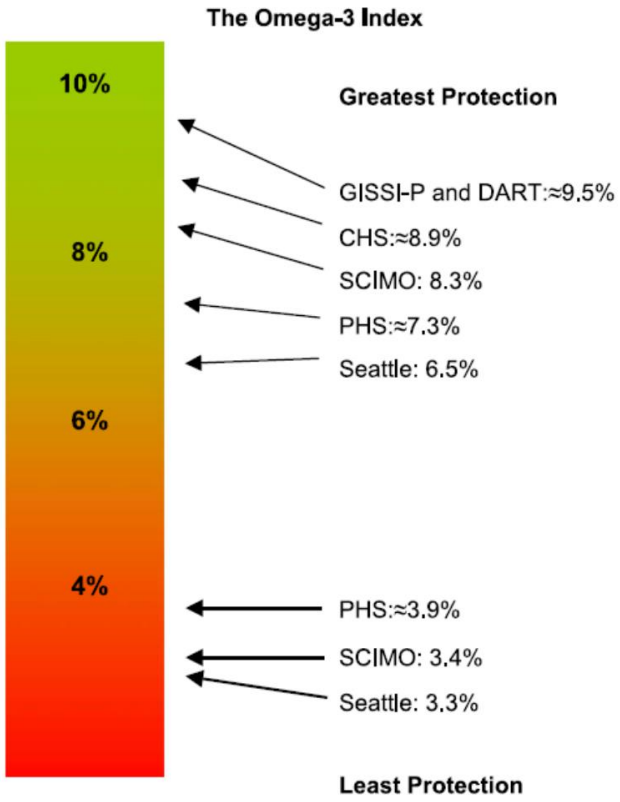


Figure 1-9 Omega-3 Index cut points show that risk of coronary heart disease mortality was inversely associated with the omega-3 Index.
Values in the vertical bar are the omega-3 index (%) from lowest to highest (4%, 6%, 8%, and 10%). In the right side, summary of omega-3 index data sets are represented from different studies: the Seattle study (Seattle) (Siscovick et al., 1995), the Physicians Health Study (PHS) (Albert et al., 2002), the Cardiovascular Health Study (CHS) (Lemaitre et al., 2003), the Diet and Reinfarction Trial (DART) (Burr et al., 1989), the study on the prevention of coronary atherosclerosis by intervention with marine omega-3 fatty acids (SCIMO) (Von Schacky et al., 1999), and the GISSI-Prevenzione study (Trial, 1999). Figure taken from(Harris and von Schacky, 2004).

Substantial benefits are reported in for omega-3 fatty acids in relation to diseases of the cardiovascular system and some guidelines recommend use of these agents in some cardiac disorders (Saravanan et al., 2010). Mechanisms proposed to explain the reduction in blood pressure by omega-3 fatty acids include reduced production of the vasoconstrictor thromboxane A2, increased synthesis of the vasodilator nitric oxide, improved vascular reactivity and compliance, and an effect on autonomic nerve function (Saravanan et al., 2010).

Populations with high consumption of omega-3 fatty acids like Alaska, Northern Canada and Native population in Greenland have 10% rate of predicted CVD mortality. Furthermore, in Western populations many prospective and case-control studies provid a substantial evidence of the role of EPA and DHA in the reduction of CVD risk and outcome. This protective effect of omega-3 fatty acids against CVD development relates to beneficial effects of EPA and DHA on many risk factors; these include reduction of blood triacylglycerols, inflammation and blood pressure (Calder, 2014). The influence of omega-3 fatty acids on the factors involved in cardiovascular risk is summarised in Table1-4.The cardioprotective benefits are reduction in plasma triacylglycerol concentration (fasting and post-prandial), reduction in production of chemoattractants, reduction in production of growth factors, reduction in cell surface expression of adhesion molecules, reduction in production of inflammatory eicosanoids and cytokines, reduction in blood pressure, reduction in thrombosis, and improvement in endothelial relaxation and overall vascular function, and increasing plaque stability, increasing high-density lipoprotein (HDL)-cholesterol (Calder, 2014, Minihane, 2013).

Table 1-4 Factors involved in cardiovascular risk that are influenced by omega-3 fatty acids.

Table adapted from (Calder, 2014)

Factor	Effect of omega-3 fatty acids
Plasma triacylglycerol concentration (fasting and post-prandial)	↓
Production of chemoattractants	↓
Production of growth factors	↓
Cell surface expression of adhesion molecules	↓
Production of inflammatory eicosanoids and cytokines	↓
Blood pressure	↓
Endothelial relaxation	↑
Thrombosis	↓
Cardiac arrhythmias	-/↓
Heart rate variability	↑
Atherosclerotic plaque stability	↑

According to Calder (2012) a key role of arachidonic acid in inflammatory cell membranes is as a precursor of eicosanoids. These are long recognised mediators and regulators of inflammation and immunity and include prostaglandins (PG), thromboxanes, leukotrienes (LT) and other oxidised derivatives produced by the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 pathways. In general arachidonic acid-derived eicosanoids act in a pro-inflammatory way, although this is an oversimplification since it is now recognised that PGE_2, for example, has both pro- and anti-inflammatory effects, and that another eicosanoid produced from arachidonic acid, lipoxin A4, is anti-inflammatory. However, typically the increase in content of omega-3 fatty acids in cell membranes occurs at the expense of omega-6 fatty acids, especially arachidonic acid. Such changes in membrane phospholipid fatty acid composition might be expected to influence the function of cells involved in inflammation through alterations in the physical properties of the membrane such as membrane order ("fluidity") and raft structure; effects on cell signalling pathways, either through modifying the expression, activity or avidity of membrane receptors or modifying intracellular signal transduction mechanisms that alter transcription factor activation and gene expression; and alterations in the pattern of the lipid mediators produced.

Epidemiological and clinical evidence suggests that an increased intake of omega-3 fatty acids protects against mortality from coronary artery disease (CAD) and cardiovascular events. For example, in Seattle and suburban King County, an increase the omega-3 fatty acids intake was related to reduction in the risk for CHD death (Siscovick et al., 1995). A total of 334 case patients with primary cardiac arrest, aged 25 to 74 years, attended by paramedics during 1988 to 1994 and 493 population-based control cases and controls were studied. All cases and controls were free of prior clinical heart disease, major comorbidity, and use of fish oil supplements; cases and control subjects were interviewed to quantify dietary omega-3 fatty acid intake from seafood during the prior month and other clinical

characteristics. The results of this study were compared with no dietary intake of EPA and DHA: an intake of 5.5 g of n-3 fatty acids per month (one fatty fish meal per week) was associated with a 50% reduction in the risk of primary cardiac arrest. A red blood cell omega-3 fatty acid level of 5.0% of total fatty acids was associated with a 70% reduction in the risk of primary cardiac arrest (Siscovick et al., 1995).

Further evidence in CVD prevention was observed in a high-fish-consuming population in Japan. A large-scale clinical trial, the Japan EPA Lipid Intervention Study (JELIS) (Yokoyama et al., 2007) included 18 645 Japanese patients with a total cholesterol of 6·5 mmol/L or greater (14,981 patients with no history of coronary artery disease and 3664 patients with a history), all on statin (LDL cholesterol-lowering drugs) treatment, who were randomized to 1.8 g/day EPA with statin (EPA group; n=9326) or statin only (controls; n=9319) and followed for 4.6 years for major coronary events, including sudden cardiac death, fatal and non-fatal myocardial infarction, and other non-fatal events including unstable angina pectoris, angioplasty, stenting, or coronary artery bypass grafting . Compared with the control group, the EPA group demonstrated a 19% reduction in major coronary events ($p = 0.011$). The beneficial effects of EPA on CHD events was not associated with changes in the levels of total cholesterol, TG, HDL, or LDL, indicating that non-lipid factors played a major role in a cardio-protective effect of EPA. Among the proposed factors that may account for the cardio-protective effects of omega-3 fatty acids are antiarrhythmic effects, blood-pressure lowering, stabilization of atherosclerotic plaques, and decreased platelet aggregation (Harris et al., 2008, Yokoyama et al., 2007).

Albert et al (2002) conducted a prospective, nested case–control analysis among apparently healthy men who were followed for up to 17 years as part of the Physicians' Health Study. The red blood cell fatty-acid composition was measured

for 94 men in whom sudden death occurred as the first manifestation of cardiovascular disease and for 184 controls matched with them for age and smoking status. They found that base-line blood levels of red blood cell omega-3 fatty acids were strongly associated with a reduced risk of sudden death among men without evidence of prior cardiovascular disease (Albert et al., 2002).

Lemaitre et al (2003) investigated the associations of plasma phospholipid concentrations of DHA, EPA, and α-linolenic acid as biomarkers of intake with the risk of incident fatal ischemic heart disease and incident nonfatal myocardial infarction in older adults. They conducted a case-control study nested in the Cardiovascular Health Study, a cohort study of adults aged ≥ 65 y. Cases experienced incident fatal myocardial infarction and other ischemic heart disease death (n = 54) and incident nonfatal myocardial infarction (n = 125). Matched controls were randomly selected (n = 179). They found that a higher concentration of combined DHA and EPA was associated with a lower risk of fatal ischemic heart disease, and a higher concentration of α-linolenic acid with a tendency to lower risk. However they found that omega-3 fatty acids were not associated with nonfatal myocardial infarction (Lemaitre et al., 2003).

In a prospective intervention study, Burr et al (1989) examined the effects of EPA and DHA (derived from fatty fish) intervention in the secondary prevention of myocardial infarction in patients with known CHD. In this study, 2033 men were randomized to either receive or not receive advice to increase their oily fish intake to about 300 g/week. After 2 years of follow up, those receiving the fish advice experienced a 29% reduction in all-cause mortality and a 32% decrease in ischemic heart disease mortality compared to controls. They conclude that a modest intake of fatty fish (two or three portions per week) may reduce mortality in men who have recovered from myocardial infarction (Burr et al., 1989).

The Gruppo Italian per lo Studio della Sopravvivenza nell'Infarto miocardico Prevenzione (GISSI-P) study tested the effects of EPA + DHA supplementation on death from CHD. Myocardial infarction patients (n = 11,324) receiving cardiac pharmacotherapy were randomized to 850 mg/day of EPA + DHA (n=2836), 300 mg/day of vitamin E (n=2830), or the control group (n=2828) with no supplements. The primary combined efficacy endpoint was death, non-fatal myocardial infarction, and stroke. After 3.5 years of follow up, dietary supplementation with EPA + DHA, but not vitamin E, led to a clinically important and statistically significant benefit. The group given just the EPA + DHA experienced a 20% reduction in all-cause mortality, a 35% decrease in cardiac death, and a 45% reduction in sudden death (all $P< 0.01$) compared to the control group (Trial, 1999).

The effects of omega-3 fatty acids supplementation on the course of coronary artery atherosclerosis have been studied. The study on the prevention of coronary atherosclerosis by intervention with marine omega-3 fatty acids (SCIMO), was a randomized, double-blind, placebo-controlled, clinically controlled trial in which quantitative coronary angiography was utilized to follow the effects of omega-3 fatty acids supplementation (3 g EPA + DHA/day for 3 months followed by 1.5 g/day for 21 months) on plaque progression and regression in 223 patients with angiographically proven coronary artery disease over 2 years. This trial, resulted in modestly reduced course of coronary atherosclerosis progression ($p = 0.04$) and a trend towards fewer cardiovascular events (6.25% vs. 1.8%) with omega-3 fatty acid treatment. In addition, the omega-3 index (red blood cell fatty acids EPA + DHA)increased from baseline values of 3.4% to 8.3% after 2 years (Von Schacky et al., 1999).

However, some doubt exists about some established benefits and assumed mechanisms of action, whereas new areas of use and mechanisms are being

identified (Saravanan et al., 2010). Some studies report no effect of omega-3 fatty acids on cardiovascular risk factors in healthy people. (Conquer et al., 1999) investigated the effect of consuming 20 g of encapsulated seal oil containing EPA, DPA; and DHA or 20 g of vegetable oil (control) per day for 42 days, in 19 healthy, normocholesterolemic subjects. Levels of selected cardiovascular risk factors were determined. Ingestion of seal oil raised the coagulant inhibitor, protein C by 17% and decreased plasma fibrinogen by 18%. No alterations in total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglyceride, glucose and systolic and diastolic blood pressures were observed in either group.

1.6 Effect of age and sex on CVD risk, NAFLD, and omega-3 fatty acids

Sex and age are considered to influence both risk factors for CVD (Oyama et al., 2008, Jousilahti et al., 1999) and omega-3 fatty acid status (Bakewell et al., 2006, Childs et al., 2008, Walker et al., 2014, Rees et al., 2006); CVD risk is greater and NAFLD is more common in men than women (Williams et al., 2011) while both CVD risk and NAFLD increase with age.

In both sexes, the risk of CHD increased markedly with age. In most populations, serum total cholesterol increases as age increases. In men, this increase usually levels off around the age of 45 to 50 years, whereas in women, the increase continues sharply until the age of 60 to 65 years. Among young subjects, the overall risk factor level was more advantageous in women. With age, however, this advantage of women decreased markedly.

CVD risk is lower in women than men. Among women, estrogen is the predominant sex hormone. The decrease in estrogen production after menopause changes lipid metabolism in females toward a more atherogenic form by

decreasing the HDL cholesterol level and by increasing low-density lipoprotein (LDL) and total cholesterol, triglyceride, and lipoprotein(a) levels (Matthews et al., 1989). Even though sex differences in serum cholesterol levels and blood pressure disappeared with age, it is possible that the cumulative effects of these risk factors on atherosclerosis remain larger in men than in women, because of the longer exposure time in men. The HDL/total cholesterol ratio was the major determinant of the sex difference in CHD risk, and the increase in risk factor levels, particularly in serum cholesterol and blood pressure, explained a substantial part of the age-related increase in CHD incidence and mortality (Jousilahti et al., 1999). In addition to the lipid effect, estrogen may have cardioprotective effects through glucose metabolism and the haemostatic system, and it may also have a direct effect on endothelial cell function (Shahar et al., 1996).

According to (Jousilahti et al., 1999) the World Health Organization (WHO) MONICA Project and ARIC Study researchers analysed the contribution of sex differences in cardiovascular risk factors to sex differences in CHD mortality among 46 communities. Sex differences in CHD mortality between communities were correlated with sex differences in the following risk factors: smoking, obesity, high blood pressure, high total cholesterol, and low HDL cholesterol. Approximately 40% of the variation in the sex ratios of CHD mortality could be explained by differences in the sex ratios of the 5 risk factors examined (Jackson et al., 1997).

In addition, as for cardiovascular disease, female gender is not a risk for NAFLD, in fact men outnumber women in most of the published series. A prospective study of the prevalence of NAFLD (Williams et al., 2011) within the outpatients of Brooke Army Medical Centre in US showed that in 18 to 70 years old who were recruited; patients with of NAFLD were more likely to be male (58.9%), and older ($p = 0.004$). In China, the peak prevalence of NAFLD occurs earlier (40–49 years) in men than in women (over 50 years) (Fan and Farrell, 2009).

In addition, age and sex influence omega-3 fatty acids status. Rees et al (2006) reported that healthy older male subjects (53-70 years) have significantly higher concentrations of EPA, DPA, and DHA than do younger (18-42 year) healthy male subjects (Rees et al., 2006).

Women have higher levels of omega-3 fatty acid than men. (Childs et al., 2008, Walker et al., 2014). Some human studies show that females have a higher ability to synthesise EPA and DHA from alpha-linolenic acid than males (Burdge and Wootton, 2002, Burdge et al., 2002, Bakewell et al., 2006a). When dietary alpha-linolenic acid, EPA, and DHA intakes were controlled for, women had a greater proportion of DHA in plasma compared with men. This could be related to hormonal regulation of the omega-3 fatty acid biosynthetic pathway. Estrogen cause higher DHA concentrations in women than in men, probably by upregulating synthesis of DHA from vegetable-derived precursors (Giltay et al., 2004).

Thus, effect of sex and age has to be considered in the analysis and interpretation of the data in this research.

1.7 Knowledge gaps

Up to 30% of the global adult population is affected by NAFLD and there is no licensed treatment for this condition. NAFLD has a strong link with CVD and the majority of deaths among NAFLD patients are from cardiovascular causes.

Both the severity and mortality increase with the progression of NAFLD. Therefore, CVD risk is increased as NAFLD becomes more severe. NAFLD has many stages, fat builds up in the liver, which can cause inflammation and, eventually, lead to permanent scarring and this links the severity of the disease with the increased risk of CVD and death. It is therefore imperative that we treat people in the early stages of the disease through diet or use of specific bioactive nutrients before their condition becomes more severe. Omega-3 fatty acids may play an important role

in preventing and improving NAFLD. In addition, benefits are reported for omega-3 fatty acids in diseases of the cardiovascular system and some guidelines recommend their use. The influence of omega-3 fatty acids in control of CV risk in patients with NAFLD is not known.

The omega-3 index, defined as the sum of EPA and DHA in red blood cells, has been proposed as both a risk marker and risk factor for CHD death. The desirable value for omegas-3 index that associated with the lowest CVD risk is ≥ 8% and the undesirable level is ≤ 4% witch associated with a poorer clinical condition (e.g. high risk or incidence of coronary heart disease).

There has been research into the effect of intervention with omega-3 fatty acids on cardiovascular risk and on NAFLD. However, to my knowledge, no prior study has reported the actual status of omega-3 fatty acids and the omega-3 index value in NAFLD patients. In addition, no prior study has related omega-3 fatty acid status to CVD risk in NAFLD patients without increasing the oral intake of omega-3 fatty acids. It would seem important to understand the link between omega-3 fatty acid status and CV risk factors in patients with NAFLD before embarking on intervention trials to increase omega-3 fatty acid intake. This is the focus of the research described in this thesis.

1.8 Research questions and hypotheses

Research question 1:

Are the NAFLD patients studied here at increased cardiovascular risk?

Hypothesis to be tested:

a) That patients with NAFLD will have an elevated risk of cardiovascular disease but that the degree of risk will vary among them.

Research question 2

What is the status of red blood cell omega-3 fatty acids in patients with NAFLD?

Hypotheses to be tested:

a) That patients with NAFLD will have a low omega-3 fatty acid status, as assessed by the omega-3 index.
b) That age and sex will influence omega-3 fatty acid status in patients with NAFLD (higher in women and increased with age).
c) That patients with NAFLD will not have an omega-3 index that provides a high cardio-protection level.

Research question 3:

What is the relationship between red blood cell omega-3 fatty acids and cardiovascular risk factors (blood lipids, blood pressure, hyperglycaemia and insulin resistance, body fatness, and inflammatory markers) in patients with NAFLD?

Hypotheses to be tested:

a) That patients with NAFLD will have an inverse relationship between omega-3 fatty acid status and blood lipids.

b) That patients with NAFLD will have an inverse relationship between omega-3 fatty acid status and blood pressure.

c) That patients with NAFLD will have an inverse relationship between omega-3 fatty acid status and body mass index and waist circumference.

d) That patients with NAFLD will have an inverse relationship between omega-3 fatty acid status with hyperglycaemia and insulin resistance.

e) That patients with NAFLD will have an inverse relationship between omega-3 fatty acid status and inflammatory markers.

Chapter 2: **Methods**

Methods

2.1 Study design

This study is a sub-study of the INSYTE trial; as a result of slow recruitment and because INSYTE is a double blind, randomised trial that is not yet complete, my study has concentrated on the data collected only at baseline from 63 participants.

INSYTE Study design

Investigation of synbiotic treatment in non-alcoholic fatty liver disease (INSYTE) is a double blind, randomised, placebo-controlled trial testing the effects of a synbiotic intervention over 12 to 14 months in 100 patients with non-alcoholic fatty liver disease (NAFLD). Ethical approval for the INSYTE trial was obtained from the Southampton and South West Hampshire Research Ethics Committee (12/SC/0614); a copy of the ethics approval letter is in the Appendix A. The INSYTE trial took place in the Southampton NIHR Biomedical Research Centre (BRC) and the NIHR Wellcome Trust Clinical Research Facility, University Hospital Southampton NHS Foundation Trust. The study protocol number is RHMMED 1071 'INSYTE'.

The research described in this thesis is a sub-study of the INSYTE trial. The thesis does not consider the effects of the intervention with synbiotics but focusses on data collected at baseline before the synbiotic intervention started. The focus of this sub-study is to investigate the relation between omega-3 fatty acid levels in red blood cells and cardiovascular risk factors and cardiovascular risk at baseline in the patients enrolled in the INSTYE trial.

2.1.1 Summary of the INSYTE trial

INSYTE is a randomised, controlled, single centre, double-blind clinical trial addressing the hypothesis that treatment with a synbiotic will have a beneficial effect in NAFLD compared to placebo, as measured by change in liver fat and in serum biomarkers of liver disease. Patients were aged > 18 years with NAFLD, which was either biopsy-proven or confirmed by non-invasive imaging. To be enrolled alcohol consumption needed to be ≤ 14 units/week for women or ≤ 21 units/week for men.

The synbiotic used was fructo-oligosaccharide with a degree of polymerization < 10 at 4 g/twice a day (two sachets a day) plus *Bifidobacterium animalis* subsp. lactis BB-12 at a minimum of 10 billion colony forming units/day (1 capsule a day). The placebo was also provided in a capsule and as powder in a sachet, comprising a total of 4 g/twice a day of maltodextrin. The synbiotic and placebo components were supplied in identical packaging (Figure 2-1). The powder of synbiotic/placebo could be spread on food or stirred into a cold drink (i.e. < 25 degrees), and consumed soon after mixing and certainly within two hours.

Figure 2-1 Packaging of synbiotic and placebo.
The probiotic is packaged as a capsule and the prebiotic as a sachet. Placebo (maltodextrin) was provided as an identical capsule and sachet.

Participants were required to attend clinic visits at the NIHR Wellcome Trust Clinical Research Facility on 6 occasions: visits 1, 2 and 3 at 0 month for baseline measurements, visit 4 at 5-6 months for follow-up measurements, and visits 5 and 6 at 12-14 months for end of study measurements (Figure 2-2). However, data presented in this thesis was from the baseline visits only.

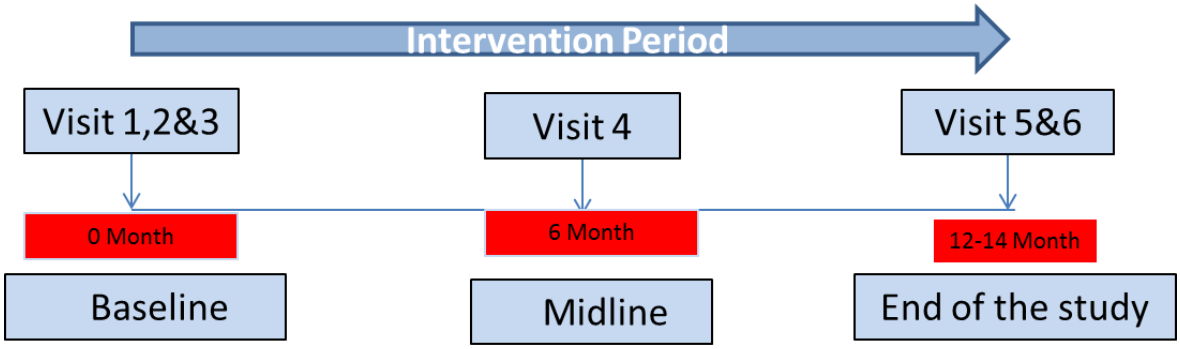


Figure 2-2 Summary of participant's visits during the INSYTE trial.

The primary outcomes of the INSYTE trial were a) liver fat; b) serum biomarkers of liver disease; and c) gut microbiota composition. Secondary outcomes of the INSYTE trial were a) liver fibrosis determined by transient elastography; b) insulin and glucose concentrations and hepatic insulin sensitivity; c) microvascular function; d) plasma cardiovascular risk markers; e) adipose tissue and blood markers of metabolism and inflammation; f) hepatic de novo lipogenesis; and g) satiety and satiety factors.

2.1.2 Patient recruitment

At University Hospital Southampton NHS Foundation Trust, male and female patients with a diagnosis of NAFLD, established as part of their attendance at hospital clinics were contacted by the research team. Contact occurred at their hospital clinic attendance or by letter of invitation from the research team.

Outside Southampton, at Poole Hospital NHS Foundation Trust, Portsmouth Hospitals NHS Trust, Royal Bournemouth and Christchurch Hospitals NHS Foundation Trust, Hampshire Hospitals NHS Foundation Trust, and the Isle of Wight NHS Trust, collaborators of the study (medical doctors responsible for the care of people with NAFLD) acted as 'post boxes' and informed potential participants about the study, providing them with a patient information sheet and asking them if interested to get in touch with the research team members at Southampton listed on the patient information sheet.

After discussion of their diagnostic liver biopsy test, or CT scan, or ultrasound, or MRI results, patients were invited to participate in the research study, by direct contact from a member of the research team, or by letter of invitation.

Each patients had approximately 2 weeks, or longer if needed, to decide upon their participation after initial discussion with the research team doctor or nurse. Consent was obtained by a participating medical doctor or nurse within the research team (see consent form in appendix A). Translators are provided by the NHS Trust for non-English speaking participants.

Both men and women were included. Inclusion criteria were age > 18 years; liver fat diagnosed on normal clinical grounds including in most cases liver assessed by Kleiner scoring system (Kleiner et al., 2005) to classify severity, with no known aetiological factors for underlying liver disease (e.g. exclusion of hepatitis A, B and C, primary biliary cirrhosis, autoimmune hepatitis, haemochromatosis); last liver biopsy (if there had been one) within 3 years prior to recruitment to the study; liver fat diagnosed by ultrasound, CT or magnetic resonance imaging (MRI) within 3 years in patients who also had either diabetes and/or features of the metabolic syndrome, alcohol consumption < 14 units/week for women or 21 units/week for men (Armstrong et al., 2012). Exclusion criteria were alcohol consumption >15 units/week for women and or 22 units/week for men; decompensated acute or chronic liver disease; a history of viral hepatitis, diarrhoea, diverticulosis, actively symptomatic irritable bowel syndrome, inflammatory bowel diseases, coeliac

disease (seropositivity for antiendomysial immunoglobulin A antibodies; IgA EMA); previous bariatric or other abdominal surgery; continuous use of antibiotics that may change gut microbiota or probiotics within the 2 months preceding enrolment, or evidence of immunoglobulin A or immunoglobulin deficiency (both of which produce confounding effects during assessments of intestinal permeability and small intestinal bacterial overgrowth); or body weight above 155 kg as this is the maximum weight capacity for the magnetic resonance imager.

Participants were block randomized according to standardized procedures (computerized randomization) by a person from the University of Southampton who was not connected to the INSYTE study. Stratified randomisation by age and sex to synbiotic or placebo was used. Investigators will remain blinded as to treatment allocation until cessation of the trial in 2018.

2.1.3 Liver fat measurement

Liver fat estimation by magnetic resonance (MR) was undertaken by amember of the Southampton General Hospital radiography staff (Michelle Walkden) at baseline and at the end of the study by both MR imaging and MR spectroscopy, as the techniques provide an estimation of liver fat using different methodologies. Both techniques are quick and non-invasive, and the total scanning time for both techniques combined was ~40 minutes for each participant.

Mean liver fat percentage

The methodology for assessing liver fat is described by Scorletti et al. (2014a). Participants underwent MR spectroscopy (MRS) of the liver to measure the quantity of liver fat accumulated in three discrete liver zones. Three 20 × 20 × 20 mm^3 spectroscopic volumes of interest (VOI) were positioned within segments 3 (inferior sub-segment of the lateral segment), 5 (inferior sub-segment of the anterior segment) and 8 (superior sub-segment of the anterior segment) of the liver, avoiding major blood vessels, intra-hepatic bile ducts, and the lateral margin of the liver. For the second visit scan, these VOI positions were copied from the

first scan, to ensure consistency. A PRESS (point resolved spectroscopy) spin echo pulse sequence was used to acquire the spectroscopic data. The pulse sequence used a TR = 1500 ms, TE = 3 ms, flip angle = 90°, bandwidth = 1000 Hz, 8 averages and acquisition duration of 1024 data points, with no water suppression. The acquisition was obtained in a breath hold examination of 18 s. Spectra were post-processed using Siemens scanner software. This was a fully automated process and involved several steps, starting with filtering the data using a Hanning filter, zero-filling the data, baseline correction, phase correction and finally curve fitting was performed (with 4 iterations) to identify the water and lipid peaks. Values for the lipid and water peak integrals were produced for each VOI and recorded for each subject.

2.1.4 The candidate's involvement in the INSYTE trial

The candidate's work in the INSYTE trial was as follows:

- Blood sample processing, delivery and storage (see table in appendix A);
- Urine sample processing, delivery and storage (see table in appendix A);
- Stool sample processing, delivery and storage (see table in appendix A);
- Laboratory analysis of red blood cell fatty acids and calculation of the omega-3 index;
- Laboratory analysis of inflammatory markers;
- Scanning of carotid intima-media thickness (CIMT) and image analysis;
- Calculation of cardiovascular risk;
- Identification of metabolic syndrome;
- Calculation of HOMA insulin resistance;
- Calculation of body mass index (BMI);

- Collation of data and statistical analysis;
- Bagged up sachets of synbiotic and placebo.

Blood, urine and stool samples were collected with reference to appropriate flow charts. The appropriate sample processing sheet was used to record all details including time and date of sample collection and its delivery to the laboratory, staff ID and patient ID. Where samples were stored frozen they were stored in labelled boxes in a -70/80ºC freezer and details of all aliquots were recorded in a freezer works sheet.

2.1.5 Data used in this thesis (sub-study of INSYTE)

This research uses the baseline measurements from 63 patients with NAFLD to investigate their cardiovascular risk and the omega-3 fatty acid status and the relationship between the two. The outcome measures reported in this thesis are as follows:

- History of reported cardiovascular diseases, hypertension or diabetes, medication use and smoking (data collected and entered in the INSYTE database by INSYTE study nurse and analysed by the candidate) ;
- Anthropometric measurements: weight and height for body mass index (BMI) calculation, and waist circumference for assessment of abdominal obesity (data collected by INSYTE study nurse and entered in the INSYTE database by INSYTE study nurse and analysed by the candidate);
- Red blood cell omega-3 fatty acids (blood samples collected by INSYTE study nurse and processed, stored and analysed by the candidate);
- Blood pressure: systolic and diastolic (data collected by INSYTE study nurse and prepared for analysis and analysed by the candidate);
- Blood markers of cardiovascular risk (measured in plasma or serum or generated from an algorithm) including blood lipids (total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride concentrations) and insulin and

glucose concentrations for calculation of insulin sensitivity as HOMA; (blood samples collected by INSYTE study nurse, processed and stored by the candidate, and analysed by Pathology laboratory staff of Southampton General Hospital);

- Blood inflammation markers: TNF-α, IL-8, IL-6, IL-10, MCP-1, E-Selectin, P-Selectin, VCAM-1, RANTES and ICAM-1 concentrations (blood samples collected by INSYTE study nurse, processed, stored, and analysed by the candidate);
- Carotid artery intima-media thickness (CIMT) (scanning carried out by Dr Eleonora Scorletti and the candidate and images analysed by the candidate);
- Presence of metabolic syndrome (data collected by INSYTE study nurse, processed, stored and analysed by the candidate).

2.2 Methodology for red blood cell fatty acid analysis

2.2.1 Processing of samples for analysis

Blood (6 ml) was taken into lithium heparin. After inversion several times, the blood was centrifuged at 2000 rpm (800 x g) for 15 minutes at room temperature (RT) with no brake. Plasma and 0.5 ml of red blood cell (RBC) pellet were collected separately. Phosphate buffered saline (PBS) (15 ml) was added to the RBCs and the tube mixed by inversion. The tube was centrifuged at 1500 rpm (450 x g) for 10 minutes at room temperature and low brake. Then the PBS was removed and discarded. The cell pellet was re-suspended in 15 ml PBS and centrifuged again at 1500 rpm (450 x g) for 10 minutes at room temperature and low brake. The PBS wash was removed and 0.5 ml PBS was added to the cell pellet which was re-suspended and transferred to a 2 ml aliquot tube. Samples were stored in a -80 ^{o} C freezer until assayed.

2.2.2 Lipid extraction

Defrosted packed RBCs (1 ml) were mixed vigorously with 5 ml chloroform:methanol (2:1 vol/vol); butylated hydroxytoluene (50 mg/L) was included in the chloroform:methanol as an antioxidant. The sample was centrifuged at 1,000 rpm for 2 minutes at RT. After centrifugation the lower organic phase that includes the extracted total lipid was collected. The sample was dried down under nitrogen at 40°C and dissolved in 0.5 ml toluene.

2.2.3 Formation of fatty acid methyl esters

Fatty acid methyl esters (FAMEs) were formed by incubation of the entire lipid extract with 1 ml methanol containing 2% (vol/vol) sulphuric acid at 50°C for 2 hours. After cooling at RT, samples were neutralized by addition of 1 ml of a neutralising solution (0.25 M $KHCO_3$ and 0.5 M K_2CO_3. Then 1 ml hexane was added to the sample and FAMEs were extracted into it and then dried under nitrogen. The sample was diluted in a small volume (150 µl) of hexane, and transferred to a gas chromatography vial for analysis by gas chromatography (GC).

2.2.4 Principle of gas chromatography

The main principle underlying the separation of FAMEs by GC is that fatty acids differ in the temperature at which they become volatile. This temperature depends on carbon chain length, and number and position of double bonds. Increasing chain length increases the temperature at which FAMEs enter the vapour phase (i.e. boil). In contrast, the greater the number of double bonds, the lower the boiling point.

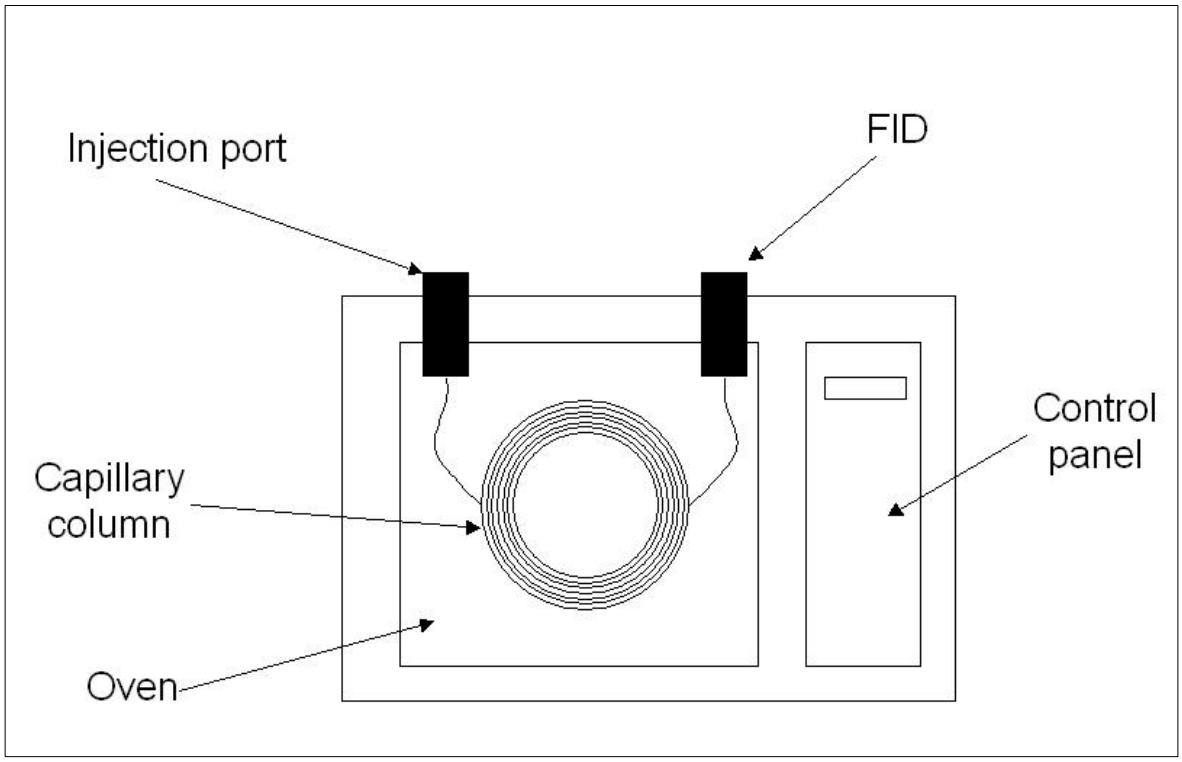


Figure 2-3 Schematic diagram of a gas chromatograph.

FAMEs are injected into the injection port which enables their application onto a capillary column (Figure 2-3). Helium gas is used to carry the FAMEs through the column. The column is kept at a lower temperature than that of the injection port to allow the FAMEs to concentrate on the column lining. Then the column is heated up gradually and the FAMEs sequentially leave the column according to their affinity to the silica lining. At the end of the column, a flame ionization detector is held at 250-300°C. Combustion of a FAME produces an ion current symmetrical to the amount of the FAME in the sample. This analysis results in a chromatogram containing a peak; each peak is unique and corresponds to a different FAME detected by the FID. The area of each peak allows each FAME to be quantified.

2.2.5 Gas chromatography analysis

In this study, the FAMEs were separated and analysed by GC on a Hewlett Packard 6890 gas chromatograph (Hewlett-Packard; Avondale, PA, USA) fitted with a Hewlett Packard system autoinjector. A BPX-70 (30 m × 0.22 mm × 0.25 µm) capillary column was used and the FID was held at a temperature of 300°C. Inlet temperature was 300°C. Oven temperature was initially 115°C and this was maintained for 2 min post-injection. Then the oven temperature was programmed to increase to 200°C at the rate of 10°C/min, to hold at 200°C for 16 min, and then to increase to 240°C at the rate of 60°C/min and then to hold at 240°C for 2 min. The total run time was 37 min. The instrument was controlled by, and data were collected using, HPChemStation (Hewlett Packard) software. FAMEs were identified by comparison of retention times with standards menhaden oil fatty acid methyl esters) and (Institute of Human Nutrition fatty acid methyl esters mixture (IHN) (see Figures 2-4 and 2-5). Figure 2-6 shows a typical GC trace of human RBC fatty acids. The within-run coefficient of variation (CoV) for the analysis of EPA and DHA as methyl esters was 3% and 2%, respectively. Between run CoV for analysis of EPA and DHA as methyl esters was 5% and 2.5%, respectively.

2.2.6 Omega-3 index calculation

Omega-3 index was calculated using concentrations of eicosapentaenoic acid and docosahexaenoic acid in the RBCs according to the equation:

Omega-3 index % = EPA % + DHA %

Harris et al. (2004b) established the correlation between omega-3 fatty acid levels in heart tissue and omega-3 fatty acid levels in RBCs. They showed that the omega-3 index, calculated from (EPA + DHA) per total fatty acid content in RBCs, and myocardial tissue were highly correlated (Harris et al., 2004).

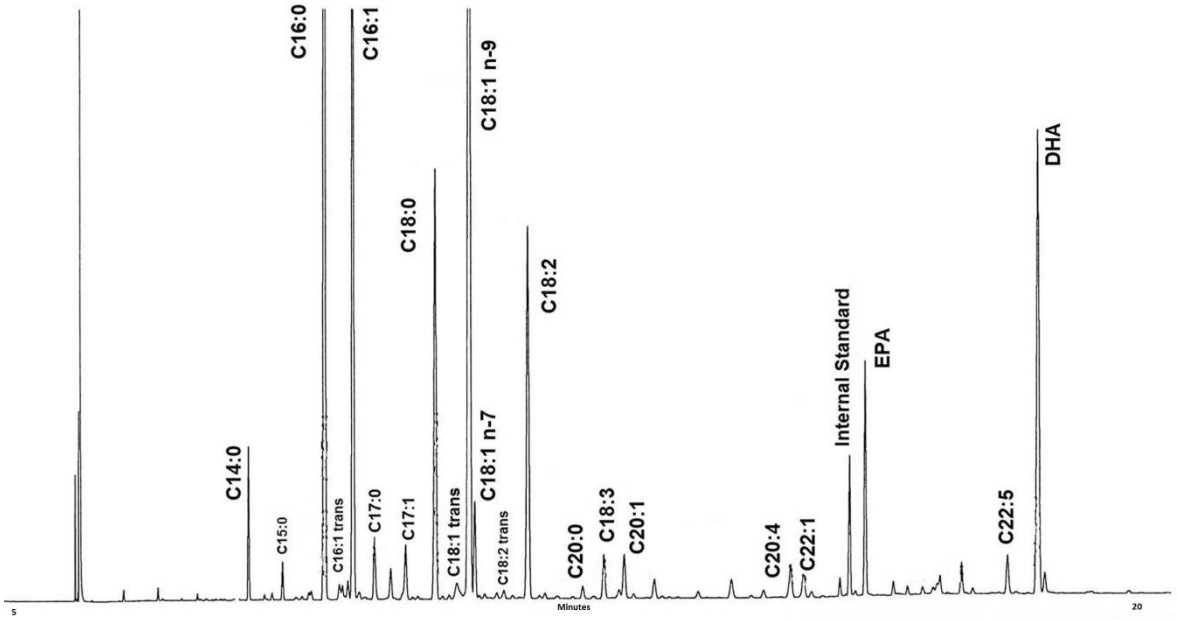


Figure 2-4 Chromatograph of typical GC profile of calibration standard: omega-3 fatty acids (the menhaden oil).

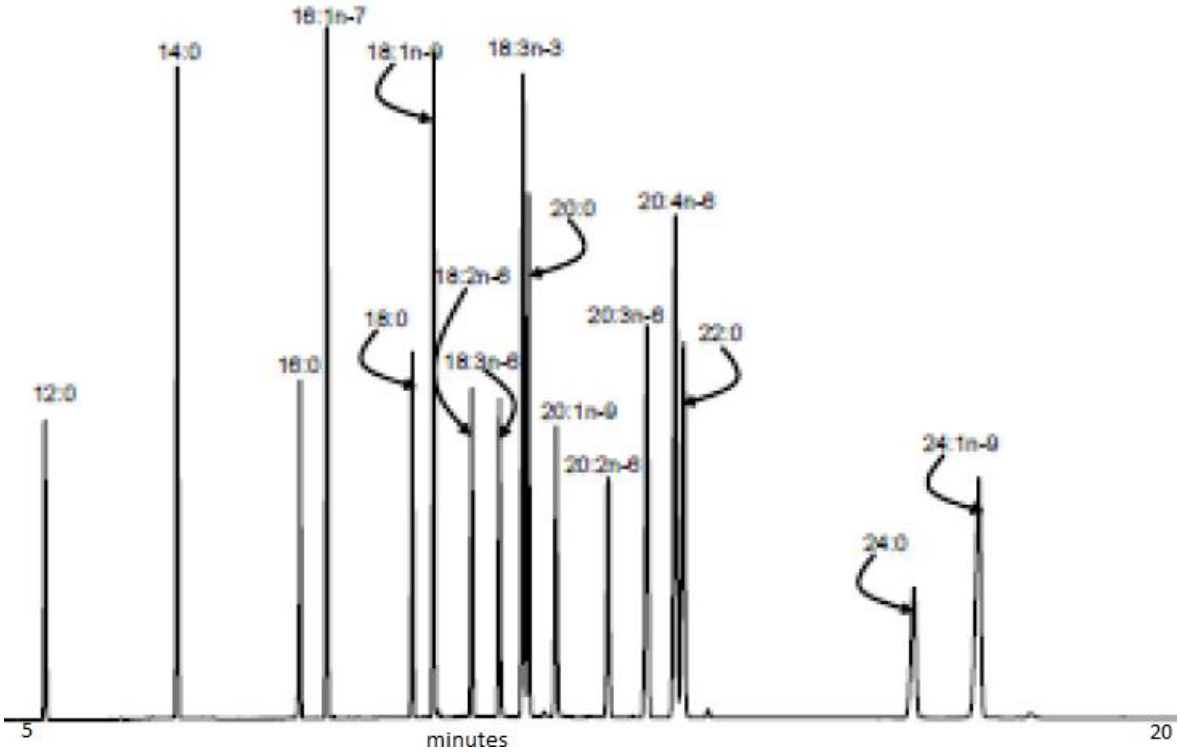


Figure 2-5 Chromatograph of typical GC profile of calibration standard: omega-3 fatty acids (the Institute of Human Nutrition standard mix).

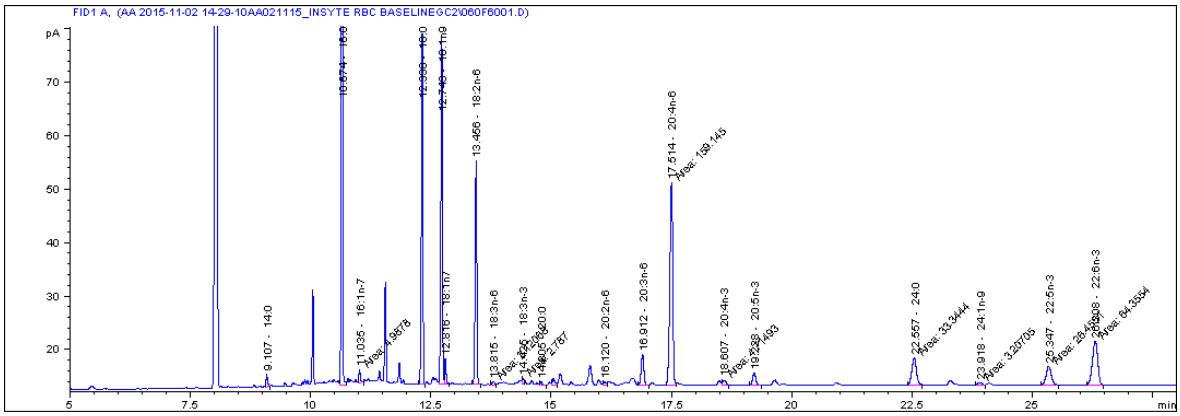


Figure 2-6 Typical GC profile of human red blood cells. A total of 100% fatty acids are usually identified

2.3 Anthropometry

2.3.1 Height

Participant's height was measured using The Leicester Height Measure Stadiometer, following the "Standard Operating Procedure for Measuring Adult Height" in the Southampton Centre for Biomedical Research. Two practitioners were required for each measurement, one to hold the participant's head in the correct position, the other to read the value. The participant was asked to stand on the stadiometer, facing forwards as tall and straight as possible with their arms hanging loosely at their sides (Figure 2-7). Their feet were flat on the base plate of the stadiometer and positioned slightly apart, in line with their hips, to aid balance. It was ensured that the participant's head was in the "Frankfort plane" (an imaginary line from the centre of the ear hole to the lower boarder of the eye socket; this is a midline position (Figure 2-7). The participant was asked to take a deep breath and hold, and then the measurement was taken. The measurement was repeated three times and these three readings were averaged.

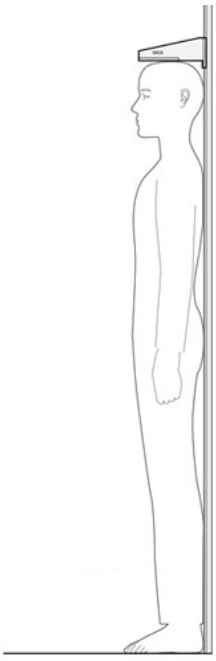


Figure 2-7 Height measurement using a stadiometer.

2.3.2 Weight

Participant's weight was measured using electronic weighing scales (Seca Medical 780 digital scale), following the "Standard Operating Procedure for Measuring Adult Weight" in the Southampton Centre for Biomedical Research. For this measure, the participant was asked to ensure shoes and any heavy clothing and objects were removed, and to stand still on the centre of the scales with their arms hanging loosely at their sides. They were required to look straight-ahead and remain as still as possible. The measurement was repeated three times and these three readings were averaged.

2.3.3 Body Mass Index (BMI) calculation

Body mass index was calculated by the standard formula (dividing body weight in kilograms by height in metres squared (kg/m^2).

2.3.4 Waist circumference measurement

Participant's waist circumference was measured using a metal tape (Lufkin Executive Tape Measure W606PM), following "The Standard Operating Procedure for Measuring Adult Weight" in the Southampton Centre for Biomedical Research. The practitioner stood behind the participant and palpated the iliac crest (the large curving pelvic bone, just below the waist). The skin was marked on both sides with a horizontal line at its highest point. Next, the lower rib margin was palpated and the skin marked on both sides with a horizontal line at the lowest point. A tape measure was used to identify the mid-point between the marks made at the iliac crest and the lower rib margin on both sides and the mid-point was marked (Figure 2-8). The tape measure was applied to the mid-point marks. The participant was asked to relax (i.e. not to deliberately hold him/herself in or out) and to look

straight ahead with arms relaxed at his/her sides. Then the participant was asked to breathe in and then out. The measurement was made and the tape read during the pause at the end of expiration. The measurement was repeated three times and these three readings were averaged.

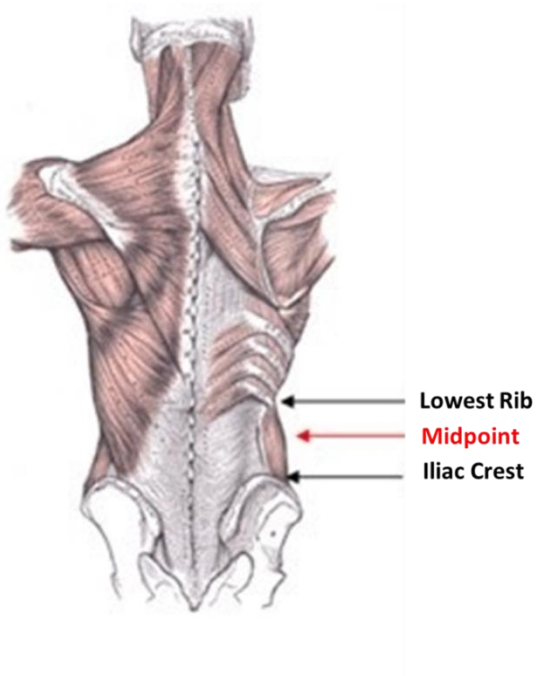


Figure 2-8 Waist circumference measurement:the mid-point between the iliac crest and the lower rib margin.

2.4 Metabolic syndrome presence assessment

The presence of metabolic syndrome (MS) was assessed following the criteria established by the International Diabetes Federation (IDF) (Alberti et al., 2009), as listed in Figure 2-9.

The factors included were:

Abdominal obesity (male waist ≥ 94 cm; female waist ≥ 80 cm) (1 point)

Triglycerides ≥ 1.7 mmol/L or on TG lowering therapy (1 point)

HDL-cholesterol (male < 1.03 mmol/L; female < 1.29 mmol/L) or on HDL improving therapy (1 point)

Blood pressure > 130/> 85 mm Hg or on BP lowering therapy (1 point)

Fasting glucose > 5.6mmol/L or on glucose lowering therapy (1 point)

0-2 Points: Absence of MS

3-5 Points: Presence of MS.

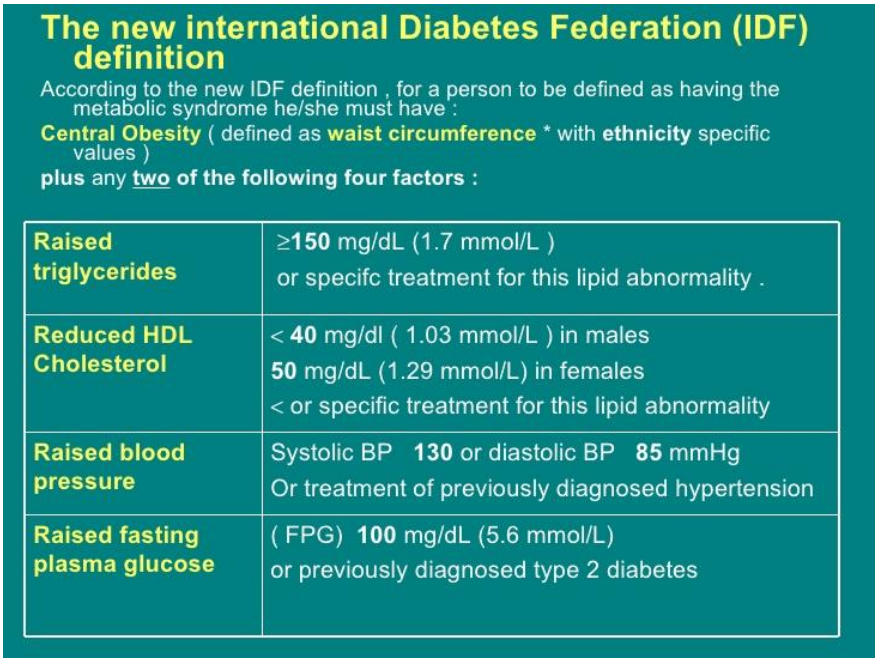


Figure 2-9 The International Diabetes Federation definition of metabolic syndrome.

2.5 Measurement of serum total cholesterol, HDL-cholesterol, and triglyceride concentrations and calculation of LDL-cholesterol concentration

2.5.1 Sample collection

Plastic 5 ml Serum Separator Tubes (SST) were used for blood collection to produce serum for the analysis of total cholesterol, HDL-cholesterol and triglyceride concentrations. Blood was collected in the fasting state (at least 12 hours without food) by a trained INSYTE study nurse (Sanchia Triggs, Andria Staniford, Gemma Rood, JiJo Thomas, Norma Diaper) or a physician (Eleonora Scorletti). All details were recorded including time and date of sample collection and samples were delivered to the pathology laboratory of Southampton General Hospital along with staff ID and patient ID. All the analyses were performed automatically using enzymatic methods on a Beckman AU5800 analyser. LDL-cholesterol concentration was calculated by the Friedewald equation (see below).

2.5.2 Total cholesterol measurement

The total cholesterol concentration of serum was measured using a colourimetric enzymatic assay; the cholesterol reagent was OSR6516 supplied by Beckman Coulter®

Principle:

In this procedure cholesteryl esters in a sample are hydrolysed by cholesteryl esterase:

$$\text{2 Cholesteryl esters} + 2\ H_2O \xrightarrow{\text{Cholesterol esterase}} \text{2 Cholesterol} + \text{2 Fatty acids}$$

The free cholesterol produced plus any free cholesterol already in the sample is oxidised by cholesterol oxidase to cholestene-3-one with the simultaneous

production of hydrogen peroxide (H_2O_2), which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromophore

$$2\ \text{Cholesterol} + 2\ O_2 \xrightarrow{\text{Cholesterol oxidase}} 2\ \text{Cholestene-3-one} + 2\ H_2O_2$$

A red quinoneimine dye is then formed and this was measured spectrophotometrically at 540/600 nm:

$$2\ H_2O_2 + \text{4-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4\ H_2O$$

The intensity of the colour produced is directly proportional to the total cholesterol concentration in the sample. The coefficient of variation of the assay is 2.13-2.17% for total cholesterol levels of 2.55-6.24 mmol/L.

2.5.3 HDL-cholesterol measurement

HDL-cholesterol concentration of serum was measured using colourimetric enzymatic assay; the HDL-cholesterol reagent was OSR6587 supplied by Beckman Coulter®

Principle:

Anti-human-β-lipoprotein antibody in the OSR6187 reagents binds to lipoproteins other than HDL (LDL, VLDL and chylomicrons) causing them to precipitate. The cholesterol concentration in the remaining soluble HDL is measured in an analogous way to total cholesterol as outlined above:

$$\text{HDL-cholesterol} + H_2O + O_2 \xrightarrow[\text{Cholesterol oxidase}]{\text{Cholesterol esterase}} \text{Cholest-4-en-3-one} + \text{Fatty acids} + H_2O_2$$

A blue dye is then formed and this was measured spectrophotometrically at 540/600 nm:

$$H_2O_2 + 4\text{-}AA + F\text{-}DAOS \xrightarrow{\text{Peroxidase}} \text{Blue dye} + F^- + 2\,H_2O$$

The intensity of the colour produced is directly proportional to the HDL-cholesterol concentration in the sample. The coefficient of variation of the assay is 3.68-3.97% for HDL-cholesterol levels of 0.72-1.63 mmol/L.

2.5.4 Triglyceride measurement

Triglyceride concentration of serum was measured using colourimetric enzymatic assay; the triglyceride reagent was OSR 61118 supplied by Beckman Coulter®.

The triglyceride measurement procedure is based on a series of coupled enzymatic reactions. The triglycerides in the sample were hydrolyzed by a combination of microbial lipases to give glycerol and fatty acids:

$$\text{Triglycerides} + 3\,H_2O \xrightarrow{\text{Lipases}} \text{Glycerol} + 3\ \text{Fatty Acids}$$

The glycerol was phosphorylated by adenosine triphosphate (ATP) in the presence of glycerol kinase to produce glycerol-3-phosphate.

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase, } Mg^{2+}} \text{Glycerol-3-phosphate} + \text{ADP}$$

The glycerol-3-phosphate was oxidized by molecular oxygen in the presence of glycerol phosphate oxidase to produce hydrogen peroxide and dihydroxyacetone phosphate.

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{Glycerol phosphate oxidase}} H_2O_2 + \text{Dihydroxyacetone phosphate}$$

The H2O2 formed reacts with 4-aminophenazone and N,N-bis(4-sulfobutyl)-3,5-dimethylaniline, disodium salt (MADB) in the presence of peroxidase to produce a blue chromophore, which was read at 660/800nm.

$$2\ H_2O_2 + MADB + 4AAP \xrightarrow{\text{Peroxidase}} \text{Blue Dye} + OH^- + H_2O$$

The intensity of the colour produced was directly proportional to the triglyceride concentration in the sample. The coefficient of variation of the assay is 2.34-3.40% for TG levels of 075-2.57 mmol/L.

2.5.5 LDL-cholesterol calculation

LDL-Cholesterol was estimated by using the Friedewald equation. This calculation includes total cholesterol, HDL-cholesterol and triglyceride concentrations:

LDL-cholesterol = Total cholesterol - HDL-cholesterol – 0.45(Triglycerides)

All concentrations were expressed in mmol/l.

There is a limitation to this method, as LDL-cholesterol cannot be calculated accurately if plasma triglyceride is > 4.52 mmol/L (400 mg/dL); therefore in this case LDL-cholesterol was considered as missing data.

2.6 Measurement of carotid intima-media thickness (CIMT)

Carotid intima-media thickness (CIMT) is a well validated screening tool for the prediction of cardiovascular disease, being a marker of subclinical atherosclerosis. CIMT measures the internal lining (intima-media) layer of the carotid arteries.

The baseline scanning of the carotid artery was performed by Dr Eleonora Scorletti and the candidate and analysed with software by the candidate.

Scaning of the carotid arteries was carried out with a duplex scanner using a 7.5 MHz linear array transducer (Philips IE33, Koninklijke Philips N.V., Netherlands) with ECG monitoring (Appendix B). All scans were carried out according to a standardised protocol of the American Society of Echocardiography (Stein et al., 2008). Briefly, participants lay face upward with the neck slightly rotated, the head leaning slightly towards the opposite of the examined side and a transverse scan was performed as a screening measure (Figure 2-10). Multiple images of the common carotid artery were recorded and stored digitally in the DICOM format for later analysis. The images were exported to a computer for analysis with Philips QLAB Quantification software version 8 (Koninklijke Philips N.V., Netherlands) (Figure 2-11). The operator identified the area to measure; the software programme automatically detected the intima-lumen and the media-adventitia interfaces (Figure 2-11). An average of three different cardiac image measurements of IMT from each of the left and right common carotid arteries was calculated. The coefficient of variation was 2.8%, which is within the limit of 6% recommended by the American Society of Echocardiography (Stein et al., 2008).

Figure 2-10 Scanning of carotid intima-media thickness.
Scanning examination of the carotid arteries located on each side of the neck.

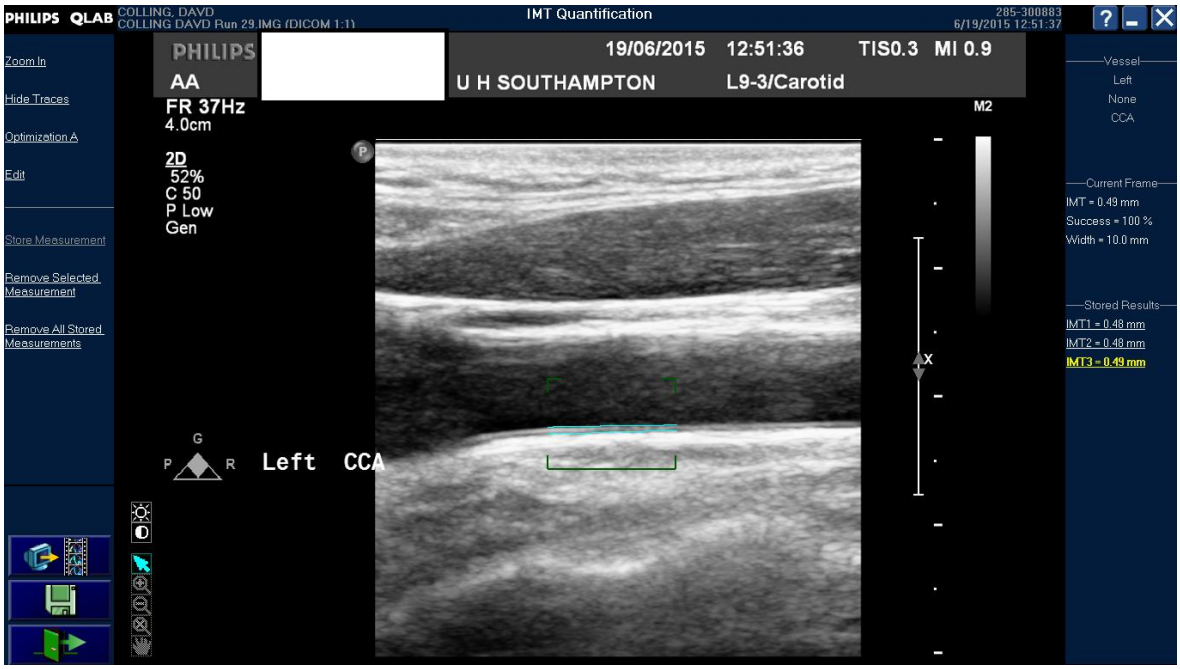


Figure 2-11 Screen shot of the image analysis of carotid intima-media thickness with Philips QLAB Quantification software; taken by the candidate.

2.7 Measurement of blood pressure

Blood pressure was measured after 5 minutes of rest and in the sitting position using an electronic monitoring device (The Dash 3000 monitor) following “The Standard Operating Procedure for Measuring Adult Blood pressure” in the Southampton Centre for Biomedical Research. The measurement was repeated three times and the three readings were averaged.

2.8 Calculation of cardiovascular risk

General cardiovascular (10-year risk) prediction was assessed following “Framingham General Cardiovascular Risk Score” (Anderson et al., 1991) using Excel file Risk Score Calculators. The data for several cardiovascular risk predictors (sex, age, diabetes, smoking, treated and untreated systolic blood pressure, total cholesterol, and HDL-cholesterol) were entered into the risk score calculators for each participant. The risk score was calculated as a percent as shown in Figure 2-12.

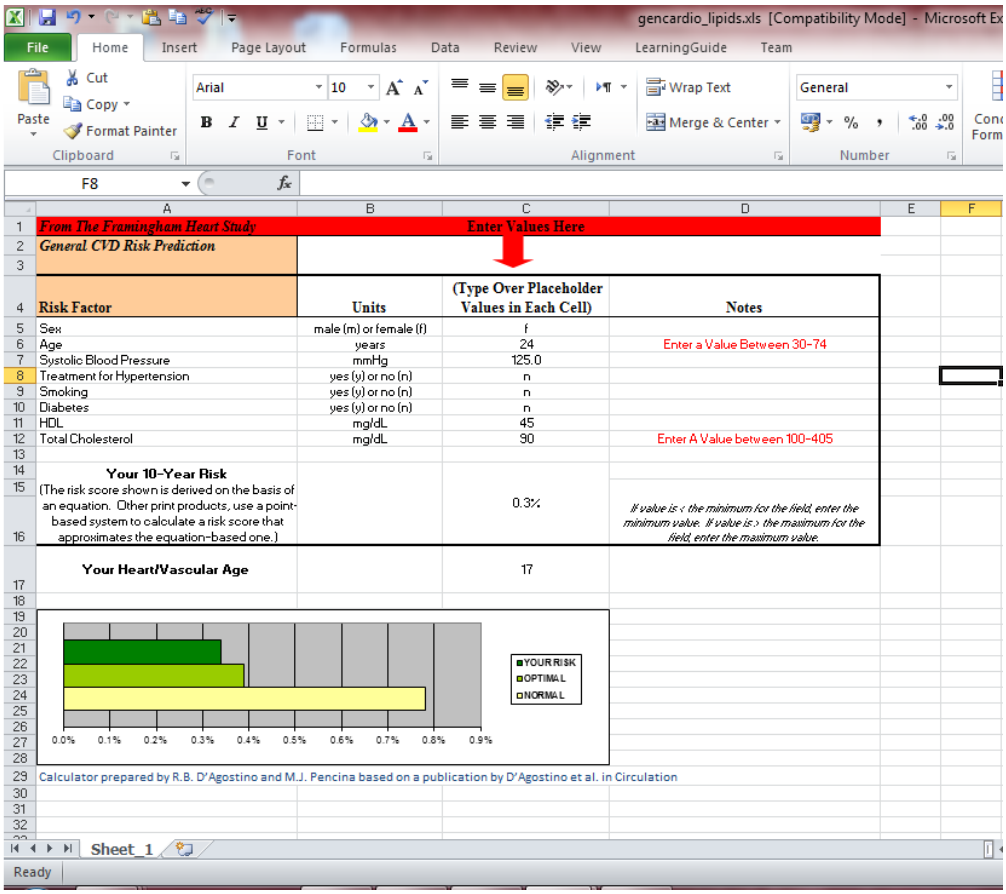


Figure 2-12 Sample of Excel file to calculate the Framingham General Cardiovascular risk score.

2.9 Fasting glucose measurements

2.9.1 Sample collection

Plastic 5 ml blood fluoride oxalate vacutainer blood bottles were used for blood collection to obtain plasma for the analysis of blood glucose. Blood was collected in the fasting state (at least 12 hours without food) by a trained INSYTE study nurse (Sanchia Triggs, Andria Staniford, Gemma Rood, JiJo Thomas, Norma Diaper) or by a physician (Eleonora Scorletti). All details were recorded including time and date of sample collection and samples were delivered to the laboratory along with staff ID and patient ID. Blood samples were transported to the Pathology laboratory of Southampton General Hospital for the analysis. All the analyses were performed automatically using enzymatic methods on a Beckman Coulter AU analysers.The glucose reagent was OSR6521 and supplied by Beckman Coulter®.

2.9.2 Principle:

Glucose is phosphorylated by hexokinase in the presence of adenosine triphosphate (ATP) and magnesium ions to produce glucose-6-phosphate and adenosine diphosphate (ADP).

$$\text{Glucose + ATP} \xrightarrow{\text{Hexokinase, ATP, } Mg^{2+}} \text{Glucose-6-phosphate + ADP}$$

Glucose-6-phosphate dehydrogenase specifically oxidises glucose-6-phosphate to gluconate-6-phosphate with the concurrent reduction of NAD+ to NADH.

$$\text{Glucose-6-Phosphate + } NAD^{+} \xrightarrow{\text{Glucose-6-phosphate dehydrogenase}} \text{Gluconate-6-P + NADH + } H^{+}$$

The increase in absorbance at 340 nm is proportional to the glucose concentration in the sample. The coefficient of variation of the assay is 1.11-1.25% for glucose levels of 3.24-16.36 mmol/L.

2.10 Insulin measurement

2.10.1 Sample collection

Plastic 3 ml ethylenediaminetetraacetic acid (EDTA) vacutainer blood tubes were used for blood collection to obtain plasma for the analysis of insulin. Blood was collected in the fasting state (at least 12 hours without food) by a trained INSYTE study nurse (Sanchia Triggs, Andria Staniford, Gemma Rood, JiJo Thomas, Norma Diaper) or a physician (Eleonora Scorletti). All details were recorded including time and date of sample collection and samples were delivered to the laboratory along with staff ID and patient ID. Blood samples were transported to the Pathology laboratory of Southampton General Hospital for the analysis. All the analyses were performed automatically using enzymatic methods on a Beckman Beckman DXI analyser.

2.10.2 Principle

The Beckman Coulter (Access) Ultrasensitive Insulin assay is a simultaneous one-step immunoenzymatic “sandwich” assay (Figure 2-13). A sample was added to a reaction vessel along with mouse monoclonal anti-insulin alkaline phosphatase conjugate and paramagnetic particles coated with mouse monoclonal anti-insulin antibody. The insulin in the sample binds to the antibody on the solid phase, while the conjugate reacts with a different antigenic site on the insulin molecule. After incubation, materials bound to the solid phase are held in a magnetic field while the unbound materials are washed away. Then, the chemiluminescent substrate Lumi-Phos 530 is added to the vessel and the light generated is directly

proportional to the concentration on insulin in the sample. The amount of insulin in the sample is determined from a stored, multi–point calibration curve. The coefficient of variation of the assay is 2.6-8.4% for insulin levels of 1.1-106.5 mU/l.

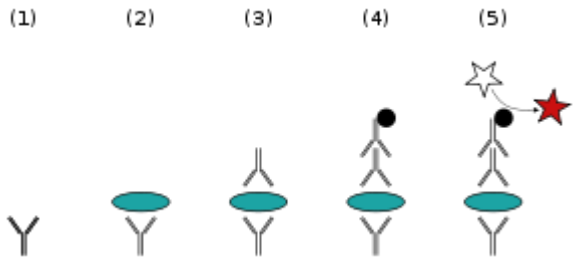


Figure 2-13 The principle of a sandwich ELISA.
(1) Plate is coated with a capture antibody; (2) Sample is added, and any antigen (in this case insulin) present binds to capture antibody; (3) Detecting antibody is added and binds to antigen; (4) Enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) Substrate is added, and is converted by enzyme to a detectable form.

2.10.3 Insulin sensitivity estimation

Insulin sensitivity was estimated using the homeostatic model assessment to quantify insulin resistance (HOMA-IR) using fasting insulin and glucose concentrations according to the equation:

HOMA-IR = (Glucose mmol/L x Insulin IU/L) / 22.5

2.11 Measurement of inflammatory markers: CRP, TNF-α, IL-6, IL-8, IL-10, MCP-1, RANTES, VCAM-1, ICAM-1, E-Selectin and P-Selectin

2.11.1 C-reactive protein measurements

2.11.1.1 Sample collection

Plastic 5 ml Serum Separator Tube (SST) vacutainer blood bottles were used for blood collection to produce serum for the analysis of C-reactive protein (CRP). Blood was collected in the fasting state (at least 12 hours without food) by a trained INSYTE study nurse (Sanchia Triggs, Andria Staniford, Gemma Rood, JiJo Thomas, Norma Diaper) or a physician (Eleonora Scorletti). All details were recorded including time and date of sample collection and samples were delivered to the pathology laboratory of Southampton General Hospital along with staff ID and patient ID. Serum C-reactive protein (CRP) concentrations were measured using an Immuno-turbidimetric test on Beckman Coulter AU analysers; the reagent used was OSR 6299 supplied by Beckman Coulter®.

2.11.1.2 Principle

When a sample was mixed with OSR6147 (4 x 14 mL) buffer and OSR6147 (4 x 6 mL) antiserum solution, CRP reacts specifically with anti-human CRP antibodies to yield insoluble aggregates. The absorbance of these aggregates is proportional to the CRP concentration in the sample. The coefficient of variation of the assay is 2.14-3.72% for CRP levels of 3.85 - 29.52 mg/L.

2.11.2 Measurement of inflammatory markers: TNF-α, IL-6, IL-8, IL-10, MCP-1, RANTES, VCAM-1, ICAM-1, E-Selectin, and P-Selectin

2.11.2.1 Sample collection

Plastic 8.5 ml Serum Separator Tube (SST) vacutainer blood bottles were used for blood collection to produce serum for the analysis of inflammatory markers. Blood was collected in the fasting state (at least 12 hours without food) by a trained INSYTE study nurse (Sanchia Triggs, Andria Staniford, Gemma Rood, JiJo Thomas, Norma Diaper) or a physician (Eleonora Scorletti). All details were recorded including time and date of sample collection and samples were delivered to the laboratory of Southampton Centre for Biomedical Research with staff ID and patient ID. Blood tubes were centrifuged at 2000 g for 10 mins at ambient temperature. Serum aliquots were stored at -80 ^{o} C until assay.

2.11.2.2 Principle of Luminex Assay

Luminex Performance Assay multiplex kits from R&D Systems Europe were used to simultaneously assess the concentrations of several inflammatory markers. Luminex kits contain colour-coded microparticles pre-coated with antibodies specific to different analytes. Microparticales, standard and samples are pipetted into wells and the immobilized antibodies on each particle bind to the analyte of interest. After washing away any unbound substances a biotinylated antibody cocktail specific to an analyte was added to each well. Wells were then washed to remove any inbounded biotinylated antibodies. Finally the microparticles were incubated with a streptavidin-phycoerythrin conjugate (Streptavidin-PE) which binds to the captured biotinylated detection antibodies; then a final wash removed unbound Streptavidin-PE. The microparticles were resuspended in buffer and read using a Luminex platform (Biorad Luminex 200), which determines the magnitude

of the phycoerythrin-derived signal which is in direct proportion to the amount of analyte bound.

Reagents, working standards and samples dilutions were prepared as recommended by manufacturer. A filter-bottomed microplate was prewet with 100 µL of wash buffer for each well, liquid was removed using a vacuum manifold designed to accommodate a microplate; 25 µL of the microparticle mixture were added to each well of the prewet filter-bottomed microplate. Then 100 µL of sample or standard were loaded into the plate and incubated for 2 – 3 h at room temperature on a horizontal orbital microplate shaker at 200 rpm. After washing away unbound substances, 50 µL of a biotin antibody cocktail was added to each well. The plate was securely covered with a foil plate sealer and incubated for 1 h at room temperature on the shaker at 200 rpm. The microplate was washed and 50 µL of streptavidin-PE cocktail was added to all wells and incubated for 30 min at room temperature on the shaker at 200 rpm. Finally, the microplate was washed to remove unbound streptavidin-PE and wells were filled with 75 µL of wash buffer. The microplate was incubated for 2 min on the shaker set at 500 rpm and analysed within 90 minutes on the Biorad Luminex 200 employing the Bio-Plex Manager TM 6.1. Coefficients of variation for each analye, provided by the manufacturer, are listed in Table 2-1.

Table 2-1 The coefficients of variation for the analysis of inflammatory markers

Inflammator marker	CoV for inter assay	CoV for intra assay
CRP	3.72-3.85%	2.14-29.52%
TNF-α	8.0-10.7%	4.1-6.8%
IL-6	9.3- 10.5%	3.6-6.0 %
IL-8	8.3-15.7 %	4.6-11.8 %
IL-10	9.7-12.5 %	3.8-7.3%
MCP-1	5.6-11.1 %	3.8-6.4%
RANTES	8.0-13.1 %	6.6-12.2 %
ICAM-1	6.4-11.5 %	3.0-5.2 %
VCAM-1	8.8-12.1 %	4.6-5.9%
E-Selectin	14.5-16.9 %	3.6-8.3 %
P-Selectin	9.4-11.9 %	2.8-5.9%
Abbreviations: CRP, C-reactive protein; TNF-α, tumour necrosis factor alpha; IL-6, Interleukin 6; IL-8, Interleukin-8; IL-10, Interleukin-10; MCP-1,monocyte chemoattractant protein1; VCAM-1, vascular adhesion molecule1; ICAM-1, intracellular adhesion molecule1.		

2.12 Power calculations

Power calculation for effect of synbiotic on liver fat

There is little published literature to date upon which to base a sample size calculation to test the effects of the synbiotic treatment on the primary INSYTE end point of change in liver fat. A sample size of 50 in each group, with a 14% drop out during the study, will have 85% power to detect a difference of 40% in liver fat (in the synbiotic arm compared with the placebo), assuming that the common standard deviation is 62%, using a power calculation test with a 0.05 two-sided significance level. In a previous randomised controlled trial (the WELCOME study) (Scorletti et al., 2014a) over a longer period of intervention, 5% of the randomised cohort withdrew between randomisation and end of study measurements. In the INSYTE trial, the mean percentage fat content was 28.5% (n = 63) with a similar standard deviation to that presented (62%). MRS can reproducibly detect liver fat content as low as 5%.

Power calculation for omega-3 fatty acids as a predictor of cardiovascular risk

This sub-study investigates associations between two continuously, approximately normally distributed variables (i.e., a RBC fatty acid and a cardiovascular risk factor) in a population of size n = 63 and using a statistical test at the 5% level of significance. One variable is considered as an outcome (the cardiovascular risk factor) and the other as a predictor (the omega-3 fatty acid). With the sample size of 63 there is 80% power to detect a change of 0.353 standard deviations in the outcome variable for every standard deviation change in the predictor, 90% power to detect a change of 0.408 standard deviations in the outcome variable for every standard deviation change in the predictor, and 95% power to detect a change of 0.454 standard deviations in the outcome variable for every standard deviation change in the predictor.

2.13 Statistical analysis

The research addresses the hypothesis that there is a relation between RBC omega-3 fatty acids and cardiovascular risk factors in patients with NAFLD (see section 1.8). All raw data of all participants were entered into an Excel database and transferred to SPSS for analysis. The normal distribution of the data was tested by the Shapiro–Wilk and Kolmogorov–Smirnov tests. Outlier data were not excluded from the analysis. Data of all participants are reported as mean and standard deviation (SD) for normally distributed variables, or as median and interquartile range (IQR) for non-normally distributed variables.

To obtain a symmetrical distribution befor proceeding with data analysis, data that were not distributed normally were normalised by log transformation and if data did not normalise by this procedure then they were normalised by rank transformation.

Initially, univariate correlations between RBC omega-3 fatty acids and cardiovascular risk factors were assessed as Spearman's or Pearson's correlations. Subsequently, multivariate analysis was performed using each risk factor as the outcome and fatty acids and other variables (e.g. age, sex) as explanatory or confounding factors. All statistical analyses were performed using IBM SPSS Statistics for Windows (version 21. .0; SPSS, Inc., Chicago, IL). A value of $P < 0.05$ was considered to be statistically significant for all analyses.

Chapter 3: **Cardiovascular risks factors and omega-3 fatty acids status of patients with non-alcoholic fatty liver disease**

Chapter 3

3.1 Introduction, research questions and hypothesis

3.1.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of fat-related conditions ranging from simple fatty liver, to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis. Up to 30% of the global adult population is affected by NAFLD (Brea and Puzo, 2013, Marchesini et al., 2003, Bhatia et al., 2012a) and there is no licensed treatment for this condition. As described in section 1.3.1, NAFLD has a strong link with cardiovascular disease (CVD), and there are suggestions that NAFLD itself promotes atherogenesis (Bhatia et al., 2012a, Munteanu and Mircea, 2014).

NAFLD can be regarded as the liver manifestation of the metabolic syndrome (MS), which is a condition defined as combinations of hypertension, type 2 diabetes, visceral obesity, insulin resistance and dyslipidaemia (Scorletti et al., 2011, Alberti et al., 2009). Many of these features co-exist and some studies report that 30% of NAFLD patients have MS (Brea and Puzo, 2013, Marchesini et al., 2003, Bhatia et al., 2012a). Four-times more people with MS have NAFLD than not (Bhatia et al., 2012b). The mortality risk of CVD is doubled in patients with MS (Bhatia et al., 2012a) and NAFLD itself has a strong link with CVD (Munteanu and Mircea, 2014), suggesting that NAFLD itself might promote atherogenesis (Targher et al., 2010). Moreover, NAFLD involves pathological dysfunction of fat tissue (Diaz et al., 2015 ,Bhatia et al., 2012a), which itself is correlated to CVD risk (Munteanu and Mircea, 2014, Bhatia et al., 2012a). The presence of inflammation along with steatosis in NASH greatly increases risk of CVD and of mortality compared with simple steatosis (Mann et al., 2015). Obesity and the presence of visceral adipose

tissue (VAT) are positively and independently associated with liver fat (Diaz et al., 2015). Plasma free fatty acids are the main source of fatty acids for synthesis of liver triglycerides (TGs) in NAFLD (Tarantino and Caputi, 2011), arising in part by increasing lipolysis from insulin resistant adipose tissue. Adipose tissue is also metabolically active, secreting different adipokines, cytokines, and hormones that serve to regulate hepatic fat, inflammation, insulin resistance (IR), and affect CVD outcome (Bhatia et al., 2012a, Bhatia et al., 2012b).

Omega-3 fatty acids are very long chain polyunsaturated fatty acids (PUFA). Eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) are the most important omega-3 fatty acids. They have been shown to protect against CVD in some population groups as described in section 1.5.5. Substantial evidence from case-control and prospective studies has indicated that high intakes or blood levels of EPA and DHA are associated with reduced risk of CVD (Harris and von Schacky, 2004). Beneficial modification of a cardiovascular risk factors is most likely responsible for the protective effect of omega-3 fatty acids (Calder, 2004).

The oral intake of the omega-3 fatty acids is the major determinant of the levels of these fatty acids in blood lipids and in cells and tissues (Walker et al., 2014). However, other factors, such as age and sex, may also influence the incorporation and levels of omega-3 fatty acids. Understanding the different determinants of omega-3 fatty acid statu is important for correlating health benefits of these fatty acids with particular dietary intakes (Walker et al., 2014). There is evidence, predominantly from cross-sectional analyses, suggesting that females have higher

plasma DHA concentrations than males (Childs et al., 2008, Walker et al., 2014). Tracer studies suggest that females may be more efficient than males at converting precursor n-3 PUFA such as α-linolenic acid into more biologically active omaga-3 fatty acids like EPA and DHA (Walker et al., 2014).

(Bakewell et al., 2006a) tested the hypothesis that sex-related differences in omega-3 fatty acid metabolism are reflected in the concentrations of omega-3 fatty acids in plasma lipids. The subjects studied were healthy men (n 13) and women (n 23) aged 18–35 years consuming their habitual diet. There were no significant differences between men and women in their consumption of protein, carbohydrate, total fat, alcohol, individual fatty acids and selected micronutrients. DHA concentration was significantly higher in women compared with men. There were no significant differences between men and women in the concentrations of any other fatty acids measured. These results support the suggestion that greater DHA synthesis in women than men results in a higher DHA concentration in plasma lipids. Red blood cell (RBC) omega-3 fatty acids are a strong long-term marker (averaged over the red blood cell lifespan ≥3 months) of omega-3 fatty acid intake (Harris and von Schacky, 2004), and therefore RBCs are a potentially useful marker of omega-3 status. In contrast, plasma concentrations reflect intake over only the last few days to weeks (Arab, 2003). In addition, RBC membrane omega-3 fatty acid composition is more biologically stable than plasma concentrations (Harris and Thomas, 2010), and has been shown to be highly correlated with omega-3 fatty acid concentrations in tissues such as the heart (Harris et al., 2004a).

This chapter describes the baseline characteristics of patients with NAFLD including status of omega-3 fatty acids (level of red blood cell eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) and omega-3 index), liver fat, medical history, sex, age, anthropometric characteristics (body weight, body mass index, waist circumference), blood pressure (systolic, diastolic), blood lipids (total cholesterol, HDL-cholesterol, total cholesterol /HDL-

cholesterol ratio, LDL-cholesterol, TGs), glucose, insulin, HOMA insulin resistance, presence of the metabolic syndrome, and concentrations of inflammatory markers (C-reactive protein, TNF-α, IL-6, IL-8, IL-10, RANTES, ICAM-1, VCAM-1, MCP-1, P-selectin, E-Selectin and VCAM-1) in plasma, carotid intima-media thickness , and cardiovascular 10-year risk prediction. These baseline results will be compared with the normal range, where this is available, and CV risk. The relationship between CV risk factors and omega-3 status will be described in detail in the next two chapters.

3.1.2 Research questions

Do the NAFLD patients studied here have cardiovascular risk?

What is the status of red blood cell omega-3 fatty acids in patients with NAFLD?

What is the omega-3 index in patients with NAFLD?

3.1.3 Hypotheses

The hypotheses to be tested in this chapter are:

a) That patients with NAFLD will have an elevated risk of cardiovascular disease but that this will vary among the patients.

b) That patients with NAFLD will have a low omega-3 fatty acid status.

c) That age and sex will influence omega-3 fatty acid status in patients with NAFLD (higher in women and increased with age).

d) That patients with NAFLD will not have an omega-3 index that provides a high cardioprotection level.

3.2 Methods

3.2.1 Study design

This research is a sub-project of the INSYTE study which is a randomised, controlled, double-blind clinical trial of a synbiotic in patients with NAFLD. The focus of this sub-project is to study the relation between cardiovascular risk factors (and cardiovascular risk) and the omega-3 fatty acids status at baseline in 63 patients enrolled in the INSTYE study. Participants attend a clinic visit at the NIHR Wellcome Trust Clinical Research Facility, Southampton General Hospital, at INSYTE study entry for baseline measurements (as described in section 2.1). To confirm findings made with the INSYTE patients, this research will also use the baseline data set of the WELCOME study. The WELCOME study was a randomised double blind placebo controlled trial of omega-3 fatty acids in 105 patients with NAFLD (Scorletti et al., 2014a). The WELCOME study was approved by the local ethics committee (REC: 08/H0502/165). The patient inclusion criteria and the methods of analysis were identical in the WELCOME study as in the INSYTE study.

3.2.2 Analyses performed

The status of omega-3 fatty acids was determined by analysing red blood cell fatty acid composition using gas chromatography (as described in section 2.4). The following are presented: EPA content, DHA content, DPA content, and omega-3 index (the sum of EPA+DHA).

General characteristics and cardiovascular risk were determined using the following:

Patient history (as described in section 2.3)

Body weight, height and body mass index and waist circumference (as described in section 2.5).

Systolic and diastolic blood pressure measured using an electronic monitoring device (as described in section 2.9).

Fasting blood lipid concentrations (total cholesterol, HDL-cholesterol and TGs analysed using a colourimetric enzymatic assay and (calculated) LDL-cholesterol) (as described in section 2.7).

Fasting blood glucose and insulin concentrations analysed using enzymatic methods (as described in sections 2.11 and 2.12), and insulin resistance estimated by the homeostatic model assessment (as described in section 2.12.1).

Fasting blood concentrations of inflammatory markers (TNF-α, IL-8, IL-6, MCP-1, E-Selectin, and MMP-9 measured using a Luminex Assay (as described in section 2.13.2) and CRP measured using an Immuno-turbidimetric assay (as described in section 2.13.1).

Cardiovascular risk scoreassessed following the “Framingham General Cardiovascular Risk Score” (as described in section 2.10).

Carotid intima-media thickness measured by scanning of the carotid arteries (as described in section 2.8).

Metabolic syndrome presence assessed following the criteria established by the International Diabetes Federation (IDF) (as described in section 2.6).

3.2.3 Statistical analysis

All data were entered into an Excel database and transferred to SPSS for analysis. Outlier data were not excluded from the analysis. The normal distribution of the data was tested by the Shapiro–Wilk and Kolmogorov–Smirnov tests. All data are reported as mean and SD for normally distributed variables, or as median and interquartile range (IQR) for non-normally distributed variables. Non-normally distributed variables were log base 10 transformed to obtain a symmetrical data distribution before proceeding with data analysis. Two-way ANOVA was used to identify main effects and any interaction between subgroups (age, sex). Where there were effects of age or sex, data were further analysed according to subgroup. Univariate correlations were used to study the relationships between red blood cell omega-3 fatty acids and age as Pearson's correlations. Significance was considered when P was < 0·05. All statistical analyses were performed using IBM SPSS Statistics for Windows (version 21.0; SPSS, Inc., Chicago, IL).

3.3 Results

3.3.1 Characteristics and cardiovascular risk in patients with NAFLD

This section presents the characteristics and cardiovascular risk factors of the patients with NAFLD studied in this research.

3.3.1.1 Liver fat, age, sex, alcohol and smoking habit, ethnicity, medical history and medication use in 63 NAFLD patients in the INSYTE study

Table 3-1 shows liver fat percentage, age, sex, alcohol consumption, cigarette smoking and ethnicity for the 63 participants included in this sub-project of the INSYTE study. Liver fat determined by magnetic resonance imaging was an average of 27 % of liver weight (range 4 to 99%), as would be expected in patients with NAFLD. The participants had a mean age of 51.5 years and around 60% were male. The participants were considered to be no or low alcohol consumers. The participants can be considered as non-smokers. The majority of participants were white Caucasian.

Table 3-1 Characteristics of patients with NAFLD included in the INSYTE study

Variable	Total N	N (%)	Mean (SD) or Median (IQR 25,75)	Min - Max
Liver fat (%)	63		27 (16-41)	4 - 99
Age (years)	63		51.5 (12.4)	21 - 67
< 50 years		29 (46)	45 (34, 47)	21- 49
≥ 50 years		34 (54)	59.5 (53, 67)	50 - 67
Sex	63			
Male		37 (59)		
Female		26 (41)		
Alcohol consumption				
No		36 (56)		
Yes		28 (44)		
Alcohol (-units /week)	63		0.0 (0-4)	0 - 20
Cigarettes smoking:	63			
Never smoked		35 (55)		
Past smoker		28 (44)		
Current smoker		8 (12.5)		
Number of cigarettes /day	63		0.0 (0-0)	0 - 20
Ethnicity	63			
White		60 (94)		
Indian		1 (1.6)		
Other Asian		2 (3)		
Other		1 (1.6)		
Data are expressed as mean or median with minimum and maximum values, or as number and percentage. Variables that were normally distribution are expressed as mean (standard deviation). Variables that were not normally distributed are expressed as median (25th and 75th percentiles). Abbreviations: N, number.				

Table 3-2 shows the medical history of the participants. The data describes the presence of hypertension, hyperlipidaemia, diabetes type 1, diabetes type 2, ischemic heart disease and family history (parent and sibling) of diabetes and ischemic heart disease. Hypertension was defined as a blood pressure reading between 120/80mmHg and 140/90mmHg. Hyperlipidaemia was defined as an

increase in the concentrations of cholesterol, triglycerides or both. Half of the participants had hypertension, and around 40% had hyperlipidaemia. Only one participant had diabetes type 1, while 40% had diabetes type 2. All participants were apparently free of diagnosed ischemic heart disease. Around half of the participants had a family history of diabetes and more than half of the participants had a family history of ischemic heart disease.

Table 3-2 Medical history of the patients with NAFLD included in the INSYTE study

Medical history	Total N	N (%)
Hypertension	63	
Y		31 (49)
N		32 (51)
Hyperlipidaemia	63	
Y		26 (41)
N		37 (59)
Diabetes	63	
No Diabetes		37 (59)
Diabetes type 1		1 (1.6)
Diabetes type 2		25 (39)
Ischemic heart disease	63	
Y		0 (0)
N		63 (100)
Family History of Disease		
Diabetes	63	
Y		26 (46)
N		31 (54)
Ischemic heart disease	63	
Y		33 (56)
N		27 (44)
Data are expressed as number and percentage. Abbreviations: N, number; Y, yes; N,no.		

Table 3-3 shows the use of medication to treat blood lipid abnormalities (other than the use of statins), hypertension and diabetes. Half of the participants were using antihypertensive medication, and 65% were using lipid lowering medication. Around a third of participants were using statins. Only 8% of participants used insulin injection to control diabetes and while 60 % used oral antidiabetics.

Table 3-3 Medication use of the patients with NAFLD included in the INSYTE study

Medications using	Total N	N (%)
Lipid lowering medication (include statins)	63	
yes		22 (35)
no		41 (65)
Antihypertensive medication	63	
yes		34 (53)
no		29 (46)
Oral antidiabetic medication	63	
yes		26 (59)
no		37 (41)
Insulin injections	63	
yes		5 (8)
no		58 (92)
Statins	63	
yes		21 (33)
no		42 (67)
Data are expressed as number and percentage. Abbreviations: N, number		

3.3.1.2 Liver fat, age, sex, alcohol and smoking habit, ethnicity, medical history, and medication use in 105 NAFLD patients in the WELCOME study

Table 3-4 shows liver fat percentage, age, sex, alcohol consumption, cigarette smoking and ethnicity for the 105 participants included in the WELCOME study. Liver fat was 23 % of liver weight (range 1.7 to 85%), as would be that is expected

in patients with NAFLD. The participants had a mean age of 51 years and around 60% were male. The participants were considered to be no or low alcohol consumers and around 90% were non-smokers.

Table 3-4 Characteristics of patients with NAFLD in the WELCOME study

Variable	Total N	N (%)	Mean (SD) Median (IQR 25,75)	Min - Max
Liver fat (%)	101		23 (13, 36)	2 - 85
Age (years)	105		51 (11)	23 - 76
< 50 years		42 (40%)	41 (6)	23 - 49
≥ 50 years		63 (60%)	58 (54, 62)	50 - 76
Sex	105			
Male		60 (57)		
Female		45 (43)		
Alcohol (-units/week)	105		1 (0, 6)	0 - 40
Cigarette smoking:	105			
Never smoked		54 (51)		
Past smoker		37 (35)		
Current smoker		14 (13)		
Data are expressed as mean or median with minimum and maximum values. Variables that were normally distribution are expressed as mean (standard deviation). Variables that were not normally distributed are expressed as median (25th and 75th percentiles). Abbreviations: N, number.				

Table 3-5 shows the medical history of for the 105 participants included in the WELCOME study. The data describes the presence of hypertension, hyperlipidaemia, diabetes type 1, diabetes type 2, ischemic heart disease and family history (parent and sibling) of diabetes and ischemic heart disease. Hyperlipidaemia was defined as an increase in the concentrations of cholesterol, triglycerides or both ; more than half of the participants had hypertension, and half of the participants had hyperlipidaemia. Only two percent had diabetes type 1, while one third of the participants had diabetes type 2. Majority of participants

were free of diagnosed ischemic heart disease. Around 40% of the participants had a family history of diabetes and ischemic heart disease.

Table 3-5 Medical history of the patients with NAFLD in the WELCOME study

Medical history	Total N	N (%)
Hypertension	105	
Y		54 (51)
N		51 (49)
Hyperlipidaemia	101	
Y		50 (49.5)
N		51 (50.5)
Diabetes	105	
No Diabetes		70 (67)
Diabetes type 1		2 (2)
Diabetes type 2		33 (31)
Ischemic heart disease	105	
Y		4 (4)
N		101 (96)
Family History of Disease		
Diabetes	103	
Y		39 (37)
N		64 (61)
Ischemic heart disease	103	
Y		40 (38)
Y		63 (60)
Data are expressed as number and percentage. Abbreviations: N, number; Y,yes; N, no.		

Table 3-6 shows the use of medication to treat blood lipid abnormalities (other than the use of statins), hypertension and diabetes. Less than half of the participants were using antihypertensive medication, and half of them were using lipid lowering medication. More than 40% of participants were using statins. While one third of the participants used oral antidiabetics to control diabetes, only 9% of participants used insulin injection

Table 3-6 Medication use of the patients with NAFLD in the WELCOME study

Medications using	Total N	N (%)
Lipid lowering medication (include statins)	101	
Y		50 (48)
N		51 (49)
Antihypertensive medication	101	
Y		39 (37)
N		62 (59)
Oral antidiabetic medication include forstatins	101	
Y		33 (31)
N		68 (65)
Insulin injection	105	
Y		9 (8.6)
N		96 (91)
Statins	101	
Y		44 (42)
N		57 (54)
Data are expressed as number and percentage. Abbreviations: N, number; Y,yes; N, no		

3.3.1.3 Anthropometric measurements in patients with NAFLD

3.3.1.3.1 Body weight, height, body mass index and waist circumference in patients in the INSYTE study

Table 3-7 shows anthropometric measurements for the 63 participants in the INSYTE study. The average waist circumference was 111 cm, indicating the presence of abdominal obesity in both males and females. Mean body mass index for all participants was 33.5 kg/m². As shown in Figure 3-1 only 1.6% participants were of normal weight, while 23% were overweight, 38% were moderately obese, 27% were severely obese and 9% were very severely obese.

Table 3-7 Anthropometric measurements in patients with NAFLD included in the INSYTE study

Variable	Total N	Mean (SD)	Min - Max	Normal Range*
Height (cm)	63	98 (17)	58 - 136	
Body weight (kg)	63	171 (11)	152 - 196	
Body mass index (kg/m²)	63	33.5 (5)	23 - 50	18.5 -24.9
Waist circumference (cm)	63	111 (11)	87 - 137	
Males	26	112 (11)	96 - 136	< 102
Females	37	111 (11)	87 - 137	< 88

Data are expressed as mean and standard deviation with minimum and maximum values. *Normal ranges are according to NHS Foundation Trust. Abbreviations: N, number.

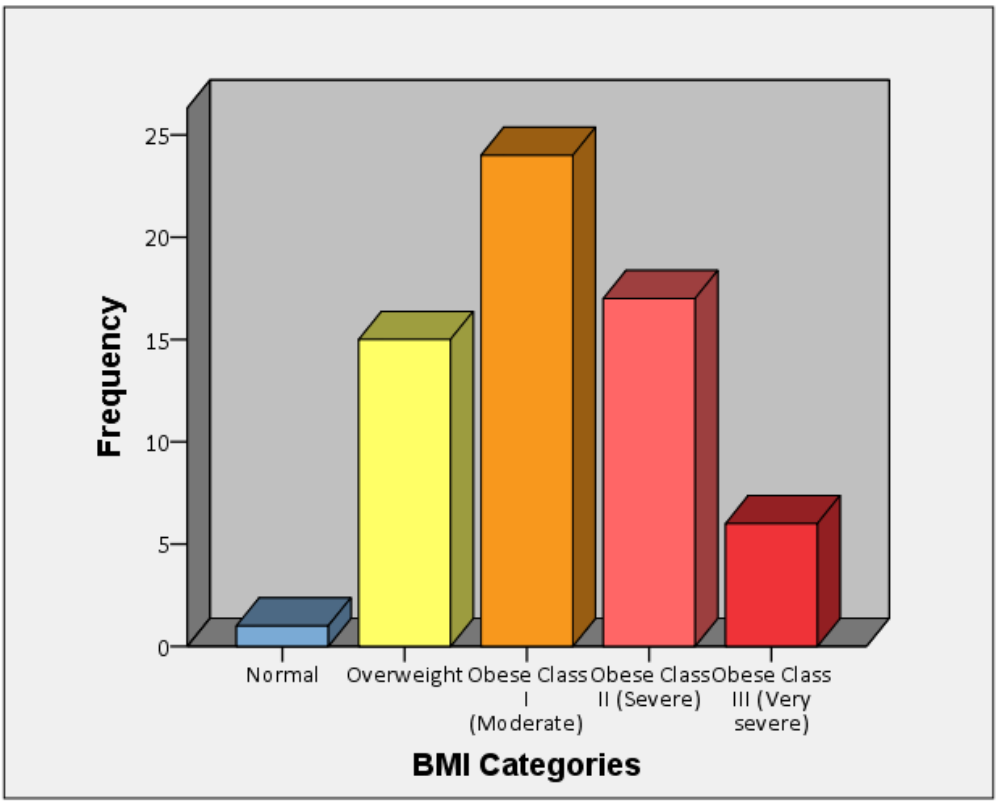


Figure 3-1 Body mass index category (BMI) in 63 patients with NAFLD included in the INSYTE study.
BMI categories: normal weight =18.5-24.9 kg/m^2, overweight = 25-29.9 kg/m^2, obese class I (moderately obese) = 30-34.9 kg/m^2, obese class II (severely obese) = 35-39.9 kg/m^2, obese class III (very severely obese) >= 40 kg/m^2.

3.3.1.4 Body weight, height, body mass index and waist circumference in the WELCOME study

Table 3-8 shows anthropometric measurements for the 105 participants in the WELCOME study. The average waist circumference was 110 cm, indicating the presence of abdominal obesity in both males and females.. Mean body mass index for all participants was 31 kg/m². As shown in Figure 3-2 only 1% participants were of normal weight, while 28% were overweight, 43% were moderately obese, 23% were severely obese and 10% were very severely obese

Table 3-8 An anthropometric measurements in NAFLD patients with in the WELCOME study

Variable	Total N	Mean (SD)	Min- Max	Normal Range*
Height (cm)	105	170 (10)	147 - 200	
Body weight (kg)	105	95 (86, 104)	59 - 166	
Body mass index (kg/m²)	105	31 (30, 32)	23 - 52	18.5 -24.9
Waist circumference (cm)	105	110 (103, 119)	86 - 154	
Males	60	110 (104, 116)	87 - 152	< 102
Females	45	109 (101, 121)	86 - 154	< 88
Data are expressed as mean and standard deviation with minimum and maximum values. *Normal ranges are according to NHS Foundation Trust. Abbreviations: N, number.				

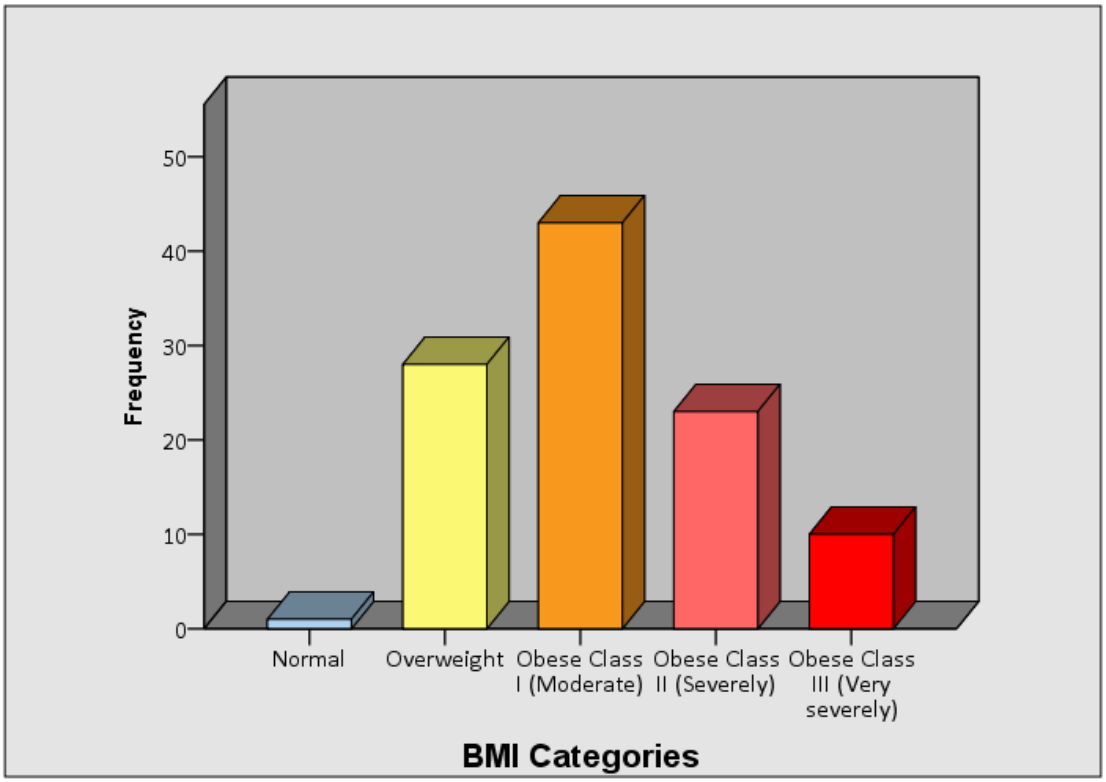


Figure 3-2 Body mass index category in 105 patients with NAFLD in the WELCOME study.
BMI categories: normal weight =18.5-24.9 kg/m^2, overweight = 25-29.9 kg/m^2, obese class I (moderately obese) = 30-34.9 kg/m^2, obese class II (severely obese) = 35-39.9 kg/m^2, obese class III (very severely obese) >= 40 kg/m^2

3.3.1.5 Blood pressure in patients with NAFLD

3.3.1.5.1 Systolic and diastolic blood pressure in patients with NAFLD included in the INSYTE study

Table 3-9 shows the systolic and diastolic blood pressure for 63 patients with NAFLD included in the INSYTE study. Even though half of the participants were using antihypertensive medication, mean systolic blood pressure of all participants was high at 133 mm Hg.

Table 3-9 Systolic and diastolic blood pressure in patients with NAFLD included in the INSYTE study

Variable	Total N	Mean (SD)	Min- Max	Normal range*
Systolic blood pressure (mm Hg)	63	133 (15)	99 - 173	< 130
Diastolic blood pressure (mm Hg)	63	74 (8)	55 - 94	< 85
Data are expressed as mean with minimum and maximum values. *Normal ranges are according to NHS Foundation Trust. Abbreviations: N, number				

3.3.1.5.2 Systolic and diastolic blood pressure in patients with NAFLD included in the WELCOME study

Table 3-10 shows the systolic and diastolic blood pressure for the 105 participants with NAFLD included in the WELCOME study. Mean systolic blood pressure of all participants was high at 137 mm Hg, although around 40% of all participants were takeing antihypertensive medications. Mean diastolic blood pressure was at the border of the normal range.

Table 3-10 Systolic and diastolic blood pressure in patients with NAFLD included in the WELCOME study

Variable	Total N	Mean (SD) or Median (IQR 25,75)	Min - Max	Normal range*
Systolic blood pressure (mm Hg)	105	137 (126, 148)	111 - 194	< 130
Diastolic blood pressure (mm Hg)	105	85 (11)	58 - 112	< 85

Data are expressed as mean or median with minimum and maximum values.
*Normal ranges are according to NHS Foundation Trust. Abbreviations: N, number.

3.3.4.1 Fasting blood lipid concentrations in patients with NAFLD

3.3.1.5.3 Fasting total cholesterol, HDL-cholesterol, triglycerides and LDL-cholesterol in patients with NAFLD included in the INSYTE study

Table 3-11 shows fasting blood lipid concentrations for the 63 participants. Mean total cholesterol was in the desirable range (< 5.0 mmol/l) while 40% of participants had a high cholesterol concentration. Mean HDL-cholesterol was below the desirable concentration and 35% of patients had an HDL-cholesterol concentration below the desirable range. Median LDL-cholesterol was within the normal range, but 29% of patients had an LDL-cholesterol concentration above the desirable range. Mean total cholesterol/HDL-cholesterol ratio was higher than the normal range. Mean TG concentration was higher than the normal range and 65% of patients had a TG concentration above the desirable range.

Table 3-11 Fasting blood lipid concentrations in patients with NAFLD included in the INSYTE study

Variable	Total N	Mean (SD) or Median(IQR 25,75)	Min - Max	Normal Range*
Total cholesterol (mmol/L)	63	5.0 (1.2)	2.9 - 8.7	< 5.0
HDL-cholesterol (mmol/L)	63	1.2 (0.25)	0.7 - 1.8	1.2-1.6
Males	37	1.14 (0.25)	0.7 - 1.8	> 1.04
Females	26	1.35 (0.2)	1.1 - 1.7	> 1.3
LDL-cholesterol (mmol/L)	58	2.6 (2.0 – 4.0)	1.11 - 5.07	< 3.34
Total cholesterol/ HDL	63	4.1 (0.8)	2.6 - 6.4	< 4.0
Triglyceride (mmol/L)	63	1.8 (0.1)	0.7 - 4.9	< 1.7
Data are expressed as mean or median with minimum and maximum values. Variables that were normally distribution are expressed as mean (standard deviation). Variables that were not normally distributed are expressed as median (25th and 75th percentiles). *Normal ranges are according to NHS Foundation Trust. Abbreviations: N, number.				

3.3.1.5.4 Fasting total cholesterol, HDL-cholesterol, triglycerides and LDL-cholesterol of the patients with NAFLD in the WELCOME study

Table 3-12 shows fasting blood lipid concentrations for the 105 participants of the WELCOME study. Mean total cholesterol was in the desirable range while 40% of participants had a high cholesterol concentration. Mean HDL-cholesterol was below the desirable concentration. and 45% of patients had an HDL-cholesterol concentration below the desirable range. Median LDL-cholesterol was within the normal range, but 28% of patients had an LDL-cholesterol concentration above the desirable range. Mean total cholesterol/HDL-cholesterol ratio was higher than the normal range. Mean triglyceride concentration was higher than the normal range and 49.5% of patients had a TG concentration above the desirable range.

Table 3-12 Fasting blood lipid concentrations in patients with NAFLD included in the WELCOME study

Variable	Total N	Mean (SD) or Median(IQR 25,75)	Min - Max	Normal Rang*
Total cholesterol (mmol/L)	105	4.7 (4.0, 5.5)	2.5 - 10	< 5.0
HDL-cholesterol (mmol/L)	105	1.06 (0.26)	0.5 - 1.8	1.2-1.6
Males	60	0.99 (0.2)	0.52 - 1.65	> 1.04
Females	45	1.15 (0.3)	0.67 - 1.8	> 1.3
LDL-cholesterol (mmol/L)	88	2.9 (0.9)	1.0 - 5.5	< 3.34
Total cholesterol/ HDL ratio	105	4.9 (4.0, 6.0)	2.3 - 8.7	< 4.0
Triglycerides (mmol/L)	99	1.7 (1.2, 2.4)	0.6 - 8	< 1.7
Data are expressed as mean or median with minimum and maximum values. Variables that were normally distribution are expressed as mean (standard deviation). Variables that were not normally distributed are expressed as median (25th and 75th percentiles). *Normal ranges are according to NHS Foundation Trust. Abbreviations: N, number.				

3.3.1.6 Fasting blood concentrations of insulin, glucose and calculation of insulin resistance in NAFLD patients

3.3.1.6.1 Fasting blood concentrations of insulin, glucose and calculation of insulin resistance in patients with NAFLD included in the INSYTE study

Table 3-13 describes the fasting blood glucose, fasting blood insulin and HOMA insulin resistance of the 63 participants in the INSYTE study. Blood glucose and insulin concentrations were high. HOMA insulin resistance score was double the normal range. As shown in Figure 3-3 only around 20% of the participants were in the normal range, while 80% had high insulin resistance.

Table 3-13 Fasting blood glucose, fasting blood insulin and HOMA insulin resistance in patients with NAFLD included in the INSYTE study

Variable	Total N	Median (IQR 25,75)	Min – Max	Normal Range*
Glucose (mmol/L)	61	6 (5.4-7.7)	4.3 - 16.5	4.1- 6.0
Insulin (µUnit/ml)	63	15 (9.6-20)	2.4 - 81	An interpretation of the results will depend on the glucose result
HOMA Insulin Resistance	61	4 (2.6-6.0)	0.46 - 53	< 2.41
Data are expressed as median (25th and 75th percentiles) with minimum and maximum values. *Normal ranges are according to NHS Foundation Trust. Abbreviations: N, number				

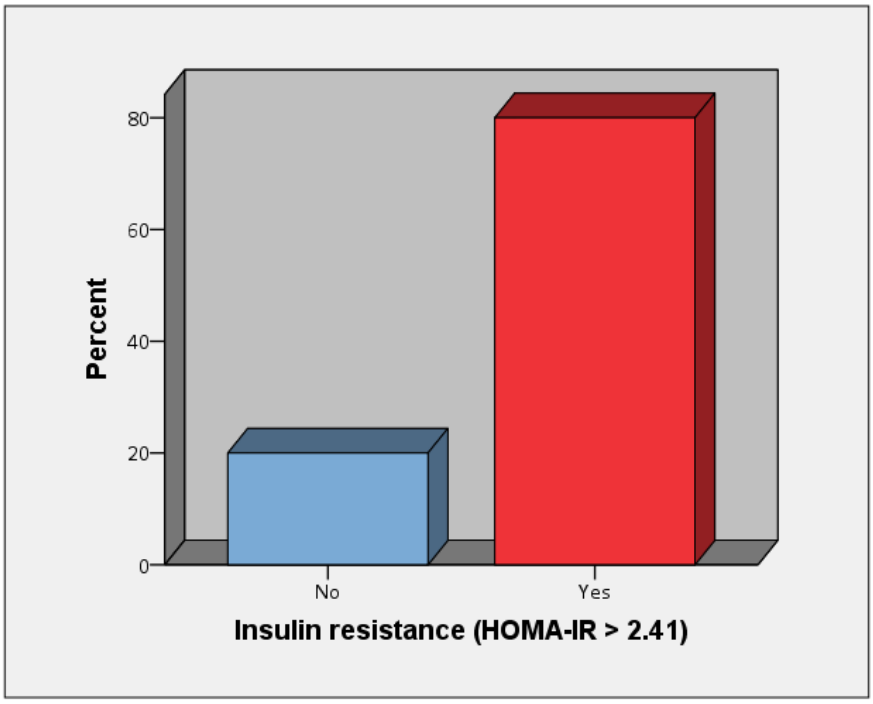


Figure 3-3 Percentage of patients included in the INSYTE study showing low or high insulin resistance according to the HOMA insulin resistance score.

3.3.1.6.2 Fasting blood concentrations of insulin, glucose and calculation of insulin resistance in the WELCOME study

Table 3-14 describes the fasting blood glucose, fasting blood insulin and HOMA insulin resistance of the 105 participants in the WELCOME study. Blood glucose and insulin concentrations were high. HOMA insulin resistance score was higher than the normal range. As shown in Figure 3-4 only around 40% of the participants were in the normal range, while 60% had high insulin resistance.

Table 3-14 Results of fasting blood glucose, fasting blood insulin and HOMO insulin resistance of the patients with NAFLD in the WELCOME study

Variable	Total N	Median (IQR 25,75)	Min – Max	Normal Range*
Glucose (mmol/L)	105	5 (5, 7)	4 - 19	4.1 - 6
Insulin (µUnit/ml)	104	13 (8, 20)	4 - 169	An interpretation of the results will depend on the glucose
HOMA Insulin Resistance	96	3 (2, 5)	0.7 - 16	< 2.41
Data are expressed as median (25th and 75th percentiles) with minimum and maximum values. *Normal ranges are according to NHS Foundation Trust. Abbreviations: N, number				

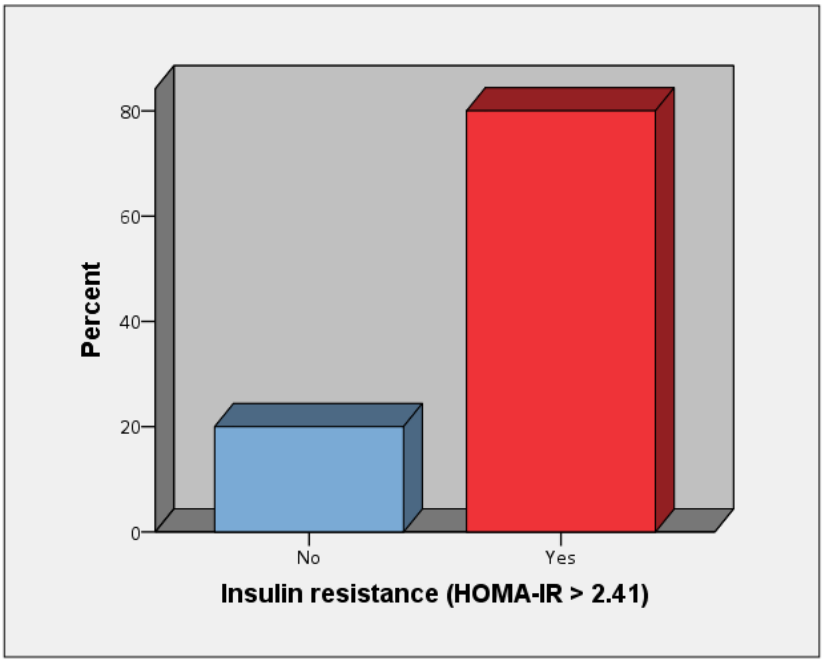


Figure 3-4 Percentage of patients included the WELCOME study showing low or high insulin resistance according to the HOMA insulin resistance score

3.3.1.7 Concentrations of inflammatory markers in NAFLD patients

3.3.1.7.1 Fasting blood concentrations of inflammatory markers patients with NAFLD included in the INSYTE study

Table 3-15 describes the fasting blood concentrations of inflammatory markers (CRP, TNF-α, IL-6, IL-8, IL10, RANTES, ICAM-1, VCAM-1, MCP-1, P-selectin, E-Selectin, ICAM-1, and VCAM-1) for the 63 participants in the INSYTE study . A concentration of CRP >3 mg/L is considered to indicate low grade inflammation. (40 %) of participants had low grade inflammation as shows in Figure 3-5.

Table 3-15 Fasting blood concentrations of inflammation markers in the INSYTE study

Variable	Total N	Mean (SD) or Median(IQR 25,75)	Min- Max	Normal range*
CRP (mg/L)	63	2 (2.0-5.0)	0.5 - 15.0	< 3.0
TNF-α (pg/ml)	63	12 (9.3-13.6)	4 - 47	
IL-6 (pg/ml)	63	3 (2.4-3.8)	1.35 - 13.5	
IL-8 (pg/ml)	63	15 (12-21.5)	7 - 35	
IL-10 (pg/ml)	63	0.9 (0.07-1.0)	0.5 - 4.6	
MCP-1 (pg/ml)	63	298 (105)	92 - 638	
RANTES (pg/ml)	63	49 (22)	14 - 127	
VCAM-1 (pg/ml)	63	852 (717-997)	176 - 2416	
ICAM-1 (pg/ml)	63	325 (121)	660 - 780	
E-Selectin (pg/ml)	63	87 (58-103)	2.4 - 240	
P-Selectin (pg/ml)	63	86 (69-104)	21 - 238	

Data are expressed as mean or median with minimum and maximum values. Variables that were normally distribution are expressed as mean (standard deviation). Variables that were not normally distributed are expressed as median (25th and 75th percentiles). *Normal ranges are according to NHS Foundation Trust. Abbreviations: CRP, C-reactive protein; TNF-α, tumour necrosis factor alpha; IL-6, Interleukin 6; IL-8, Interleukin-8; IL-10, Interleukin-10; MCP-1,monocyte chemoattractant protein1; VCAM-1, vascular adhesion molecule1; ICAM-1, intracellular adhesion molecule1; N, number.

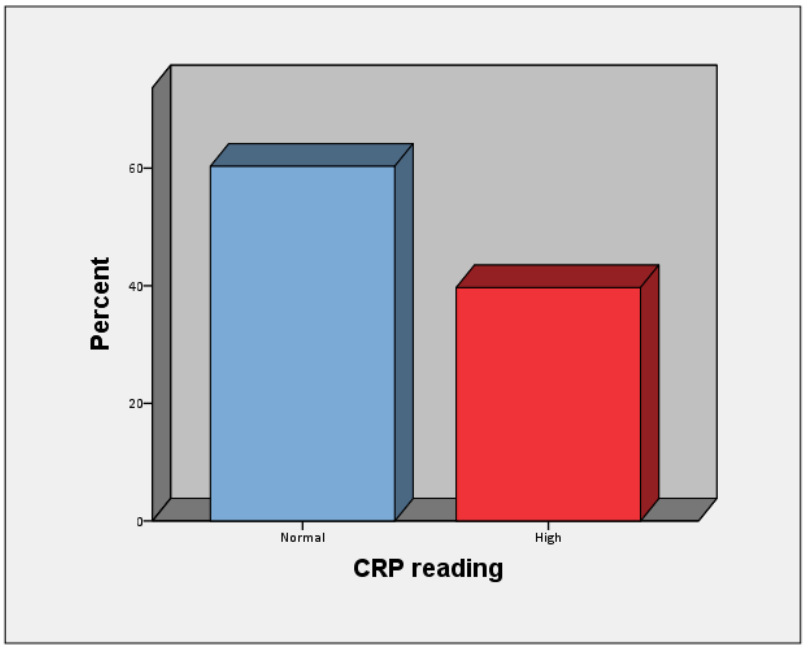


Figure 3-5 Percentage of patients with NAFLD included in the INSYTE study showing low or high CRP concentration. Abbreviations: CRP, C-reactive protein.

3.3.1.7.2 Fasting blood concentrations of inflammation markers in NAFLD patients in the WELCOME study

Table 3-16 describes the fasting blood concentrations of inflammation markers (CRP, TNF-α, IL-6, IL-8, IL10, VCAM-1, MCP-1, P-selectin, E-Selectin, ICAM-1, and VCAM-1) for the 105 participants in the WELCOME study . A concentration of CRP >3mg/L is considered to indicate low grade inflammation. Around 50 % of participants had low grade inflammation as shows in Figure 3-6.

Table 3-16 Fasting blood concentrations of inflammatory markers in the WELCOME study

Variable	Total N	Mean (SD) or Median (IQR 25,75)	Min - Max	Normal range
CRP (mg/l)	105	3 (2, 6)	1 - 35	< 3.0
TNF-α (pg/ml)	104	6 (5, 7)	2 - 19	
IL-6 (pg/ml)	53	1 (1, 2)	0.5 - 6	
IL-8 (pg/ml)	104	12 (8, 16)	1.4 - 106	
IL-10 (pg/ml)	104	4 (3, 7)	1.2 - 603	
MCP-1 (pg/ml)	104	460 (397, 526)	194 - 1349	
VCAM-1 (pg/ml)	104	464 (366, 620)	4 - 2153	
E-Selectin (pg/ml)	103	37 (27, 43)	9 - 164	
P-Selectin (pg/ml)	104	117 (35)	36 - 225	

Data are expressed as mean or median with minimum and maximum values. Variables that were normally distribution are expressed as mean (standard deviation). Variables that were not normally distributed are expressed as median (25th and 75th percentiles). *Normal ranges are according to NHS Foundation Trust. Abbreviations: CRP, C-reactive protein; TNF-α, tumour necrosis factor alpha; IL-6, Interleukin 6; IL-8, Interleukin-8; IL-10, Interleukin-10; MCP-1,monocyte chemoattractant protein1; VCAM-1, vascular adhesion molecule1; N, number.

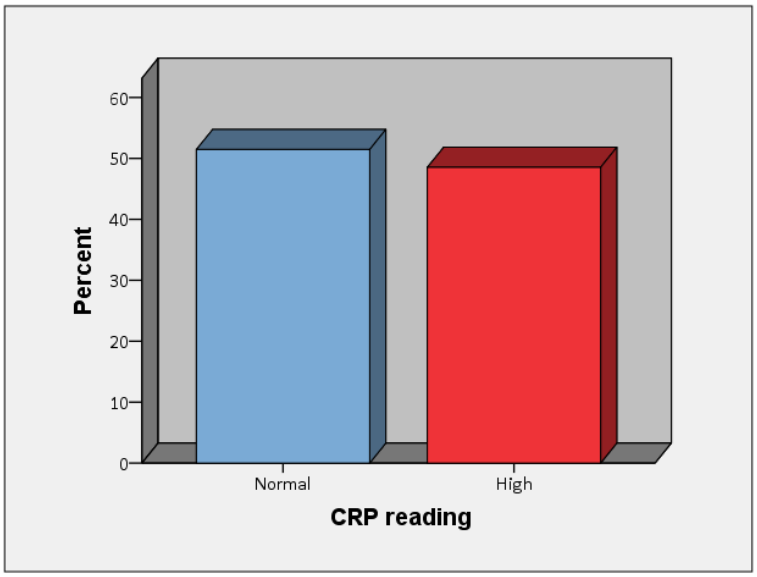


Figure 3-6 Percentage of patients with NAFLD included in the WELCOME study showing low or high CRP concentration.
Abbreviations: CRP, C-reactive protein

3.3.1.8 Carotid intima-media thickness and cardiovascular risk score in patients with NAFLD

3.3.1.8.1 Carotid intima-media thickness and cardiovascular risk score in patients with NAFLD included in the INSYTE study

Table 3-17 describes the carotid intima-media thickness and "Framinghan General Cardiovascular Risk Score " for the 63 participantsin INSYTE study. Median carotid intima-media thickness was within the normal range, however 10% of participants had high thickness as shown in Figure 3-7.

The mean 10 year cardiovascular risk was around 10.0%. Figure 3-8 shows the distribution of risk across four categories (<10%, 10- 20%, 20-30% and > 30%): all participants have risk of CVD within the next ten years with a round half having < 10% risk, 24% having 10-20% risk, 13% having 20-30% and 16% having more than 30% risk.

Table 3-17 Carotid intima-media thickness and cardiovascular risk score in patients with NAFLD include in the INSYTE study

Variable	Total N	Median (IQR 25,75)	Min- Max	Normal Range*
Carotid intima-media thickness	58	0.65 (0.58 , 0.70)	0.46 - 1.17	0.6 to 0.8
Cardiovascular Risk Score **	63	10.2 (6, 22)	2 - 30	-
Data are expressed as median (25th and 75th percentiles) with minimum and maximum values. **Framingham General Cardiovascular Risk Score. *Normal ranges are according to NHS Foundation Trust. Abbreviations: N, number..				

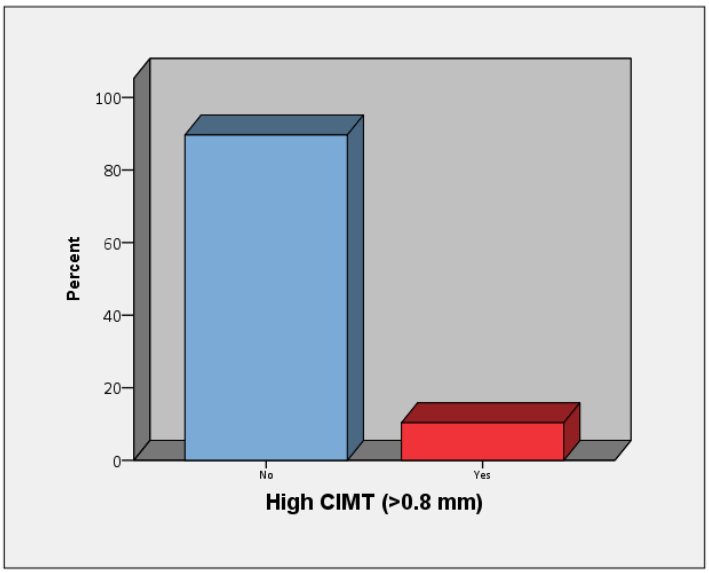


Figure 3-7 Percentage of patients with NAFLD included in the INSYTE study showing low or high CIMT.
Abbreviations: CIMT, carotid intima-media thickness.

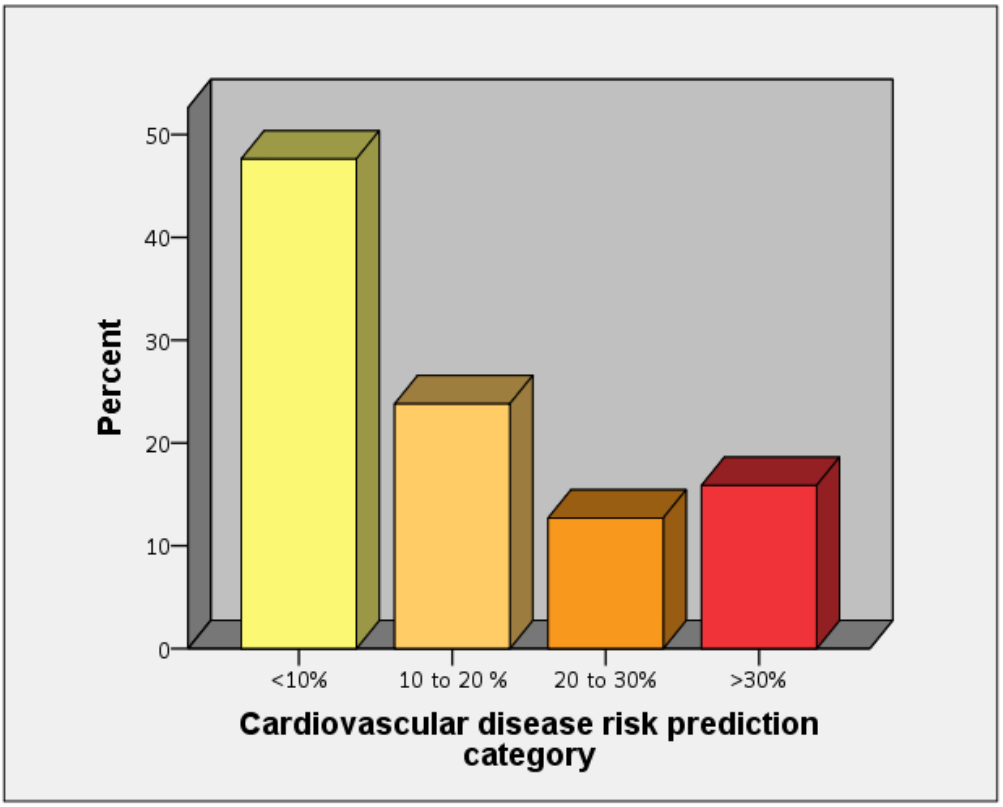


Figure 3-8 Distribution of the 63 patients with NAFLD in the INSYTE study into four cardiovascular risk categories:
(<10%, 10- 20%, 20-30% and > 30%) depending on calculation of the Framinghan general cardiovascular risk score.

3.3.1.8.2 Carotid intima-media thickness and cardiovascular risk score in NAFLD patients of the WELCOME study

Table 3-18 describes the carotid intima-media thickness and 10 year cardiovascular risk score for the 105 participants in WELCOME study. Median carotid intima-media thickness was within the normal range, however 9% of participants had high thickness as shown in Figure 3-9.

The mean 10 year cardiovascular risk was around 20%. Figure 3-10 shows the distribution of risk across four categories (<10%, 10- 20%, 20-30% and > 30%): all participants have risk of CVD within the next ten years with around half having < 10% risk, 30% having 10-20% risk, 13% having 20-30% and 5% having more than 30% risk.

Table 3-18 Carotid intima-media thickness and cardiovascular risk score in NAFLD patients of the WELCOME study

Variable	Total N	Median (IQR 25,75)	Min- Max	Normal Range*
Carotid intima-media thickness	102	0.64 (0.59, 0.71)	0.48 , 0.98	0.6 to 0.8
Cardiovascular Risk Score **	101	19 (5, 18)	0.4 , 37	
Data are expressed as median (25th and 75th percentiles) with minimum and maximum values. **Framingham General Cardiovascular Risk Score. *Normal ranges are according to NHS Foundation Trust. Abbreviations: N, number..				

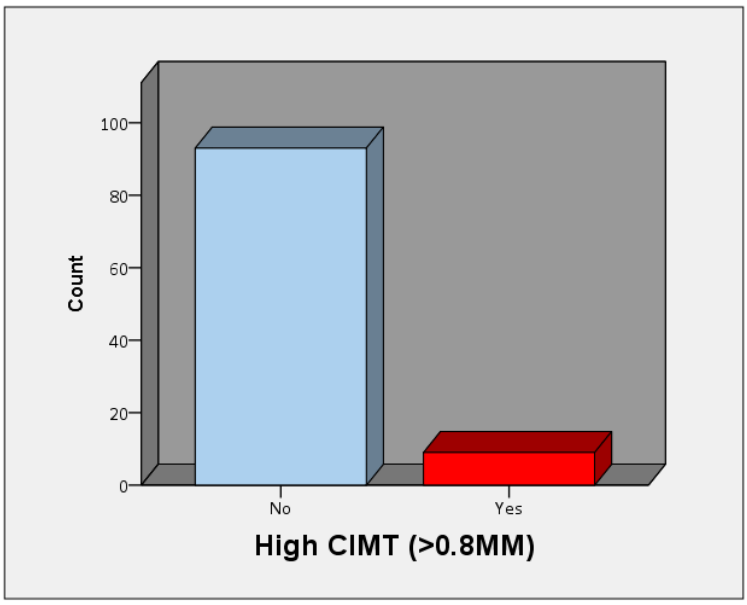


Figure 3-9 Percentage of patients with NAFLD included in the WELCOME study showing low or high CIMT.
Abbreviations: CIMT, carotid intima-media thickness.

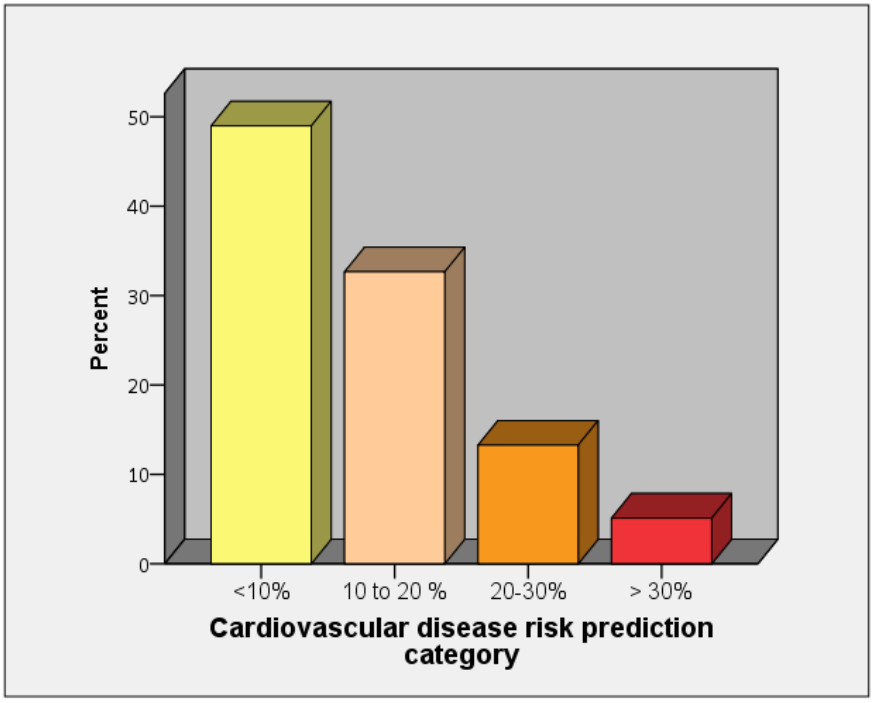


Figure 3-10 Distribution of the 105 patients with WELCOME in the INSYTE study into four cardiovascular risk categories:
(<10%, 10- 20%, 20-30% and > 30%) depending on calculation of the Framinghan general cardiovascular risk score.

3.3.1.9 Presence of the metabolic syndrome in patients with NAFLD

3.3.1.9.1 Metabolic syndrome in NAFLD patients of the INSYTE study

Table 3-19 describes the presence of metabolic syndrome components in 63 patients with NAFLD included in the INSYTE study and, as shown in Figure 3-11, almost all of the participants had metabolic syndrome.

Table 3-19 Presence of metabolic syndrome components in patients with NAFLD included in the INSYTE study

Metabolic syndrome* component					
	Abdominal obesity N (%)	High TG N (%)	Low HDL-cholesterol N (%)	Hypertension N (%)	Hyperglycaemia N (%)
Abdominal obesity	63 (100)	41 (65)	22(35)	37 (59)	41 (65)
High TG	41 (65)	41 (65)	17 (27)	26 (41)	27 (43)
Low HDL-cholesterol	22(35)	17 (27)	22(35)	15 (24)	18 (28.5)
Hypertension	37 (59)	26 (41)	15 (24)	37 (59)	25(40)
Hyperglycaemia	41 (65)	27 (43)	18 (28.5)	25(40)	41 (65)
MS components N (%)	At least one component 63 (100)	At least two components 63 (100)	At least three components 61 (97)	At least four components 17 (27)	Five components 13 (21)

Data are expressed as number and percentage. Abbreviations: MS, Metabolic syndrome ; TG,triglycerides; N, number. * according to the IDF classification (Alberti et al., 2009)

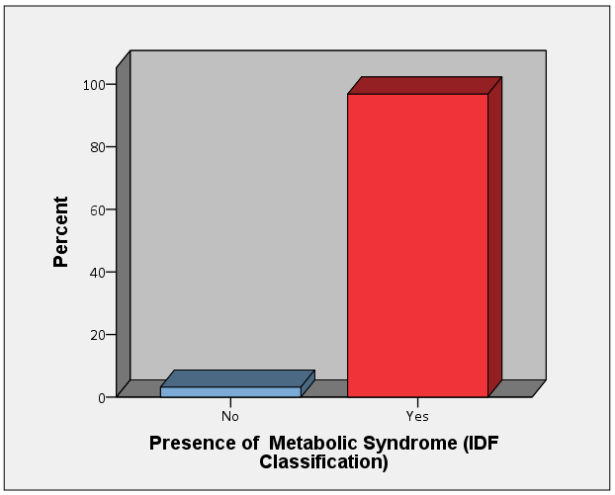


Figure 3-11 Presence of the metabolic syndrome in 63 patients with NAFLDin INSYTE study.

3.3.1.9.2 Metabolic syndrome in NAFLD patients of the WELCOME study

Table 3-20 describes the presence of metabolic syndrome components in 105 patients with NAFLD included in the WELCOME study and, as shown in Figure 3-12, almost all of the participants had metabolic syndrome.

Table 3-20 Presence of metabolic syndrome components in patients with NAFLD included in the WELCOME study

Metabolic syndrome* component					
	Abdominal obesity N (%)	High TG N (%)	Low HDL-cholesterol N (%)	Hypertension N (%)	Hyperglycaemia N (%)
Abdominal obesity	104 (99)	49 (49.6)	22(35)	37 (59)	41 (65)
High TG	45 (42)	49 (49.6)	17 (27)	26 (41)	27 (43)
Low HDL-cholesterol	47 (45)	17 (27)	47 (45)	15 (24)	18 (28.5)
Hypertension	73 (70)	26 (41)	15 (24)	73 (70)	25 (40)
Hyperglycaemia	71 (68)	27 (43)	18 (28.5)	42 (40)	71 (68)
MS components N (%)	At least one component 105 (100)	At least two components 105 (100)	At least three components 93 (89)	At least four components 26 (25)	Five components 22 (21)

Data are expressed as number and percentage. Abbreviations: MS, Metabolic syndrome ; TG,triglycerides; N, number. * according to the IDF classification (Alberti et al., 2009)

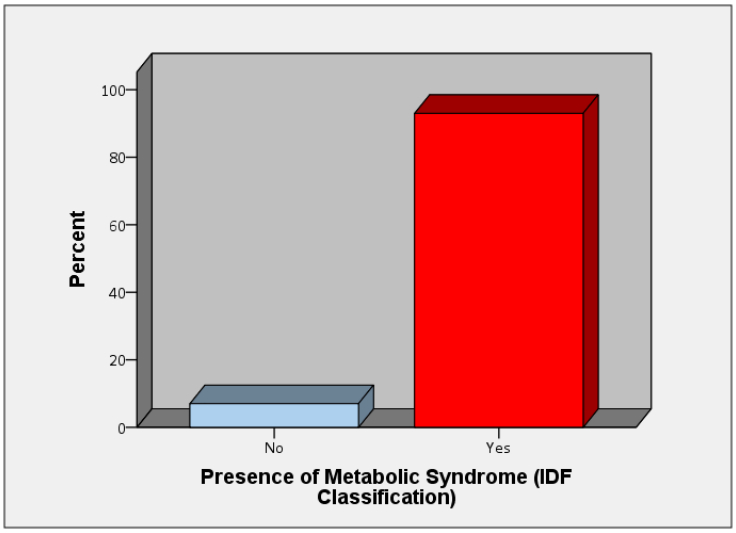


Figure 3-12 Presence of the metabolic syndrome in 105 patients with NAFLDin WELCOME study.

3.3.2 Omega-3 fatty acid status in the patients with NAFLD

3.3.2.1 Red blood cell omega-3 fatty acids status of patients with NAFLD included in the INSYTE study

Table 3-21 shows the concentration of eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) in red blood cells expressed as percentages of total fatty acids (%) and omega-3 index for the 63 participants. The mean omega-3 index was 5.7 and the range was 3.8 to 12.7. As shown in Figures 3-13 and 3-14, using omega-3 index as a cardiovascular risk factor as proposed by (Harris and von Schacky, 2004), 90% of the INSYTE study participants were in the medium level of CVD protection (4.1 to 7.9), and 3% of participants had low level of protection (≤4), while only 6% had high level of protection (≥8).

Table 3-21 Red blood cell omega-3 fatty acids (EPA content, DPA content, DHA content and omega-3 index) in patients with NAFLD include in the INSYTE study

Variable	Total N	Mean (SD) or Median (IQR 25,75)	Min - Max
RBC EPA (%)	63	0.9 (0.7, 1.2)	0.31 - 3.0
RBC DPA (%)	63	3.0 (0.3)	2.0 - 3.7
RBC DHA (%)	63	4.7 (4.0, 5.5)	3.0 - 9.6
Omega-3 Index	63	5.7 (4.8 , 6.5)	3.8 - 12.7
Data are expressed as mean or median with minimum and maximum values. Variables that were normally distribution are expressed as mean (standard deviation). Variables that were not normally distributed are expressed as median (25th and 75th percentiles). Abbreviations: N, number.			

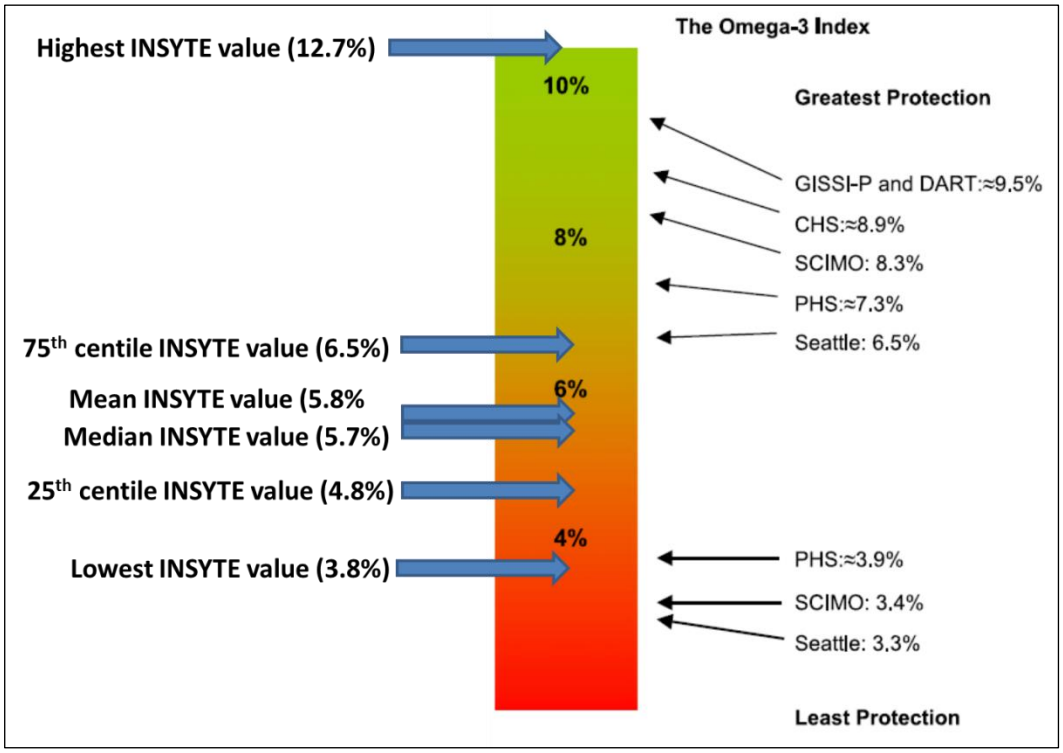


Figure 3-13 Omega-3 index cardioprotection level of the 63 patients with NAFLD in INSYTE study.
(in the left), according to the omega-3 index risk profile proposed by (Harris and von Schacky, 2004) compared to the average omega-3 index level in other studies (in the right).

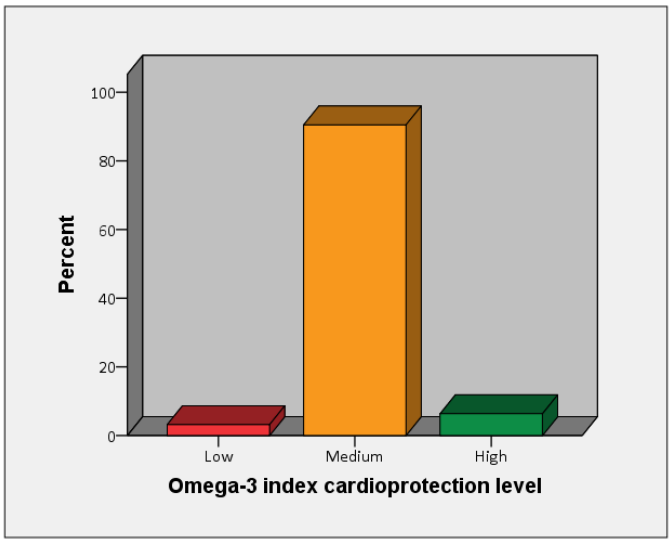


Figure 3-14 Omega-3 index cardioprotection level of the 63 patients with NAFLD in the INSYTE study.
Compared to the omega-3 index risk profile proposed by (Harris and von Schacky, 2004): low protection (omega-3 index ≤ 4), medium protection (omega-3 index = 4.1 to 7.9), high protection (omega-3 index ≥ 8).

3.3.2.2 Red blood cell omega-3 fatty acids status of patients with NAFLD included in the WELCOME study

Table 3-22 shows the concentration of eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) in red blood cells expressed as percentages of total fatty acids (%) and omega-3 index for the 105 participants in WELCOME study. The mean omega-3 index was 5.0 and the range was 3.3 to 10.6. And as shown in Figures 3-15 and 3-16, using omega-3 index as a cardiovascular risk factor as proposed by (Harris and von Schacky, 2004), 87% of the WELCOME study participants were in the medium level of CVD protection (4.1 to 7.9), and 7% of participants had low level of protection (≤4), while only 6% had high level of protection (≥8).

Table 3-22 Red blood cell omega-3 fatty acids (EPA content, DPA content, DHA content and omega-3 index) in patients with NAFLD included in the WELCOME study

Variable	Total N	Mean (SD) or Median(IQR 25,75)	Min - Max
RBC EPA (%)	104	0.9 (0.7, 1.1)	0.4 - 2.0
RBC DPA (%)	104	2.6 (0.4)	1.6 - 3.6
RBC DHA (%)	104	4.6 (1.2)	2.4 - 8.0
Omega-3 Index	104	5.0 (4.6, 6.0)	3.3 - 10.6
Data are expressed as mean or median with minimum and maximum values. Variables that were normally distribution are expressed as mean (standard deviation). Variables that were not normally distributed are expressed as median (25th and 75th percentiles(IQR)). Abbreviations: N, number.			

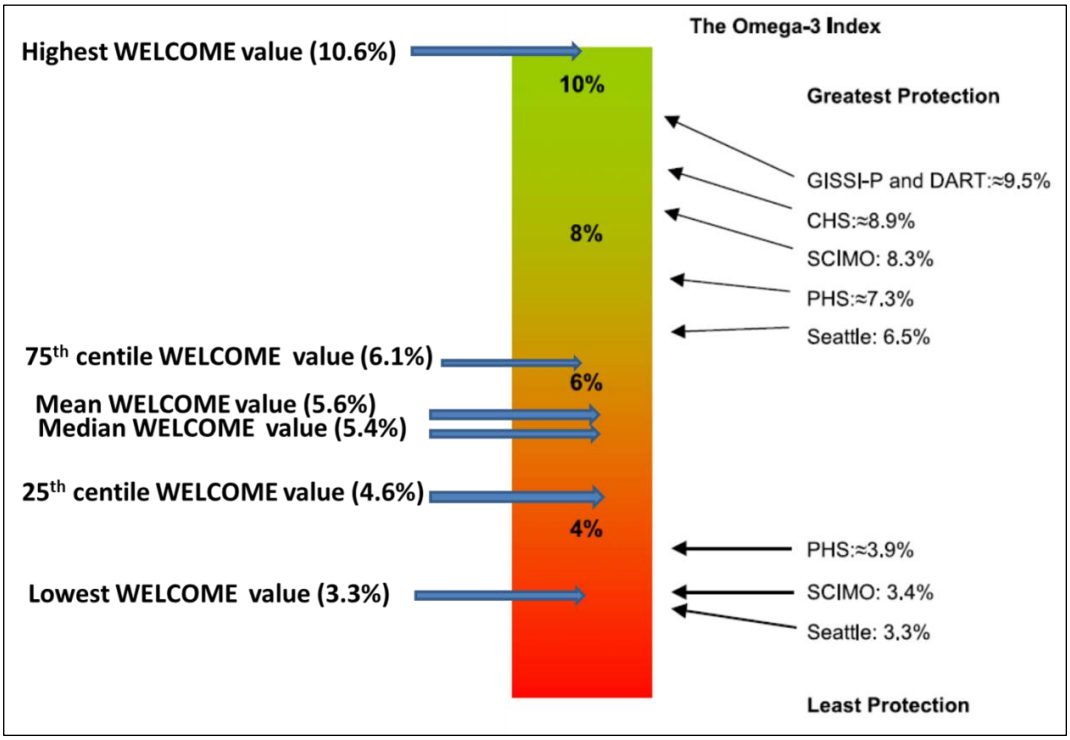


Figure 3-15 Omega-3 index cardio protection level of the 63 patients with NAFLD in WELCOME study.
(in the left) according to the omega-3 index risk profile proposed by (Harris and von Schacky, 2004) compared to the average omega-3 index level in other studies (in the right).

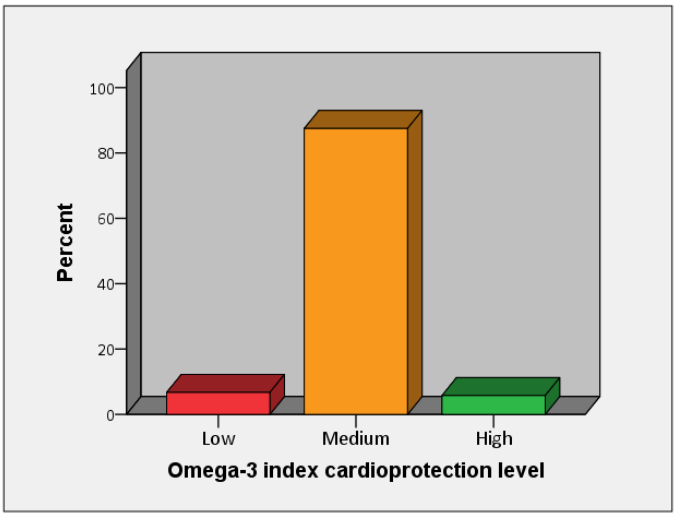


Figure 3-16 Omega-3 index cardioprotection level of the 105 patients with NAFLD in the WELCOME study.
Compared to the omega-3 index risk profile proposed by (Harris and von Schacky, 2004): low protection (omega-3 index ≤ 4), medium protection (omega-3 index = 4.1 to 7.9), high protection (omega-3 index ≥ 8).

3.3.3 Omega-3 fatty acids status in different sex and age groups in patients with NAFLD

The analysis presented in this section was conducted to examine whether the status of omega-3 fatty acids (EPA, DPA, and DHA) and omega-3 index in patients with NAFLD differed according to sex and age. This was considered important because subsequent analysis of the association between omega-3 fatty acids and CV risk factors(the primary aim of this research) may need to take into account (i.e. be adjusted for) effects of sex and age.

3.3.3.1 Omega-3 fatty acids status in different sex and age groups and the interaction between age and sex in patients with NAFLD included in the INSYTE study

3.3.3.1.1 EPA status

Table 3-23 shows the concentration of red blood cell EPA in different sex and age groups. Two-way ANOVA revealed a significant effect of age for EPA, which was higher in patients aged > 50 y compared to those < 50 y. However there was no significant effect of sex or a sex by age interaction.

Table 3-23 Concentration of EPA in red blood cells according to sex and age in 63 patients with NAFLDinclude in the INSYTE study

EPA				
		N	Mean (SD)	p
Sex				0.429
	M	37	0.9 (0.3)	
	F	26	1.1 (0.4)	
Age				0.046
	< 50 y	29	0.9 (0.2)	
	≥ 50 y	34	1.1 (0.4)	
Sex *Age				0.721
	M < 50 y	20	0.9 (0.2)	
	M ≥ 50 y	17	1.0 (0.4)	
	F < 50 y	9	0.9 (0.3)	
	F ≥ 50 y	17	1.1 (0.4)	
Two-way ANOVA was used to assess differences between sex and age groups and the sex x age interaction. Abbreviations: N,number; SD,standard deviation; M,male; F,female; p, P-value				

3.3.3.1.2 DPA status

Table 3-24 shows the concentration of red blood cell DPA in different sex and age groups. Two-way ANOVA revealed no effects of age or sex and no significant age by sex interaction.

Table 3-24 Concentration of DPA in red blood cell according to sex and age in 63 patients with NAFLD included in the INSYTE study

DPA				
		N	Mean (SD)	p
Sex				0.641
	M	37	3.05 (0.35)	
	F	26	3.0 (0.35)	
Age				0.255
	< 50 y	29	3.1 (0.3)	
	≥ 50 y	34	3.0 (0.4)	
Sex *Age				0.766
	M < 50 y	20	3.1 (0.3)	
	M ≥ 50 y	17	3.0 ()0.4)	
	F < 50 y	9	3.01 (0.3)	
	F ≥ 50 y	17	2.9 (0.3)	
Two-way ANOVA was used to assess differences between sex and age groups and the sex x age interaction. Abbreviations: N,number; SD,standard deviation; M,male; F,female; p, P-value				

3.3.3.1.3 DHA status

Table 3-25 shows the concentration of red blood cell DHA in different sex and age groups. Two-way ANOVA revealed a significant effect of age for DHA, which was higher in patients aged > 50 y compared to those < 50 y. However there was no significant effect of sex or a sex by age interaction.

Table 3-25 Concentration of DHA in red blood cells according to sex and age in 63 patients with NAFLD included in the INSYTE study

DHA				
		N	Mean (SD)	p
Sex				0.522
	M	37	4.7 (1.2)	
	F	26	5.0 (1.1)	
Age				0.006
	< 50 y	29	4.4 (0.9)	
	≥ 50 y	34	5.2 (1.2)	
Sex *Age				0.849
	M < 50 y	20	4.3 (0.8)	
	M ≥ 50 y	17	5.1 (1.4)	
	F < 50 y	9	4.45 (1.1)	
	F ≥ 50 y	17	5.3 (1.0)	
Two-way ANOVA was used to assess differences between sex and age groups and the sex x age interaction. Abbreviations: N,number; SD,standard deviation; M,male; F,female; p, P-value				

3.3.3.1.4 Omega-3 index

Table 3-26 shows the omega-3 index in different sex and age groups. Two-way ANOVA revealed a significant effect of age for omega-3 index, which was higher in patients aged > 50 y compared to those < 50 y. However there was no significant effect of sex or a sex by age interaction.

Table 3-26 Omega-3 index in red blood cells according to sex and age in 63 patients with NAFLD included in the INSYTE study

Omega-3 index				
		N	Mean (SD)	p
Sex				0.323
	M	37	5.56 (1.2)	
	F	26	6.09 (1.4)	
Age				0.005
	< 50 y	29	5.2 (1.0)	
	≥ 50 y	34	6.2 (1.4)	
Sex * Age				0.642
	M < 50 y	20	5.2 (0.9)	
	M ≥ 50 y	17	6.0 (1.4)	
	F < 50 y	9	5.4 (1.3)	
	F ≥ 50 y	17	6.5 (1.35)	
Two-way ANOVA was used to assess differences between sex and age groups and the sex x age interaction. Abbreviations: N,number; SD,standard deviation; M,male; F,female; p, P-value				

3.3.3.2 Omega-3 fatty acids status in different sex and age groups in patients with NAFLD included in the WELCOME study

3.3.3.2.1 EPA status

Table 3-27 shows the concentration of red blood cell EPA in different sex and age groups. Two-way ANOVA revealed a significant effect of age for EPA, which was higher in patients aged > 50 y compared to those < 50 y. However there was no significant effect of sex or a sex by age interaction.

Table 3-27 Concentration of EPA in red blood cells according to sex and age in 105 patients with NAFLD included in the WELCOME study

EPA				
		N	Mean (SD)	p
Sex				0.825
	M	60	0.9 (0.3)	
	F	44	1.0 (0.3)	
Age				0.001
	< 50 y	42	0.8 (0.2)	
	≥ 50 y	62	1.0 (0.3)	
Sex * Age				0.501
	M < 50 y	26	0.8 (0.2)	
	M ≥ 50 y	34	1.0 (0.3)	
	F < 50 y	16	0.8 (0.2)	
	F ≥ 50 y	28	1.0 (0.3)	
Two-way ANOVA was used to assess differences between sex and age groups and the sex x age interaction Abbreviations: N,number; SD,standard deviation; M,male; F,female; p, P-value				

3.3.3.2.2 DPA status

Table 3-28 shows the concentration of red blood cell DPA in different sex and age groups. Two-way ANOVA revealed no effects of age or sex and no significant age by sex interaction.

Table 3-28 Concentration of DPA in red blood cells according to sex and age in 105 patients with NAFLD included in the WELCOME study

DPA				
		N	Mean (SD)	p
Sex				0.225
	M	60	2.7 (0.4)	
	F	44	2.6 (0.4)	
Age				0.351
	< 50 y	42	2.6 (0.3)	
	≥ 50 y	62	2.7 (0.4)	
Sex * Age				0.912
	M < 50 y	26	2.6 (0.4)	
	M ≥ 50 y	34	2.7 (0.3)	
	F < 50 y	16	2.5 (0.2)	
	F ≥ 50 y	28	2.6 (0.4)	
Two-way ANOVA were using to assess differences between sex and age groups Abbreviations: N,number; SD,standard deviation; M,male; F,female; p, P-value				

3.3.3.2.3 DHA status

Table 3-29 shows the concentration of red blood cell DHA in different sex and age groups. Two-way ANOVA revealed a significant effect of age for DHA, which was higher in patients aged > 50 y compared to those < 50 y. However there was no significant effect of sex or a sex by age interaction.

Table 3-29 Concentration of DHA in red blood cells according to sex and age in 105 patients with NAFLD included in the WELCOME study

DHA				
		N	Mean (SD)	p
Sex				0.836
	M	60	4.6 (1.2)	
	F	44	4.6 (1.1)	
Age				0.001
	< 50 y	42	4.1 0.8)	
	≥ 50 y	62	4.9 (1.2)	
Sex * Age				0.634
	M < 50 y	26	4.1 (0.7)	
	M ≥ 50 y	34	5.0 (1.3)	
	F < 50 y	16	4.1 (0.9)	
	F ≥ 50 y	28	4.85 (1.2)	
Two-way ANOVA were using to assess differences between sex and age groups and the sex x age interaction Abbreviations: N,number; SD,standard deviation; M,male; F,female; p, P-value				

3.3.3.2.4 Omega-3 index

Table 3-30 shows the omega-3 index in different sex and age groups. Two-way ANOVA revealed a significant effect of age for omega-3 index, which was higher in patients aged > 50 y compared to those < 50 y. However there was no significant effect of sex or a sex by age interaction.

Table 3-30 Omega-3 index in red blood cells according to sex and age in 105 patients with NAFLD included in the WELCOME study

Omega-3 index				
		N	Mean (SD)	p
Sex				0.874
	M	60	5.5 (1.4)	
	F	44	5.6 (1.3)	
Age				<0.001
	< 50 y	42	4.9 (0.9)	
	≥ 50 y	62	6.0 (1.5)	
Sex * Age				0.769
	M < 50 y	26	4.9 (0.8)	
	M ≥ 50 y	34	6.0 (1.6)	
	F < 50 y	16	5 .0 (1.0)	
	F ≥ 50 y	28	5.9 (1.4)	
Two-way ANOVA was used to assess differences between sex and age groups and the sex x age interaction. Abbreviations: N,number; SD,standard deviation; M,male; F,female; p, P-value				

3.3.4 Relationship of age with red blood cell omega-3 fatty acids in patients with NAFLD

The two way ANOVA analysis presented above identified significant effects of age (> 50 y versus < 50 y) on EPA, DHA and omega-3 index in both the INSYTE and WELCOME cohorts, with higher omega-3 fatty acids in the older age group, and with no effect of age on DPA. In this section the association between age and omega-3 fatty acids in red blood cells from patients with NAFLD is further explored.

3.3.4.1 Correlations between age and red blood cell omega-3 fatty acids in patients with NAFLD

Figure 3-17 shows the correlation between age and red blood cell omega-3 fatty acids in 63 patients with NAFLD included in the INSYTE study. There were significant positive correlations between age and DHA ($p = 0.001$) and age and omega-3 index ($p = 0.002$). However, there were no significant correlations between EPA or DPA and age, although the correlation with EPA showed a positive trend ($p = 0.066$).

Figure 3-18 shows the correlation between age and red blood cell omega-3 fatty acids in 105 patients with NAFLD included in the WELCOME study. There were significant positive correlations between age and EPA, DHA and omega-3 index (all $p < 0.001$). However, there were no significant correlation between age and DPA.

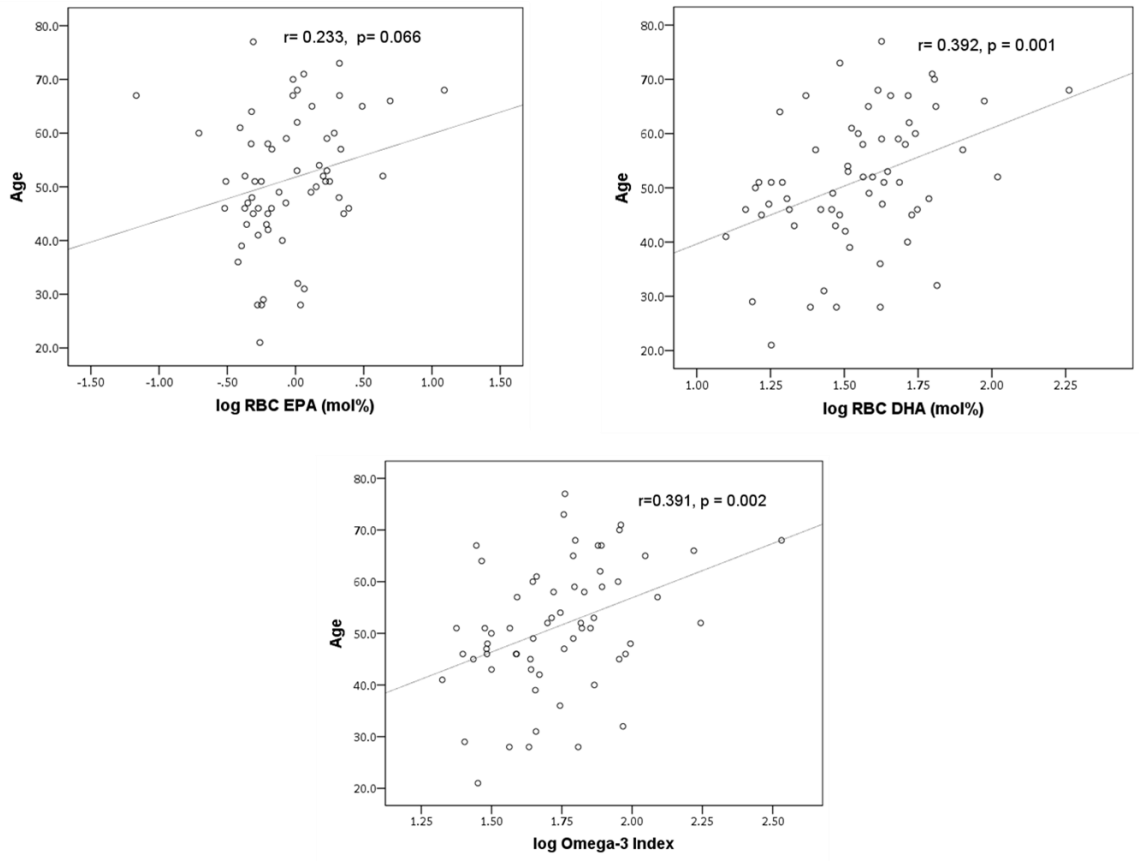


Figure 3-17 Correlations between age and red blood cell omega-3 fatty acids in 63 patients with NAFLD included in the INSTYE study.

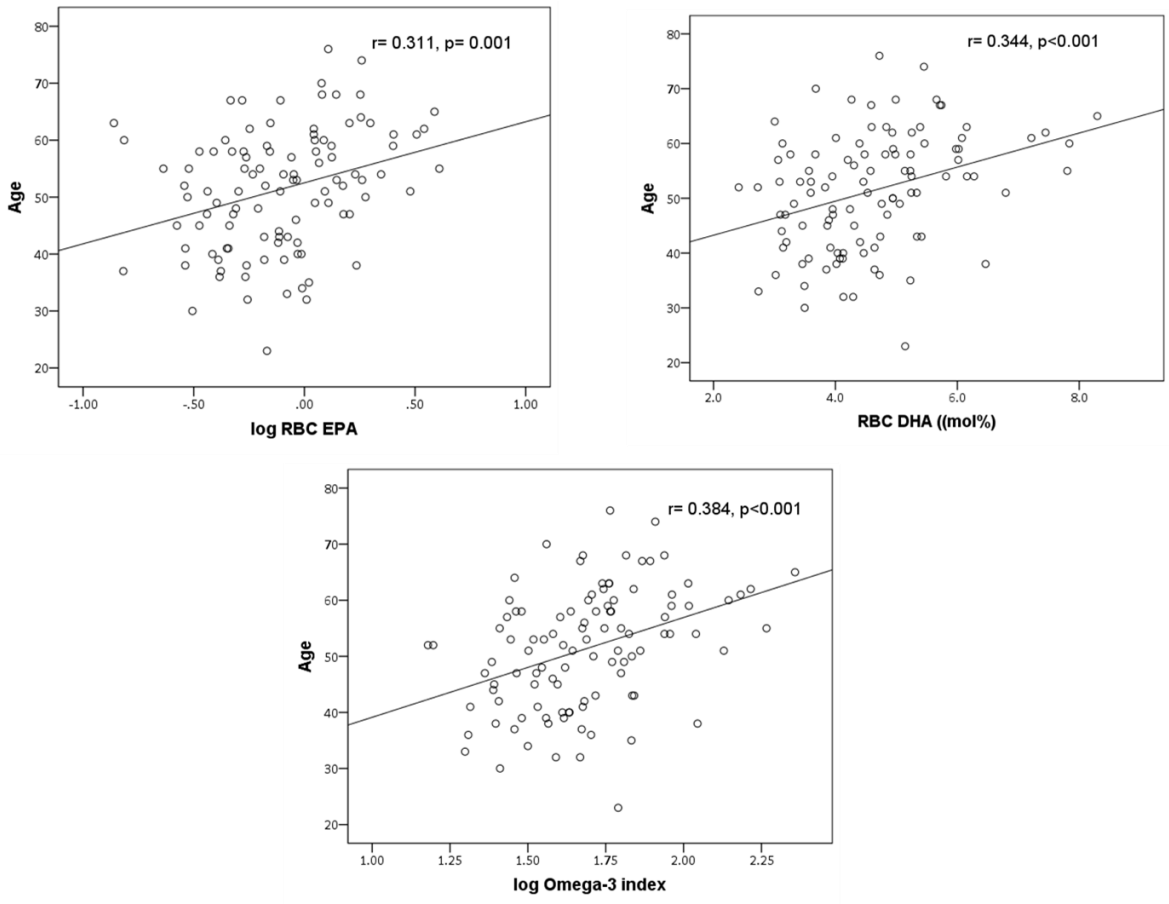


Figure 3-18 Correlations between age and red blood cell omega-3 fatty acids in 105 patients with NAFLD included in the WELCOME study.

3.3.4.2 Linear regressions between age and red blood cell omega-3 fatty acids after adjustment for sex

Tables 3-31 and 3-32 show the results of multiple linear regressions between age and red blood cell omega-3 fatty acids after adjustment for sex in NAFLD patients in the INSYTE and WELCOME studies, respectively. In both studies, age was a statistically significant predictor of EPA, DHA and omega-3 index. consistent with the findings from the two-way ANOVA and correlation analyses presented above. DPA showed no significant relationships with age after adjusting for sex.

Table 3-31 Relationship of red blood cell omega-3 fatty acids with age after adjusting for sex in patients with NAFLD in INSYTE study

Age		
Variable	Unstandardized β coefficients	*P-value*
RBC EPA	9.442	0.039
RBC DPA	-3.265	0.472
RBC DHA	3.944	0.003
Omega-3 Index	3.466	0.004
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis: between age and each individual omega-3 fatty acid. adjusting for sex		

Table 3-32 Relationship of red blood cell omega-3 fatty acids with age after adjusting for sex in patients with NAFLD in WELCOME study

Age		
Variable	Unstandardized β coefficients	*P-value*
RBC EPA	11.612	0.001
RBC DPA	3.962	0.178
RBC DHA	3.114	0.001
Omega-3 Index	2.952	0.001
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between age and each individual omega-3 fatty acidadjusting for sex		

3.3.5 Relationship among red blood cell fatty acids and omega-3 index in patients with NAFLD

The analysis in this section was conductd to present the relationship among the different omega-3 fatty acids (EPA, DPA, and DHA) and omega-3 index in patients with NAFLD after adjustment for age and sex.

3.3.5.1 Correlations among red blood cell omega-3 fatty acids and omega-3 index in patients with NAFLD

In general in NAFLD patients, EPA was significantly and positively related with DPA, DHA and omega-3 index. However, there was no significant relationship between DPA and DHA.

In the INSYTE study there were strong significant positive correlations between red blood cell EPA and red blood cell DHA ($r = 0.620$, $p < 0.001$), red blood cell DPA ($r = 0.389$, $p= 0.002$) and omega-3 index ($r = 0.980$, $p < 0.001$). Also there was a strong significant correlation between red blood cell DHA and omega-3 index ($r = 0.980$, $p < 0.001$). These correlations are shown in Figures 3-19 to 3-22. However, there were no significant correlations between red blood cell DPA and red blood cell DHA ($r = 0.086$, $p = 0.503$) , or red blood cell DPA and omega-3 index ($r = 0.163$, $p = 0.201$).

In the WELCOME study there were strong significant positive correlations between red blood cell EPA and red blood cell DHA ($r = 0.382$, $p < 0.001$), red blood cell DPA ($r = 0.448$, $p < 0.001$) and omega-3 index ($r = 0.527$, $p = 0.001$). Also there was a strong significant correlation between red blood cell DHA and omega-3 index ($r = 0.997$, $p < 0.001$). These correlations are shown in Figures 3-23 to 3-26. There were no significant correlations between red blood cell DPA and red blood cell DHA, or red blood cell DPA and omega-3 index.

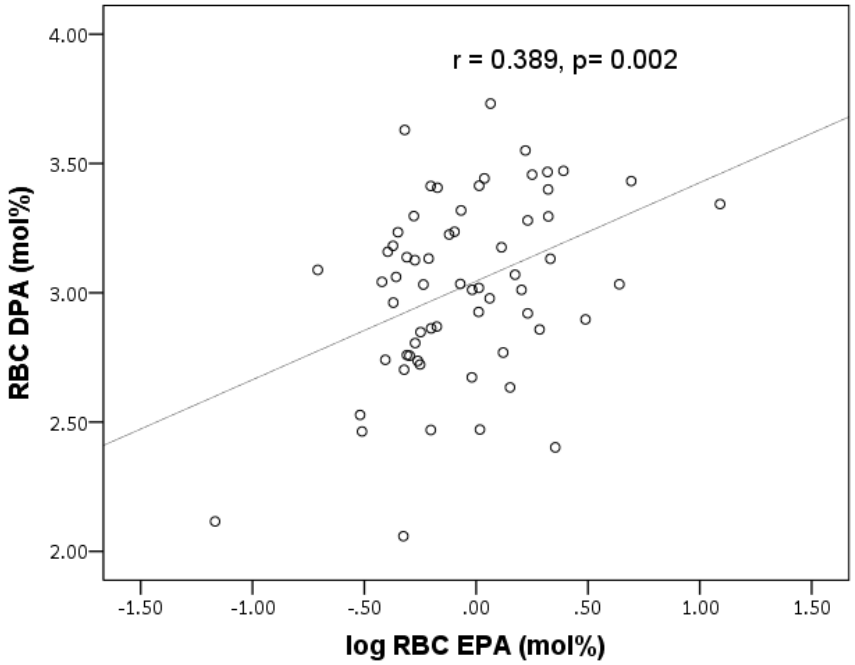


Figure 3-19 Scatter plot of correlation between red blood cell EPA and DPA in 63 NAFLD patients in the INSYTE study

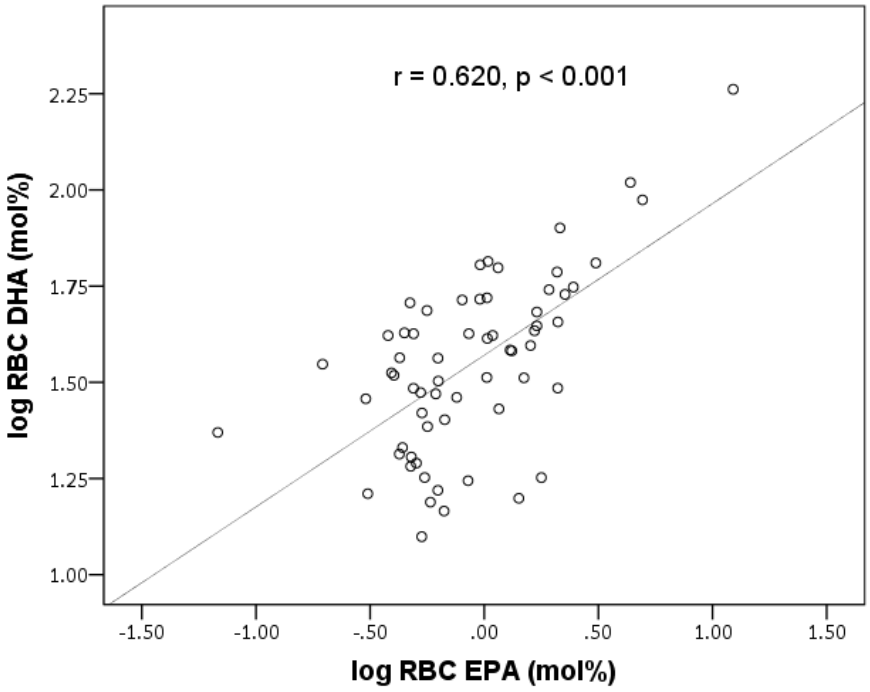


Figure 3-20 Scatter plot of correlation between red blood cell EPA and DHA in 63 NAFLD patients in the INSYTE study.

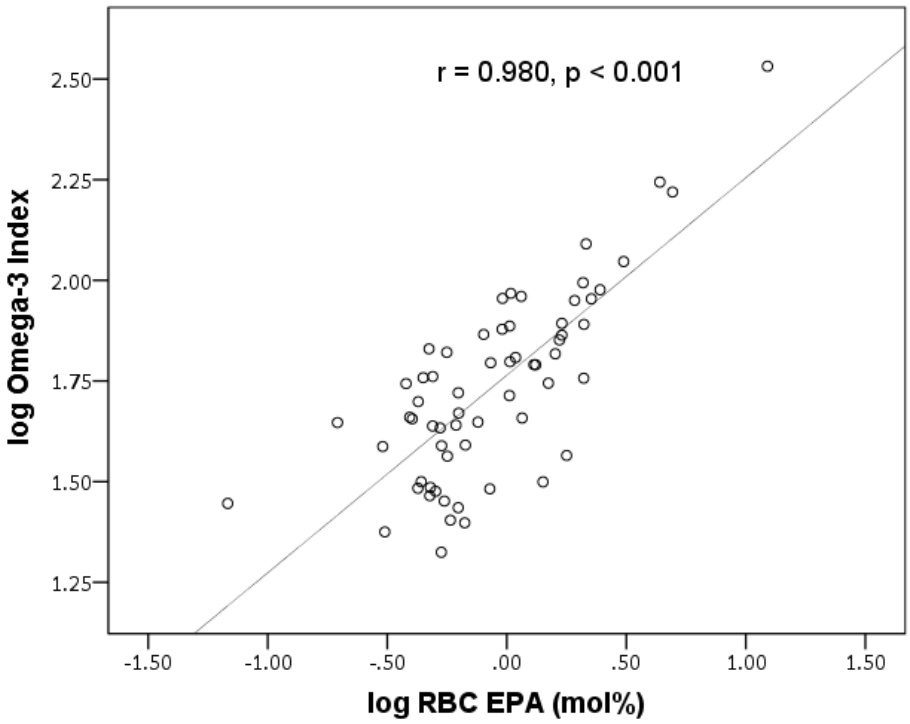


Figure 3-21 Scatter plot of correlation between red blood cell EPA and omega-3 index in 63 NAFLD patients in the INSYTE study.

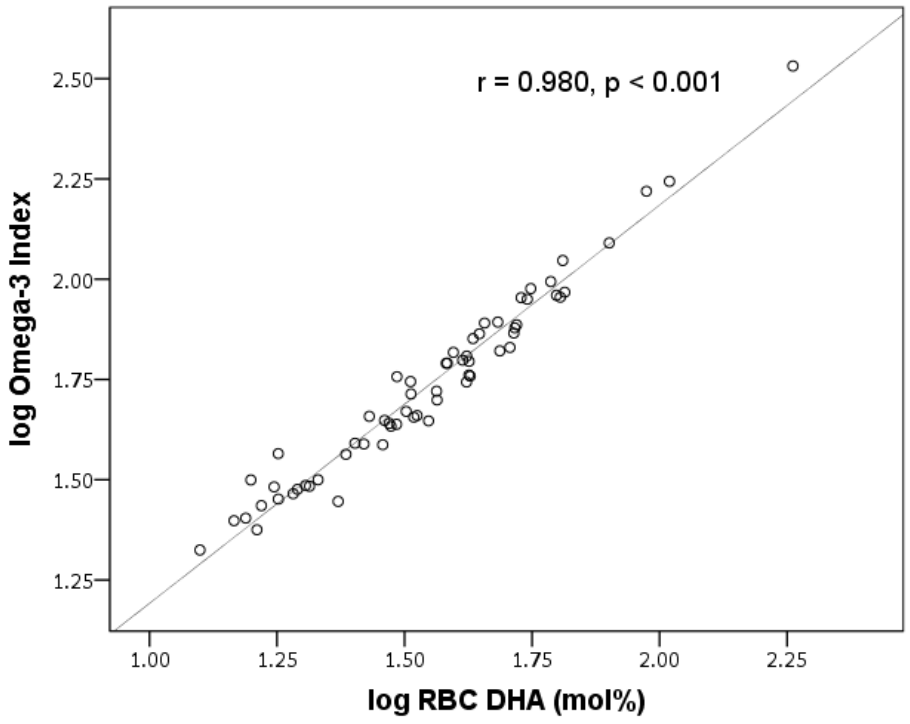


Figure 3-22 Scatter plot of correlation between red blood cell DHA and omega-3 index in 63 NAFLD patients in the INSYTE study.

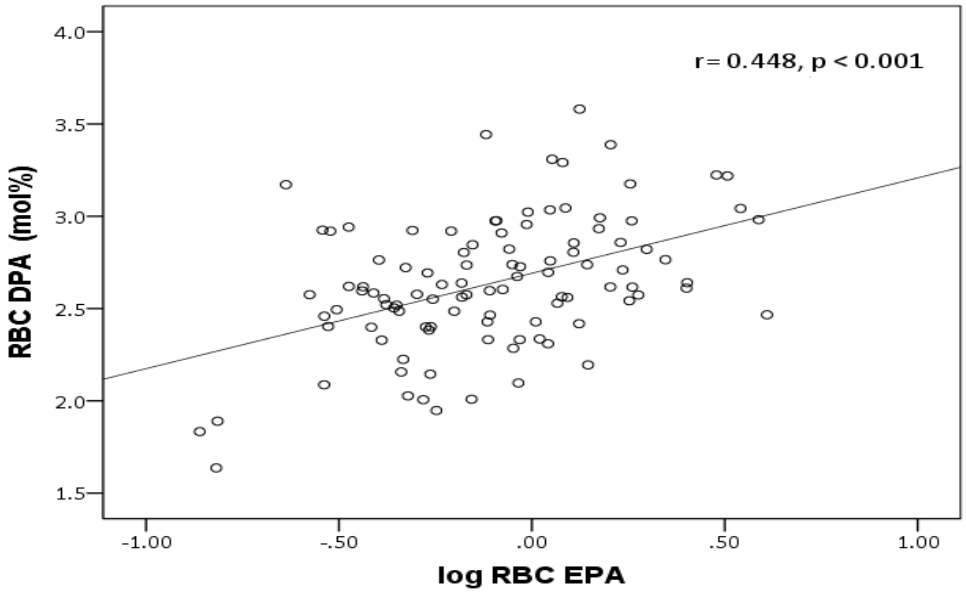


Figure 3-23 Scatter plot of correlation between red blood cell EPA and DPA in 105 NAFLD patients in the WELCOME study.

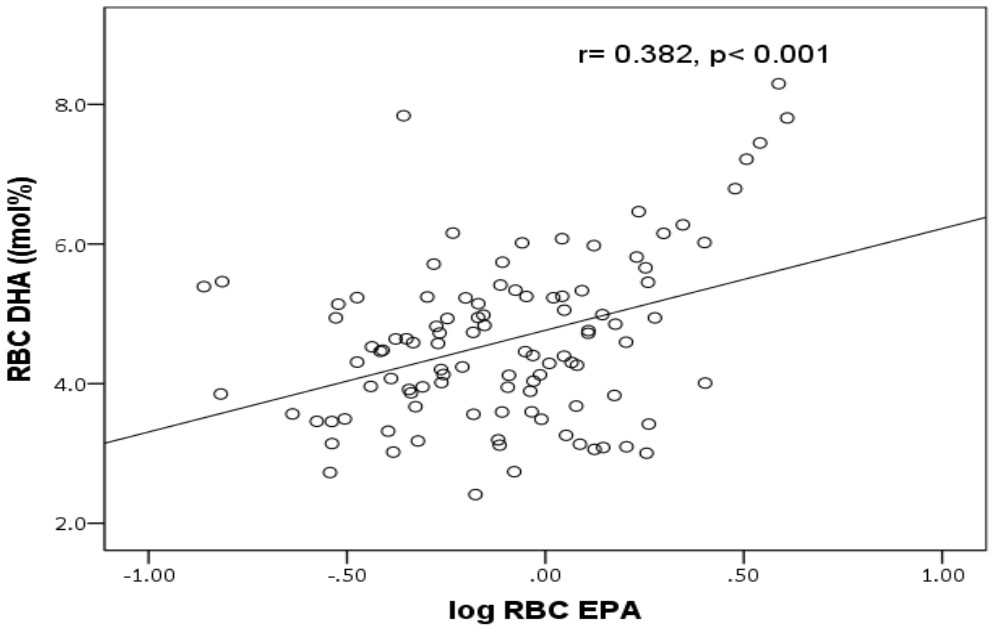


Figure 3-24 Scatter plot of correlation between red blood cell EPA and DHA in 105 NAFLD patients in the WELCOME study.

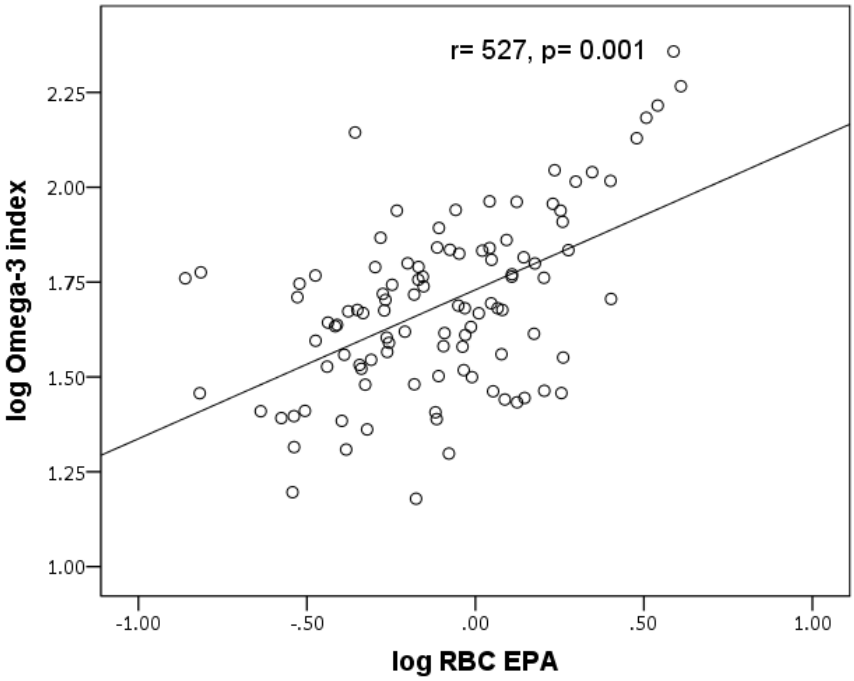


Figure 3-25 Scatter plot of correlation between red blood cell EPA and omega-3 index in 105 NAFLD patients in the WELCOME study.

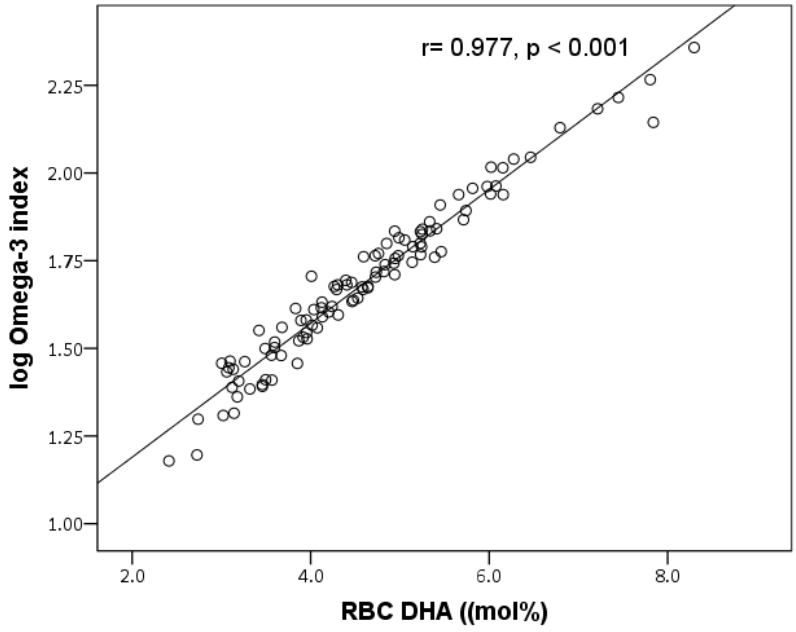


Figure 3-26 Scatter plot of correlation between red blood cell DHA and omega-3 index in 105 NAFLD patients in the WELCOME study.

3.3.5.2 Linear regressions among red blood cell fatty acids and omega-3 index in patients with NAFLD

Table 3-33 shows results of multiple linear regression analysis among red blood cell omega-3 fatty acids and omega-3 index after adjustment for age and sex in 63 NAFLD patients in the INSYTE study. In general, EPA had positive significant relationships with DPA and DHA; however, there were no significant relationships between DPA and DHA.

EPA was a statistically significant predictor of DPA, DHA and omega-3 index ($\beta = 0.443$, $p < 0.001$), ($\beta = 0.252$, $p < 0.001$), and ($\beta = 0.389$, $p < 0.001$) respectively. The beta coefficient shows that for every percent increase in red blood cell EPA the red blood cell DPA, DHA and omega-3 index will increase by 0.443, 0.252, and 0.389, respectively. Also, DHA was a statistically significant predictor of EPA and omega-3 index ($\beta = 0.352$, $p < 0.001$) and ($\beta = 0.963$, $p < 0.001$) respectively. Thus for every percent increase in red blood cell DHA the red blood cell EPA and omega-3 index will increase by 0.352 and 0.963 respectively. However, DPA showed no significant relationships with DHA or omega-3 index.

Table 3-33 Relationship among red blood cell fatty acids and omega-3 index in patients with NAFLD after adjustment for age and sex

DPA		
	β	p
RBC EPA	0.443	< 0.001
RBC DHA	0.252	0.247
Omega-3 Index	0.389	0.067

DHA		
	β	p
RBC EPA	0.352	< 0.001
RBC DPA	0.090	< 0.247
Omega-3 Index	0.963	< 0.001

EPA		
	β	p
RBC DPA	0.438	< 0.001
RBC DHA	0.974	< 0.001
Omega-3 Index	1.224	< 0.001

Omega-3 Index		
	β	p
RBC EPA	0.454	< 0.001
RBC DPA	0.143	< 0.067
RBC DHA	0.990	< 0.001

EPA, DHA, and omega-3 were log-transformed for statistical analysis. Multiple linear regression analysis between each fatty acid and other fatty acids and omega-3 was performed adjusted for age and sex. Abbreviations:β , Unstandardized β coefficient; p=P-value

3.4 Discussion

This thesis focusses on the relation between red blood cell omega-3 fatty acids and cardiovascular risk factors in patients with NAFLD. The primary cohort is patients involved in the INSYTE study, but data available from patients involved in the WELCOME study are used to confirm findings from the INSYTE patients. This chapter describes the anthropometric measurements, the cardiovascular disease risk factor profile and the status of omega-3 fatty acids in NAFLD patients from both the INSYTE and WELCOME studies. The relationships between omega-3 fatty acids and cardiovascular risk factors will be described in detail in the next two chapters. Here the results for various metabolic and cardiovascular risk factors were compared with the normal range, where this is available, and with NAFLD characteristics and overall CV risk. Although no formal statistical comparison was made, findings were very similar for all outcomes between the INSYTE and WELCOME cohorts.

The data presented are used to address the following hypotheses: a) that patients with NAFLD will have an elevated risk of cardiovascular disease but that this will vary among the patients, b) that patients with NAFLD will have a low omega-3 fatty acid status; c) that age and sex will influence omega-3 fatty acid status in patients with NAFLD (higher in women and increased with age); and d) that patients with NAFLD will not have an omega-3 index that provides a high cardioprotection level.

The data described in this chapter is in support of the hypothesis that patients with NAFLD will have an elevated risk of cardiovascular disease but that this will vary among the patients. The patients with NAFLD studied (with mean liver fat of 27% and 23% in the INSYTE and WELCOME studies, respectively) possess several characteristics indicating presence of metabolic syndrome and high cardiovascular

risk. Most patients had a BMI indicating obesity, a waist circumference indicating excess abdominal fat, and a high systolic blood pressure. Many had high blood cholesterol, triglycerides and glucose and a high cholesterol to HDL-cholesterol ratio or were being treated for these. According to HOMA-IR more than two thirds of the patients had moderate or severe insulin resistance. More than half of the patients had low grade inflammation according to CRP concentration. However, most patients did not have elevated diastolic blood pressure or CIMT. Thus it can be concluded that the patients studied had elevated risk of CVD, as indicated by the Framingham risk score, but that the risk was variable across the patients studied, again as indicated by the Framingham risk score. Therefore, the first hypothesis of the research described in this chapter can be accepted.

With regard to the second hypothesis that patients with NAFLD will have a low omega-3 fatty acid status, the mean omega-3 index, widely regarded as a good indicator of status (and CV risk) was 5.7 in the INSYTE patients and 5.0 in the WELCOME patients. Similar values are reported in both healthy subjects (Neubronner et al., 2011) and patients with CVD (Lemke et al., 2010 , Harris and von Schacky, 2004). Thus, the omega-3 status in the patients studied in this thesis is not especially low compared with other groups of individuals but is low in relation to estimated CV risk. In both cohorts studied < 6% of patients had an omega-3 index associated with a high level of CV protection. Therefore, the second hypothesis of the research described in this chapter can be accepted. Furthermore, the fourth hypothesis that patients with NAFLD will not have an omega-3 index that provides a high cardioprotection level can be accepted.

With regard to the third hypothesis that age and sex will influence omega-3 fatty acid status in patients with NAFLD (higher in women and increased with age), EPA, DHA and omega-3 index were all positively associated with age and were significantly higher in patients aged > 50 y compared to those aged < 50 y. Thus,

the part of this hypothesis that omega-3 fatty acids will be increased with age in patients with NAFLD can be accepted. These findings are consistent with other research showing that omega-3 fatty acid concentrations are higher in older individuals (Bolton-Smith et al., 1997, Rees et al., 2006), but this is the first time that this has been reported in patients with NAFLD. There was no association between sex and omega-3 fatty acids in this patient group. Thus, the part of this hypothesis that omega-3 fatty acids will be higher in women than men with NAFLD must be rejected. Several previous studies have reported higher EPA and/or DHA in women than men (Childs et al., 2008, Childs et al., 2012, Bakewell et al., 2006a).

The proportion of males included in both the INSYTE and WELCOME studies was higher (by 50%) than the proportion of females, around 60 percent and 40 percent, respectively. Patients were recruited mainly from hepatology clinics in Southampton and Portsmouth. The sex of patients included here is consistent with observations that the prevalence of the NAFLD is higher in men than in women (42% versus 24% of adults, respectively) (Brea and Puzo, 2013). The average age of patients included in both INSYTE and WELCOME studies was 51 years with a wide range (21 to 76 years across the two studies). This wide age range may itself affect CV risk because of the tracking of many risk factors with age (Targher et al., 2007). Both sex and age affect CV risk (Brea and Puzo, 2013), so the wide variability in risk factors and in calculated CV risk may in part be accounted for by the inclusion of both men and women and the wide age range of included patients.

The patients with NAFLD included in both the INSYTE and WELCOME studies were considered as non-alcoholic and non-smokers because they reported low consumption of alcohol and cigarettes. According to their medical history, around half of the patients had hypertension and around half were using antihypertensive medication. According to the British Heart Foundation, nearly 30% of adults in the

UK have hypertension and up to half are not receiving anti-hypertensive treatment (British Heart Foundation Statistics). Forty and fifty percent of participants (in INSYTE and WELCOME, respectively) had hyperlipidaemia with more than 30% in INSYTE and 50% in WELCOME using lipid lowering medications (more than 30% in INSYTE and more than 40% in WELCOME were using statins). Forty percent of patients in INSYTE and more than 30% in WELCOME had type-2 diabetes and around 60% in INSYTE and 30% in WELCOME used oral antidiabetics. Eight percent in INSYTE and 9% in WELCOME used insulin injection to control diabetes. Around 50% in INSYTE and 40% in WELCOME had a family history of diabetes and 60% in INSYTE and 40% in WELCOME had a family history of ischemic heart disease. This profile among the NAFLD patients studied here supports that NAFLD is associated with other diseases such as hypertension, hyperlipidaemia, type-2 diabetes, and metabolic syndrome. According to (Ahmed et al., 2012) hypertriglyceridemia and insulin resistance are independently associated with NAFLD. Also, according to (Targher et al., 2010) there is a close correlation among abdominal obesity, insulin resistance, NAFLD and risk of cardiovascular disease. According to British Heart Foundation Statistics having diabetes can double the risk of developing CVD.

Mean systolic and diastolic blood pressures were 133 and 74 mmHg in the INSYTE study and 137 and 85 mmHg in the WELCOME study, which is not excessive (Ha et al., 2013). However, this is most likely due to the use of medications to treat high blood pressure (Brea and Puzo, 2013): in both studies around 50% of the participants used blood pressure lowering medications.

The NAFLD patients included in this research had a high likelihood of being obese (more than 70%). In comparison around 27% of adults in the UK have obesity (El-Sayed et al., 2011). Mean body mass index of the participants in INSYTE (33.5 kg/m^2) and in WELCOME (31 kg/m^2) classified the majority of patients as class I obese (30-34.9 kg/m^2). According to (Dixon et al., 2001) prevalence of NAFLD

increased from 16% in people with normal BMI to 78% in the obese. Also the prevalence of NAFLD in bariatric surgery patients was reported as 96% (Argo and Caldwell, 2009). Waist circumference was high among patients included in the current research, with 97% having waist circumference above the generally accepted cut-off. Both obesity and abdominal obesity substantially increase the risk of CVD (Cusi, 2012).

Around 35 and 50 percent of the patients included in INSYTE and WELCOME, respectively, were taking medications to treat hyperlipidaemia. Also, around 33 and 42 percent of the those in INSYTE and WELCOME, respectively, were taking statins. In comparison about 10% of UK adults are using statins (British Heart Foundation Statistics). Even so many participants had dyslipidaemia with raised total cholesterol and TG and decreased HDL-cholesterol compared to the normal range. Median total cholesterol (5.0 and 4.7mmol/L in INSYTE and WELCOME studies, respectively) was in the higher end of the normal range (< 5mmol/L) and half of the patients were above the normal range, while mean HDL-cholesterol (1.2 and 1.0 mmol/L in INSYTE and WELCOME studies, respectively) was lower than the normal range (1.2-1.6 mmol/L). Median LDL-cholesterol (2.8 and 2.9 mmol/L in INSYTE and WELCOME studies, respectively) was in the normal range (< 3 mmol/L), but about half of the patients had LDL-cholesterol concentration above the normal range. The median ratio of total cholesterol to HDL-cholesterol (4.5 and 4.9 in INSYTE and WELCOME studies, respectively) was also higher than the normal range (< 4). Median triglycerides (1.9 and 1.7 mmol/L in INSYTE and WELCOME studies, respectively) were higher than the normal range (< 1.7 mmol/L) which is expected in the NAFLD patients (Brea and Puzo, 2013). The presence of dyslipidaemia is as expected in a group of patients with NAFLD and it is associated with increasing CV risk (Ahmed et al., 2012, Anonymous, 2010).

As expected in patients with NAFLD, median glucose concentration (6 and 5 mmol/L in INSYTE and WELCOME studies, respectively) was higher than the normal range (4.1-6 mmol/L). Even so, around 59% and 8% of the participants were taking oral medications or insulin injection to control diabetics. It was identified that 71% of the patients had insulin resistance assessed as HOMA-IR. According to (Wild and Byrne, 2005) 90% of individuals with NAFLD have insulin resistance, therefore increasing the risk of CVD.

In the NAFLD patients included in this research, the presence of metabolic syndrome was assessed. Metabolic syndrome was assessed as: abdominal obesity, triglycerides, HDL-cholesterol, blood pressure and fasting glucose. Almost all (97%) of the participants had metabolic syndrome. This is as expected because many features of MS are often shared with NAFLD (Mishra et al., 2013). The presence of metabolic syndrome will increase the risk of heart disease (Manco, 2011). A study in Italy found that NAFLD associated with most features of the metabolic syndrome and there is now general agreement that NAFLD is another clinical feature of the metabolic syndrome (Ahmed et al., 2012).

Overall a third of the INSYTE patients and around half of the WELCOME patients had low grade inflammation according to CRP concentration. CRP is an acute phase protein synthesised in the liver and it is an independent predictor of cardiovascular risk (Ridker et al., 1998). Obesity is linked to low grade inflammation and adipose tissue can release inflammatory markers, like IL-6, that promote CRP synthesis. In turn inflammation is linked to insulin resistance because inflammatory cytokines like IL-6 and TNF-α inhibit insulin signalling in metabolic tissues (Tilg and Moschen, 2008). Inflammation is also linked to increased CVD risk (Ridker et al., 1998).

In this study, CIMT were assessed. CIMT is used as a surrogate marker for atherosclerosis (Lim et al., 2008). Although NAFLD is associated with increased CIMT in the literature (Mishra et al., 2013), median CIMT of 0.65 mm in both INSYTE and WELCOME cohorts was in the normal range (0.60-0.81 mm). It is possible that the normal CIMT is related to use of blood pressure and lipid lowering medications. Increasing age was associated with CIMT consistent with the literature (Ebrahim et al., 1999).

Ten-year cardiovascular risk was calculated using an Excel file calculator that takes into account the following: sex, age, diabetes, smoking, treated and untreated systolic blood pressure, total cholesterol and HDL-cholesterol. All of the patients included here had some risk of CVD within the next ten years. Average ten-year cardiovascular risk in INSYTE and WELCOME studies, respectively, was 10.2% and 11.75 %. In both the INSYTE and WELCOME studies around half of the participants had <10% risk, 24% had 11-20% risk, 13% had 21-30% risk, and 16% had more than 30% risk to have CVD in the next 10 years. These findings are consistent with the CVD risk factor profile of the NAFLD patients involved in the studies. Also, these findings are consistent with what other investigators have reported. For example, Villanova et al. found that the 10-year probability of cardiovascular events was moderately increased in patients with NAFLD (Villanova et al., 2005).

All these findings of the characteristics of patients with NAFLD patients included in the INSYTE and WELCOME studies are in support of the hypothesis that patients with NAFLD will have an elevated risk of cardiovascular disease but that this will vary among the patients, as discussed earlier.

The bioactive omega-3 fatty acids EPA, DPA and DHA were described as % of total fatty acids present in the red blood cells. To my knowledge, this is the first study to

report the three bioactive omega-3 fatty acids (EPA, DPA and DHA) in patients with NAFLD. EPA, DPA and DHA for INSYTE participants were 0.9 (0.7-1.2)%, 3.0 (± 0.3)% and 4.7 (4-5.5)%, respectively, while for WELCOME participants they were 0.9 (0.7-1.1)%, 3.0 (± 0.35)% and 4.6 (± 1.2)% respectively. Thus, the omega-3 fatty acid status was identical for patients in both studies. In a group of healthy subjects studied in Southampton the red blood cell EPA and DHA were 0.66 (± 0.33)% and 3.3 (± 1.67)%, respectively (West et al., 2016). In the current study, and as a novel exploration, the relations among EPA, DHA and DPA were assessed. Independent of sex and age, EPA and DHA had a very strong significant positive association ($p < 0.001$). EPA and DPA also had a very strong correlation ($p < 0.001$). However, DHA and DPA had no association ($p = 0.247$). EPA and DHA are obtained preformed from the diet, mainly from seafood especially fatty fish. Fish intake is a strong determinant of EPA and DHA in red blood cells (Harris, 2007). Thus, EPA and DHA may be strongly related because they have a common dietary source. Although DPA is also found in the diet, it is commonly described that DPA is formed from EPA by elongation. Thus EPA and DPA may be associated because of this metabolic relationship. It is also well described that DPA is not well converted to DHA. Thus, the absence of a clear metabolic relationship between DPA and DHA may explain the lack of an association between these two fatty acids, as observed here.

In this research the omega-3 index of NAFLD patients was reported for the first time. Omega-3 index for NAFLD patients was 5.7% and 5.0% in INSYTE and WELCOME studies, respectively. Von Schacky and Harris have reported that an omega-3 index of ≤ 4% is associated with the highest CVD risk, while an index of ≥ 8% associated with the lowest CVD risk (Harris and von Schacky, 2004). The desirable value for omegas-3 index is ≥8% and the undesirable level is ≤ 4%.(Harris and von Schacky, 2004) (von Schacky, 2014). Overall the NAFLD patients studied here were at a medium elevated risk (4.1 to 7.9) of CVD. This finding indicates that

patients with NAFLD do not have a high cardioprotection level of omega-3 fatty acids.

The distribution of omega-3 index in various populations has been described and a low value of omega-3 index was associated with a poorer clinical condition (e.g. high risk or incidence of coronary heart disease). The omega-3 index in unselected German individuals (n=5000) was 7.15±2.12, while in German patients with congestive heart failure (n=895) it was 3.74 ±1.2. In unselected data from Europeans (n=10,000) omega-3 index was 6.96 ±2.15, while in USA patients with stable coronary heart disease (n=956) it was 4.6. However, in Asian countries which have low incidence of coronary heart disease, the omega-3 index was around 11 in healthy subjects and 8.19-12.8 in patients with different diseases (von Schacky, 2014). The findings of the current study are in general accordance with the European data reported previously.

Sex has been reported to have an effect on EPA and DHA status, but this has not been previously explored in patients with NAFLD. In this study EPA and DHA tended to be higher in females than in males (1.06 vs 0.97% and 5.0 vs 4.7%, respectively), but they were not significantly different. The omega-3 index was higher in females than in males (6.0% vs 5.7%) but this difference was not significant. (Lohner et al., 2013) and (Childs et al., 2008, Walker et al., 2014) reported higher levels of omega-3 fatty acids in females compared to males. This could be related to hormonal regulation of the omega-3 fatty acid biosynthetic pathway. Some human studies show that females have a higher ability to synthesise EPA and DHA from alpha-linolenic acid than males (Burdge and Wootton, 2002) and (Burdge et al., 2002). This is supported by the finding of (Giltay et al., 2004) that, when dietary alpha-linolenic acid, EPA, and DHA intakes were controlled, women had a greater proportion of DHA in plasma compared with men. The authors suggested that estrogens cause higher DHA concentrations

in women than in men, probably by upregulating synthesis of DHA from vegetable-derived precursors. Moreover, (Bakewell et al., 2006a) tested the hypothesis that sex-related differences in omega-3 fatty acids metabolism are reflected in the concentrations of omega-3 fatty acids in plasma lipids. The subjects were healthy men (n 13) and women (n 23) aged 18–35 years consuming their habitual diet. There were no significant differences between men and women in their consumption of protein, carbohydrate, total fat, alcohol, individual fatty acids and selected micronutrients. DHA concentration in plasma lipids was significantly higher in women compared with men. There were no significant differences between men and women in the concentrations of any other fatty acids measured. The absence of a sex effect on DHA status in the current study could be that the female participants were older than those studied by other researchers.

Age has been reported to have an effect on EPA and DHA status, but this has not been explored previously in patients with NAFLD. In this study, omega-3 fatty acid status in the participants aged < 50 and ≥ 50 years was assessed; 54% of the participants were less than 50 years old and 46% were more than 50 years old. Mean age in the < 50 and ≥ 50 years groups was 40 and 60 years, respectively. The omega-3 fatty acid status was higher in the older patients in both INSYTE and WELCOME cohorts. Furthermore, there were linear positive associations between age and EPA and DHA status. Consistent with this (Rees et al., 2006) reported that older healthy male subjects (aged 53-70 years) had significant higher concentrations of EPA and DHA than did younger healthy male subjects (18-42 years). Also, they reported that in response to supplementation older males incorporated EPA into plasma more readily than did younger. In another study higher concentrations of EPA and DHA were reported in older individuals, independent of any differences in dietary intakes (Walker et al., 2014).

In the current study EPA, DHA and omega-3 index were higher in older than younger subjects and correlated positively with age; the effect of age was

maintained after adjustment for sex. However, DPA had no relation with age. This confirms the previous finding of a positive correlation between omega-3 fatty acids and age. For example, (Walker et al., 2014) reported that DHA in plasma and adipose tissue and EPA in adipose tissue were increased with age. This significant association with age could be explained by the different intakes of omega-3 fatty acids from the diet. However, surveys do not support such differences in dietary intake. An alternative explanation is that there is a difference in bodily handling of these fatty acids with aging (Rees et al., 2006). This might relate to less oxidation of omega-3 fatty acids with increasing age due to reduced muscle mass and reduced physical activity.

3.5 Summary and conclusion

Non-alcoholic fatty liver disease is associated with other diseases and NAFLD has a strong link with cardiovascular risk. This chapter describes the elevated risk of cardiovascular disease and the status of omega-3 fatty acids in NAFLD patients. Findings observed in the INSYTE cohort of patients were all replicated in the WELCOME cohort. Overall, the results presented in this chapter confirm that NAFLD patients have elevated risk of cardiovascular disease and have a relatively low status of omega-3 fatty acids. More than one-third of the NAFLD patients were using lipid lowering, antihypertensive, or oral antidiabetic medications. Most NAFLD patients had a BMI indicating obesity and a waist circumference indicating excess abdominal fat. Many had high blood cholesterol, triglycerides and glucose and high cholesterol to HDL-cholesterol ratio. According to HOMA-IR more than two thirds of the participants had moderate or severe insulin resistance. More than one third of the participants had low grade inflammation according to CRP concentration. Most participants did not have elevated diastolic blood pressure or CIMT. Overall participants involved in INSYTE and WELCOME are at high metabolic and cardiovascular risk. Mean omega-3 index for NAFLD patients (5.5%) indicated a medium elevated risk of CVD. There was a significant positive association between age and red blood cell EPA, DHA and omega-3 index, but no significant relationship between sex and omega-3 status.

Thus, these data for NAFLD patients are very amenable to analysis of the relationship between red cell omega-3 fatty acid status and CV risk factors which will be presented in the next chapters**.**

Chapter 4: **Relationship of red cell omega-3 fatty acids with blood lipids, blood pressure, carotid intima-media thickness and cardiovascular risk score in patients with NAFLD**

4.1 Introduction, research questions and hypothesis

4.1.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterised by an atherogenic lipid profile, which includes of high blood triglycerides (TG), low high-density lipoprotein (HDL) cholesterol, an increase in small dense low-density lipoprotein (LDL) particles, increased very low-density lipoprotein (VLDL) cholesterol and elevated apolipoprotein B100 concentration (Fabbrini et al., 2010). This type of atherogenic dyslipidaemia is strongly linked to increased risk of cardiovascular disease (CVD) (Bhatia et al., 2012a).

The protective effect of omega-3 fatty acids towards CVD development most likely relates to the advantageous modification of a wide range of risk factors. Omega-3 fatty acids have a range of molecular and metabolic actions that could influence blood lipid concentrations, especially fasting TG (Oikawa et al., 2009). This effect is reached by a combination of a reduction in hepatic synthesis of TG and an increased clearance of circulating TGs (Saravanan et al., 2010, Calder, 2015a). One important action is the reduced hepatic assembly and secretion of VLDL, which are the main TG carrying lipoproteins in the fasting state. This effect comes about through a combination of actions including inhibition of hepatic TG synthesis and downregulation of synthesis of apolipoprotein B100 required for VLDL assembly, and diversion of hepatic fatty acids toward β-oxidation and away from TG synthesis (Shearer et al., 2012, Calder, 2015a).

As reviewed by Harris (1997) the effect of supplemental omega-3 fatty acids on fasting blood TG concentrations has been examined in a large number of studies. Harris concluded that omega-3 fatty acids lowered TG in subjects with starting

fasting TGs < 2 mmol/L by 25% and by 28% in those with starting fasting TGs ≥ 2 mmol/L. The doses of omega-3 fatty acids used in many studies have been high, and there is clear evidence that the TG lowering effect of omega-3 fatty acids is dose dependent. There is consistent evidence that both EPA and DHA lower fasting blood TG concentration but that DHA is a little more effective (Calder, 2015a). In subjects with type 2 diabetes, a meta-analysis of the effects of supplemental omega-3 fatty acids on blood lipids that included 18 studies (total of 823 subjects) concluded that fish oil supplementation lowers TG and raises LDL cholesterol, with no statistically significant effect on total cholesterol or HDL cholesterol. The TG-lowering effect and the elevation in LDL cholesterol were most marked in those trials that recruited hypertriglyceridemic subjects and used higher doses of fish oil (Montori et al., 2000).

The ability of omega-3 fatty acids to lower fasting TG levels reflects their effect on fatty acid, TG, and TG-rich lipoprotein metabolism. Such effects will also modify the metabolism of other lipoproteins. Consequently, omega-3 fatty acids have been reported to also alter the concentrations of cholesterol and cholesterol-carrying lipoproteins including high-density lipoprotein (HDL) and low-density lipoprotein (LDL) (Calder, 2015a). The effect of omega-3 fatty acids in NAFLD patients was investigated in the WELCOME study which used a high dose (3.6 g/day) of EPA and DHA for 15-18 months. There was a significant reduction in serum TG and a significant increase in HDL cholesterol (Scorletti et al., 2014b, Scorletti et al., 2015).

Carotid intima-media thickness (CIMT) is a well-established marker of subclinical atherosclerosis. This measurement is used to determine the presence and extent of carotid atherosclerosis. Ultrasound measurement of intima-media thickness has been reported as a procedure to detect the early stages of atherosclerosis (Kasliwal et al., 2014). Recent studies show a link between NAFLD and a

consequent increase in CIMT (Ahmed et al., 2010). Assessment of CIMT progression is widely used in intervention studies as a surrogate end-point for adverse clinical events, as well as evaluating benefits of specific atherosclerotic modifying therapies (Bhatia et al., 2016). Investigators reported a positive association between high dietary intake of omega-3 fatty acids and less carotid atherosclerotic plaque burden and improved arterial elasticity, after intervention on NAFLD patients with 4 g/day of omega-3 fatty acids for 15-18 months, the investigators concluded that improvement in two markers of NAFLD severity is independently associated with reduced CIMT progression (Bhatia et al., 2016). Also omega-3 fatty acids were demonstratred to have a plaque-stabilizing effect on carotid plaques (Thies et al., 2003).

Hypertension is an important risk factor for cardiovascular disease (CVD). The protective effect of omega-3 fatty acids towards CVD development most likely relates to the beneficial modification of a broad range of risk factors. One of these factors may be blood pressure, which is lowered by omega-3 fatty acids (Calder, 2014). Omega-3 fatty acids could act to lower blood pressure through one or more, or perhaps all of the following mechanisms: the production of eicosanoids with vasoactive effects, the secretion of aldosterone, the generation of nitric oxide by the endothelium, improved vascular reactivity, and improved cardiac haemodynamics (Calder, 2015a, Mori and Woodman, 2006). A large number of studies have tested the effect of supplemental omega-3 fatty acids on blood pressure in humans; these studies have been subjected to meta-analysis a number of times over a long period of time (Appel et al., 1993, Morris et al., 1993, Geleijnse et al., 2002). These meta-analyses, as well as a number of the individual studies, clearly show that omega-3 fatty acids can reduce blood pressure in subjects with untreated hypertension and also in other types of subject including healthy normotensives (Calder, 2015a). Many studies have indicated that high doses (3 g/day) of omega-3 fatty acids are associated with reductions in blood

pressure of the order of 3 to 5 mmHg (Appel et al., 1993, Morris et al., 1993, Geleijnse et al., 2002). The blood pressure lowering effect of omega-3 fatty acids seems to be more noticeable in people with hypertension and those who are older (≥ 45 years) than in other groups, and appear to need daily doses of more than 3 g per day (Geleijnse et al., 2002). According to (Cottin et al., 2011) evidence to date suggests that DHA is more efficient than EPA in decreasing blood pressure.

Cardiovascular risk (10-year risk) has been used for prediction of coronary events (fatal/nonfatal myocardial infarction or sudden death). CVD risk can be assessed by the Framingham score, which is calculated by use of an extensively validated algorithm (Anderson et al., 1991). The score takes into consideration sex, age, systolic blood pressure, total cholesterol, HDL cholesterol, diabetes and smoking. The Framingham score is based on data from a sample of the Framingham Heart and Offspring studies (Anderson et al., 1991). NAFLD patients have a higher 10-year CVD risk score than the general population of the same age and sex (Treeprasertsuk et al., 2012). In a South Korean community-based healthy non-obese cohort study (n=30,172), researchers identified increased prevalence of CVD risk score >10% in patients with NAFLD (NASH > simple steatosis), independent of age, BMI and smoking (Sung et al., 2009). To my knowledge, no prior study has related omega-3 fatty acid status to blood pressure or a CVD risk score in patients with NAFLD without increasing the oral intake of omega-3 fatty acids. The majority of the studies in the research field investigate the effect of increased omega-3 intake on NAFLD with different dose and duration of intervention. However, I investigated the relationship between CVD risk factor with the actual status of omega-3 fatty acids in patients with NAFLD without any intervention.

This research focused on the relation between the status of red blood cell omega-3 fatty acids and cardiovascular risk factors in patients with NAFLD. This chapter describes the relationship between blood lipids, blood pressure, CIMT and

cardiovascular 10-year risk score with omega-3 fatty acid status (level of red blood cell eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) and omega-3 index).

4.1.2 Research questions

What is the relationship between red blood cell omega-3 fatty acids status and blood lipids in patients with NAFLD?

What is the relationship between red blood cell omega-3 fatty acids status and carotid intima media thickness in patients with NAFLD?

What is the relationship between red blood cell omega-3 fatty acids status and blood pressure in patients with NAFLD?

What is the relationship between red blood cell omega-3 fatty acids status and cardiovascular 10-year risk score in patients with NAFLD?

4.1.3 Hypothesis

The hypotheses of the work described in this chapter are:

a) That patients with NAFLD will have a negative relationship between omega-3 fatty acid status and blood lipids.

b) That patients with NAFLD will have a negative relationship between omega-3 fatty acid status and carotid intima media thickness.

c) That patients with NAFLD will have a negative relationship between omega-3 fatty acid status and blood pressure.

d) That patients with NAFLD will have a negative relationship between omega-3 fatty acid status and cardiovascular 10-year risk score..

4.2 Methods

4.2.1 Study design

This research is a sub-project of the INSYTE study. The focus of this sub-project is to study the relation between cardiovascular risk and the omega-3 fatty acid status using the baseline data. The study design is described in chapter 2; in brief INSYTE study is a randomised, controlled, single centre, double-blind clinical trial addressing the hypothesis that treatment with a synbiotic will have a beneficial effect in NAFLD compared to placebo, as measured by change in liver fat and serum biomarkers of liver disease. Patients were aged >18 years with NAFLD, which was either biopsy-proven or confirmed by non-invasive imaging. To be enrolled, alcohol consumption needed to be ≤ 14 units / week for women and ≤ 21 units / week for men. Participants were required to attend clinic visits at the NIHR Wellcome Trust Clinical Research Facility. During these visit body composition, blood pressure and carotid intima-media thickness were all measured and blood samples collected for assessment of cardiovascular risk factors.

This research also used the baseline data set of the WELCOME study. The WELCOME study is a randomised double blind placebo controlled trial, 105 participants with NAFLD participants The WELCOME study was approved by the local ethics committee (REC: 08/H0502/165) (Scorletti, 2014a). In the WELCOME study the inclusion criteria, and the methods of analysis were identical to those of the INSYTE study.

4.2.2 Analyses performed

Status of omega-3 fatty acids was determined by analysing red blood cells by using gas chromatography (as described in section 2.4). The following were measured: EPA content, DHA content, DPA content, and Omega-3 index (the sum of EPA+DHA). Fasting blood lipid concentrations: total cholesterol, HDL-cholesterol and TGs were analysed by using a colourimetric enzymatic assays and LDL-cholesterol was calculated (as described in section 2.7). Carotid intima-media thickness was measured by scanning of the carotid arteries (as described in section 2.8). Systolic and diastolic blood pressure was measured by using an electronic monitoring device (as described in section 2.9). Calculation of the cardiovascular risk score was assessed following "Framingham General Cardiovascular Risk Score" (as described in section 2.10). An Excel file Risk Score Calculator was used that included these predictors: sex, age, diabetes, smoking, treated and untreated systolic blood pressure, total cholesterol, and HDL-cholesterol.

4.2.3 Statistical analysis

The chapter addresses the hypotheses that a) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and blood lipids, b) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and carotid intima media thickness, c) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and blood pressure, and d) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and cardiovascular 10-year risk score. All data were entered into an Excel database and transferred to SPSS for analysis. Non-normally distributed variables were log base 10 transformed to obtain a symmetrical data distribution before proceeding with data analysis. Univariate correlations were employed to study the relationships between red blood cell omega-3 fatty acids and CVD risk factors (blood lipids, carotid intima-media thickness, blood pressure, and

cardiovascular 10-year risk score) as Pearson's or Spearman correlations. Subsequently, multivariate liner regressions analysis was performed using each CVD risk factor (blood lipid, carotid intima-media thickness, blood pressure, and cardiovascular 10-year risk score) as the outcome and fatty acids and other variables (age, sex) as confounding factors. Also, multivariate logistic regression analysis was performed using normal and high cut-off values for each CVD risk factor as the outcome and fatty acids and other variables (age, sex) as confounding factors. Significance was considered when P-value was below 0·05. All statistical analyses were performed using IBM SPSS Statistics for Windows (version 21.0; SPSS, Inc., Chicago, IL).

4.3 Results

This section presents the result of relationship between red blood cell omega-3 fatty acids (EPA, DPA, and DHA and omega-3 index) with CVD risk factors (blood lipids, carotid intima-media thickness, blood pressure and cardiovascular risk score) in patients with NAFLD involved in the INSYTE study; data available from NAFLD patients involved in the WELCOME study are used to confirm the findings from the INSYTE patients.

The analyses in this section were performed to identify the relationships. First, correlation was performed to identify the relationships. Second, multiple linear regressions were performed to check the prediction relationship and to exclude any effect of age and sex. Third, multiple logistic regression analyses were conducted to check the relationships in two levels (normal and high) or in two categories of participant.

4.3.1 Blood lipids and carotid intima media thickness

4.3.1.1 Correlations between blood lipids and CIMT

Table 4-1 shows the correlation between blood lipids (total cholesterol, HDL-cholesterol, LDL-cholesterol, total cholesterol/HDL-cholesterol ratio and TG) and the correlation between blood lipids and CIMT after adjustment for sex and age in patients with NAFLD included in the INSYTE study. As expected there were significant positive correlations between total cholesterol, HDL-cholesterol, LDL-cholesterol, and total cholesterol/HDL-cholesterol ratio. There were also significant positive correlations between TGs and total cholesterol ($r= 0 .278$; $p < 0.001$) and TGs and TC:HDL ($r= 0 .394$; $p < 0.01$). After adjustment for sex and age there were significant positive correlations between CIMT and total cholesterol, LDL, and TC:HDL ($r= 0.216$, $p < 0.05$; $r= 0.247$, $p < 0.05$; $r= 0 .276$, $p < 0.05$) respectively.

Table 4-1 Correlation between different blood lipids and between blood lipids and CIMT in patients with NAFLD enrolled in the INSYTE study (n = 63)

Variable	TC	HDL-C	LDL-C	TC:HDL-C	TGs
TC	1	0.547***	0.904***	0.567***	0.278*
HDL-C	0.547***	1	0.349**	-0.354**	-0.080
LDL-C	0.904***	0.349**	1	0.622***	-0.117
TC:HDL-C	0.567***	-0.354**	0.622***	1	0.394**
TGs	0.278*	-0.080	-0.117	0.394**	1
CIMT	0.216*	-0.009	0.247*	0.276**	0.044

Data that were non-normally distributed were log transformed before analysis. Pearson correlation analysis between blood lipids. Multiple linear regression analysis between CIMT and any of the individual blood lipids adjusted for age and sex. *p-value < 0.05, **p-value < 0.01 and ***p-value < 0.001. Abbreviations: TC, total cholesterol; HDL-C, LDL-C, LDL-cholesterol; TC:HDL-C, total cholesterol/HDL-cholesterol ratio; TGs, triglycerides; CIMT, carotid intima media thickness.

4.3.1.2 Correlation between blood lipids and carotid intima-media thickness and age and sex

As shown in Figure 4-1 there was a strong positive correlation between age and CIMT ($r= 0.564$, $p < 0.001$) in patients with NAFLD included in the INSYTE study. There was a similar strong positive correlation between age and CIMT in the 105 patients with NAFLD in the WELCOME study ($r= 0.458$, $p < 0.001$) as shown in Figure 4-2. There was no correlation between age and TC , HDL-C, LDL-C, TC:HDL-C, and TGs. Table 4-3 shows the correlation of sex and age with blood lipids (total cholesterol, HDL-cholesterol, LDL-cholesterol, total cholesterol/HDL-cholesterol ratio and TGs) and with CIMT. Correlations with sex were after adjustment for age, and correlations with age were after adjustment for sex. There was a significant negative correlation between cholesterol/HDL-cholesterol and age ($r= -0.264$, $p= 0.036$), and sex had a positive correlation with HDL-cholesterol ($r= 0.413$, $p= 0.001$), being higher in women.

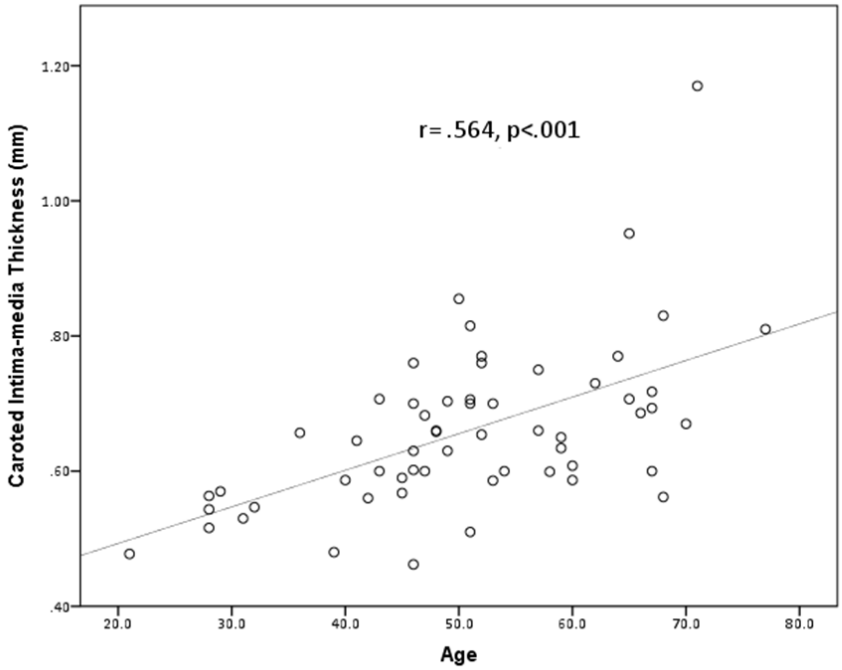


Figure 4-1 Correlation between CIMT and age in 63 patients with NAFLD in the INSYTE study.

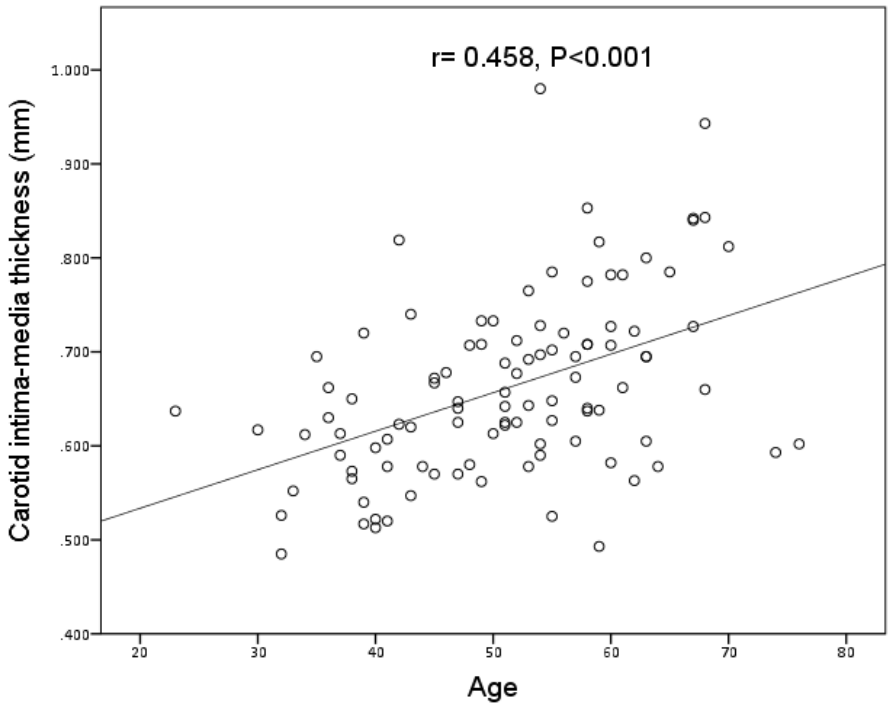


Figure 4-2 Correlation between CIMT and age in 105 patients with NAFLD in the WELCOME study.

Table 4-2 Correlations of blood lipids and CIMT with sex and with age in patients with NAFLD in the INSYTE study

In INSYTE study (n= 63)				
	Age		Sex	
Variable	Unstandardized B coefficients	*p-value*	Unstandardized B coefficients	*p-value*
Total cholesterol	- 0.047	0.715	0.181	0.156
HDL-cholesterol	0.173	0.187	0.413	0.001
LDL-cholesterol	- 0.156	0.242	0.229	0.084
TC:HDL-cholesterol	- 0.264	0.036	- 0.146	0.254
Triglycerides	- 0.047	0.715	- 0.209	0.103
CIMT	0.564	< 0.001	- 0.192	0.149
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis: between age and sex with each blood lipid and CIMT. Age data were adjusted for sex and sex data were adjusted for age. Abbreviations: CIMT, carotid intima media thickness.				

4.3.1.3 Relation between carotid intima-media thickness with blood lipids after adjustment for age and sex

Table 4-3 shows linear regressions between blood lipids (cholesterol, HDL, LDL, total TC:HDL and TGs) and CIMT after adjustment for age and sex in 63 patients with NAFLD in the INSYTE study. There was a positive linear relationship (β=0.021, p= 0.042) between cholesterol and CIMT; the beta coefficient shows that for every mmol/L increase in cholesterol, CIMT will increase by 0.21 mm. There was also a positive linear relationship (β= 0.040, p= 0.009) between LDL and CIMT; the beta coefficient shows that for every mmol/L increase in LDL, CIMT will increase by 0.04 mm.

Table 4-3 Relation between carotid intima-media thickness with blood lipids after adjustment for age and sex

Carotid intima-media thickness (CIMT)		
INSYTE study (n= 63)		
Variable	Unstandardized B coefficient	p-value
Total cholesterol	0.021	0.042
HDL-cholesterol	- 0.004	0.938
LDL-cholesterol	0.040	0.009
TC:HDL-cholesterol	0.081	0.081
Triglycerides	0.013	0.687
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between CIMT and for any of the individual blood lipids. All data were adjusted for age and sex.		

4.3.2 Red blood cell omega-3 fatty acids and total cholesterol

4.3.2.1 Correlation of total cholesterol and red blood cell omega-3 fatty acids

In 63 patients with NAFLD in the INSYTE study, there was no significant correlation between omega-3 fatty acids and total cholesterol. The correlations between EPA,

DPA, DHA and omega-3 fatty acids with total cholesterol were (r= 0.168, p= 0.189), (r= 0.049, p= 0.704), (r=0.059, p= 0.647), (r= -0.011, p= 0.931), respectively.

4.3.2.2 Relation of total cholesterol with red blood cell omega-3 fatty acids after adjustment for age and sex

Table 4-4 shows the results of multiple linear regressions between total cholesterol and red blood cell omega-3 fatty acids after adjustment for age and sex for NAFLD patients in the INSYTE and the WELCOME studies. There were no statistically significant relationships between total cholesterol and any individual fatty acid or omega-3 index except for EPA in WELCOME study where there was a positive linear relationship (β= 1.236, p= 0.002). Thus, omega-3 fatty acids are not a predictor of total cholesterol except for EPA in the WELCOME study.

Table 4-4 Relation of total cholesterol with red blood cell omega-3 fatty acids after adjustment for age and sex

Total cholesterol				
	INSYTE study (n= 63)		WELCOME study (n= 105)	
	Unstandardized B Coefficient	p-value	Unstandardized B Coefficient	p-value
RBC EPA	0.560	0.198	1.236	0.002
RBC DPA	0.203	0.641	0.059	0.858
RBC DHA	- 0.382	0.600	0.089	0.398
Omega-3 Index	- 0.085	0.906	0.130	0.160
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between total cholesterol and each fatty acid was performed adjusting for age and sex.				

4.3.2.3 Relation of total cholesterol categories (normal and high cut-off values) with red blood cell omega-3 fatty acids

Table 4-5 shows the results of multiple logistic regressions between cholesterol normal (< 5 mmol/L) and high (≥ 5 mmol/L) level categories and red blood cell omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE study. There were no significant logistic regression relationships between cholesterol categories and any individual omega-3 fatty acid or omega-3 index. Thus, omega-3 fatty acids are not a predictor of cholesterol level.

Table 4-5 Relation of total cholesterol categories (normal and high cut-off values) with red blood cell omega-3 fatty acids after adjustment for age and sex

Total cholesterol level categories		
INSYTE study (n= 63)		
	B Coefficient	p-value
RBC EPA	1.093	0.171
RBC DPA	0.446	0.548
RBC DHA	- 0.073	0.953
Omega-3 Index	0.344	0.776
Data that were non-normally distributed were log transformed before analysis. Multiple logistic regression analysis between total cholesterol cut off (normal (<5 mmol/L), high (≥ 5mmol/L)) with each fatty acid was performed adjusting for age and sex.		

4.3.3 Red blood cell omega-3 fatty acids and HDL cholesterol

4.3.3.1 Correlation of HDL cholesterol and red blood cell omega-3 fatty acids

In patients with NAFLD included in the INSYTE study, there was no significant correlation between omega-3 fatty acids and HDL-cholesterol concentration. The correlations between EPA, DPA DHA and omega-3 fatty acids with HDL were (r= 0.151, p= 0.249), (r= 0.192, p= 0.141), (r= 0.076, p= 0.562), (r= -0.096, p= 0.463) respectively.

4.3.3.2 Relation of HDL-cholesterol with red blood cell omega-3 fatty acids after adjustment for age and sex

Table 4-6 shows the results of multiple linear regressions between HDL-cholesterol and red blood cell omega-3 fatty acids after adjustment for age and sex in patients with NAFLD in the INSYTE and WELCOME studies. There were no statistically linear relationships between HDL-cholesterol and any individual omega-3 fatty acid or omega-3 index in the INSYTE study. However, in the WELCOME study there was a strong positive correlation (β= 0.244, p= 0.002) between EPA and HDL-cholesterol. Thus, omega-3 fatty acids are not a predictor of HDL-cholesterol except for EPA in the WELCOME study.

Table 4-6 Relation of HDL-cholesterol with red blood cell omega-3 fatty acids after adjustment for age and sex

	INSYTE study (n= 63)		WELCOME study (n= 105)	
	Unstandardized B Coefficient	p-value	Unstandardized B Coefficient	p-value
RBC EPA	0.041	0.662	0.244	0.002
RBC DPA	- 0.106	0.210	0.117	0.073
RBC DHA	- 0.069	0.650	- 0.004	0.860
Omega-3 Index	- 0.047	0.758	0.009	0.625
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between HDL-cholesterol and each fatty acid was performed adjusting for age and sex.				

Table 4-7 shows the results of multiple logistic regression between HDL cholesterol level categories {for male (low < 1.04 mmol/L and normal ≥ 1.04 mmol/L) and for female (low < 1.3 mmol/L and normal ≥ 1.3 mmol/L)} and red blood cell omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE study. In males and females there were no significant relationships between HDL categories and any individual omega-3 fatty acid or omega-3 index. Thus, omega-3 fatty acids are not a predictor of HDL-cholesterol level.

Table 4-7 Relation of HDL-cholesterol categories (normal and high cut-off values) with red blood cell omega-3 fatty acids after adjustment for age and sex

INSYTE study (n= 63)				
	Male		Female	
	B Coefficient	p-value	B Coefficient	p-value
EPA	1.257	0.284	- 0.363	0.787
DPA	0.745	0.481	- 2.219	0.121
DHA	2.704	0.203	0.162	0.937
Omega-3 Index	2.996	0.187	0.012	0.995
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between HDL-cholesterol categories HDL cholesterol level categories {for male (low < 1.04 mmol/L and normal ≥ 1.04 mmol/L) and for female (low < 1.3 mmol/L and normal ≥ 1.3 mmol/L)} and each fatty acid was performed adjusting for age and sex.				

4.3.4 Red blood cell omega-3 fatty acids and total cholesterol/HDL ratio

4.3.4.1 Correlations of total cholesterol/HDL ratio with red blood cell omega-3 fatty acids

There was no significant correlation between omega-3 fatty acids with cholesterol/HDL ratio in patients with NAFLD included in the INSYTE study. The correlations between EPA, DPA DHA and omega-3 fatty acids with cholesterol/HDL ratio were (r=0.010, p=0.936), (r=0.201, p=0.115), (r=-0.137, p=0.285), (r=-0.111, p =0.385) respectively.

4.3.4.2 Relation of total cholesterol/HDL ratio with red blood cell omega-3 fatty acids after adjustment for age and sex

Table 4-8 shows the results of multiple linear regressions between cholesterol/HDL ratio and red blood cell omega-3 fatty acids after adjustment for age and sex for NAFLD patients in INSYTE and WELCOME studies. There were no statistically linear relationships between cholesterol/HDL ratio and any individual fatty acid or omega-3 index. Thus, omega-3 fatty acids are not a significant predictor of cholesterol/HDL ratio.

Table 4-8 Relation of total cholesterol/HDL ratio with red blood cell omega-3 fatty acids after adjustment for age and sex

Total cholesterol/HDL-cholesterol Ratio				
	INSYTE study (n=63)		WELCOME study (n=105)	
	Unstandardized B Coefficient	p-value	Unstandardized B Coefficient	p-value
RBC EPA	0.209	0.490	0.015	0.974
RBC DPA	0.397	0.184	- 0.593	0.107
RBC DHA	- 0.094	0.852	0.074	0.532
Omega-3 Index	0.017	0.972	0.058	0.575
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between total cholesterol/HDL-cholesterol ratio and each fatty acid was performed adjusting for age and sex.				

Table 4-9 shows the results of multiple logistic regressions between total cholesterol/HDL-cholesterol ratio cut-off level categories (normal <4 mmol/L and high ≥ 4 mmol/L) and red blood cell omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE study. There were no significant logistic regression relationships between cholesterol/HDL categories and any individual omega-3 fatty acids or omega-3 index. Thus, omega-3 fatty acids are not a predictor of cholesterol/HDL ratio.

Table 4-9 Relation of total cholesterol / HDL ratio (normal and high cut-off values) with red blood cell omega-3 fatty acids after adjustment for age and sex

Total cholesterol/HDL-cholesterol ratio level categories		
INSYTE study (n=63)		
	B Coefficient	p-value
RBC EPA	0.731	0.348
RBC DPA	0.834	0.283
RBC DHA	- 1.222	0.345
Omega-3 Index	- 0.659	0.598
Data that were non-normally distributed were log transformed before analysis. Multiple logistic regression analysis between cholesterol/HDL categories and each fatty acid was performed adjusting for age and sex.		

4.3.5 Red blood cell omega-3 fatty acids and LDL cholesterol

4.3.5.1 Correlation of LDL cholesterol with red blood cell omega-3 fatty acids

In patients with NAFLD included in the INSYTE study, there was no significant correlation between LDL-cholesterol and EPA (r=0.139, p = 0.299), DPA (r=0.114, p = 0.392), DHA (r=- 0.068, p = 0.612), or omega-3 index (r=- 0.027, p = 0.840).

4.3.5.2 Relation of LDL cholesterol and red blood cell omega-3 fatty acids fatty acids after adjustment for age and sex

Table 4-10 shows the results of multiple linear regressions between LDL cholesterol and omega-3 fatty acids after adjustment for age and sex for NAFLD patients in INSYTE and WELCOME studies. There were no significant relationships between LDL cholesterol and any individual omega-3 fatty acid or omega-3 index in patients included in the INSYTE study; however in patients included in the WELCOME study there were positive correlations between LDL cholesterol and EPA (β= 0.730, p= 0.035) and between omega-3 index and LDL cholesterol (β= 0.156, p= 0.042). Thus, omega-3 fatty acids are not a significant predictor of LDL cholesterol in NAFLD patients in the INSYTE study; however, EPA and omega-3 index were a predictors of LDL cholesterol in NAFLD patients in the WELCOME study.

Table 4-10 Relation of LDL-cholesterol with red blood cell omega-3 fatty acids after adjustment for age and sex

LDL-cholesterol				
	INSYTE study (n=63)		WELCOME study (n=105)	
	Unstandardized B Coefficient	p-value	Unstandardized B Coefficient	p-value
RBC EPA	0.141	0.329	0.730	0.035
RBC DPA	0.111	0.402	0.127	0.643
RBC DHA	- 0.099	0.687	0.156	0.075
Omega-3 Index	- 0.023	0.924	0.156	0.042
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between LDL-cholesterol and each fatty acid was performed adjusting for age and sex.				

Table 4-11 shows the results of multiple logistic regressions between LDL cholesterol level categories (normal <3.34 mmol/L and high ≥ 3.34 mmol/L) and red blood cell omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE study. There were no significant logistic regression relationships between LDL cholesterol categories and any individual fatty acids or omega-3 index. Thus, omega-3 fatty acids are not a predictor of LDL cholesterol.

Table 4-11 Relation of LDL-cholesterol categories (normal and high cut-off values) with red blood cell omega-3 fatty acids after adjustment for age and sex

LDL cholesterol level categories		
INSYTE study (n=63)		
	Coefficients B	p-value
RBC EPA	0.915	0.347
RBC DPA	0.401	0.634
RBC DHA	0.409	0.788
Omega-3 Index	0.657	0.663
Data that were non-normally distributed were log transformed before analysis. Multiple logistic regressions analysis between LDL-cholesterol categories and each fatty acid was performed adjusting for age and sex.		

4.3.6 Red blood cell omega-3 fatty acids and triglyceride

4.3.6.1 Correlations of triglyceride and red blood cell omega-3 fatty acids

In patients with NAFLD included in the INSYTE study, there was no significant correlation between TGs and EPA (r=0.023, p= 0.860), DPA (r=0.023, p= 0.857), DHA (r=- 0.051, p= 0.695) or omega-3 index (r=- 0.035, p= 0.789).

4.3.6.2 Relation of triglyceride and red blood cell omega-3 fatty acids fatty acids after adjustment for age and sex

Table 4-12 shows linear regressions between TGs and omega-3 fatty acids after adjustment for age and sex for NAFLD patients in INSYTE and WELCOME studies. There were no statistically linear relationships between TGs and any individual fatty acid or omega-3 index. However in patients included in the WELCOME study there was a negative linear relationship (β= - 0.604, p= 0.043) between TGs and DPA. Thus, omega-3 fatty acids are not a significant predictor of TGs; however, in NAFLD patients in the WELCOME study, DPA was a significant predictor of TGs.

Table 4-12 Relation of triglycerides with red blood cell omega-3 fatty acids after adjustment for age and sex

Triglyceride				
	INSYTE study(n=63)		WELCOME study(n=105)	
	Unstandardized B Coefficient	p-value	Unstandardized B Coefficient	p-value
RBC EPA	0.062	0.667	0.124	0.748
RBC DPA	0.003	0.822	- 0.604	0.043
RBC DHA	- 0.024	0.920	- 0.043	0.668
Omega-3 Index	0.009	0.971	- 0.026	0.766
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between triglyceride and each fatty acid was performed adjusting for age and sex				

Table 4-13 shows the results of multiple logistic regressions between triglyceride level categories (normal < 1.7 mmol/L and high ≥ 1.7 mmol/L) and red blood cell omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE study. There were no significant logistic regression relationships between TG categories and any individual fatty acid or omega-3 index. Thus, omega-3 fatty acids are not a predictor of triglyceride level categories.

Table 4-13 Relation of triglyceride categories (normal and high cut-off values) with red blood cell omega-3 fatty acids after adjustment for age and sex

Triglyceride level categories		
INSYTE study (n= 63)		
	B Coefficients	p-value
RBC EPA	0.220	0.788
RBC DPA	- 0.139	0.862
RBC DHA	- 0.532	0.690
Omega-3 Index	- 0.241	0.855
Data that were non-normally distributed were log transformed before analysis. Multiple logistic regressions analysis between triglyceride categories and each fatty acid was performed adjusting for age and sex.		

4.3.6.3 4.3.6.2.1 Relation of presence of hyperlipidaemia and red blood cell omega-3 fatty acids in NAFLD participants

The analyses in this section were performed to present the relationship of red blood cell fatty acids and omega-3 index with hyperlipidaemia. Multiple logistic regression analysis conducted to check the relationships in two categories (presence or absence of hyperlipidaemia).

Table 4-14 shows logistic regressions between presence or absence of hyperlipidaemia and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE study. There were no statistically relationships between having or not having hyperlipidaemia and any individual fatty acids or omega-3 index. Thus, omega-3 fatty acids are not a significant predictor of hyperlipidaemia in patients with NAFLD.

Table 4-14 Relation of presence or absence of hyperlipidaemia and red blood cell omega-3 fatty acids fatty acids after adjustment for age and sex

Hyperlipidaemia		
INSYTE study (n= 63)		
	B Coefficient	p-value.
RBC EPA	0.024	0.975
RBC DPA	0.005	0.995
RBC DHA	0.192	0.881
Omega-3 Index	0.236	0.851
Data that were non-normally distributed were log transformed before analysis. Multiple logistic regression analysis between hyperlipidaemia each fatty acid was performed adjusting for age and sex.		

4.3.7 Red blood cell omega-3 fatty acids and carotid intima-media thickness

4.3.7.1 Correlations of carotid intima-media thickness and red blood cell omega-3 fatty acids

In patients with NAFLD included in the INSYTE study, there was no significant correlation of omega-3 index with CIMT. The correlations between EPA, DPA DHA and omega-3 fatty acids and CIMT were (r= 0.205,p= 0.123, (r= -0.030, p= 0.519), (r= -0.077, p= 0.260), (r= -0.091, p= 0.175) respectively.

4.3.7.2 Relation of carotid intima-media thickness with red blood cell omega-3 fatty acids after adjustment for age and sex

Table 4-15 shows the results of multiple linear regression analysis between carotid intima-media thickness and omega-3 fatty acids after adjustment for age and sex for NAFLD patients in INSYTE and WELCOME studies. There were no statistically significant linear relationships between CIMT and EPA, DPA, DHA and omega-3 index. Thus omega-3 fatty acids were not predictors of CIMT.

Table 4-15 Relation of carotid intima-media thickness with red blood cell omega-3 fatty acids after adjustment for age and sex

Carotid intima-media thickness (CIMT)				
	INSYTE study (n= 63)		WELCOME study (n= 105)	
	Unstandardized B Coefficient B	p-value	Unstandardized B Coefficient B	p-value
RBC EPA	0.044	0.215	- 0.014	0.656
RBC DPA	- 0.017	0.645	- 0.022	0.367
RBC DHA	- 0.029	0.628	0.009	0.235
Omega-3 Index	- 0.004	0.940	0.007	0.331
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between CIMT and for any of the individual blood lipids. All data were adjusted for age and sex				

4.3.7.3 Relation of carotid intima-media thickness categories (normal and high cut-off values) with red blood cell omega-3 fatty acids after adjustment for age and sex

Table 4-16 shows logistic regressions between normal and high level of carotid intima-media thickness and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE study. There were no statistical relationships between CIMT in patients with a normal range of CIMT (<= 0.8 mm) or in patients with a high CIMT (> 0.8 mm) and fatty acids or omega-3 index. Thus, omega-3 fatty acids are not a significant predictor of carotid intima-media thickness.

Table 4-16 Relation of normal and high level of carotid intima-media thickness (normal and high cut-off values) with red blood cell omega-3 fatty acids after adjustment for age and sex

Carotid intima-media thickness level categories		
INSYTE study (n=63)		
	B Coefficients	p-value
RBC EPA	1.467	0.242
RBC DPA	- 1.149	0.417
RBC DHA	- 0.234	0.916
Omega-3 Index	0.653	0.741
Data that were non-normally distributed were log transformed before analysis. Multiple logistic regression analysis between CIMT categories (<= 0.8 mm, > 0.8 mm) and each fatty acid was performed adjusting for age and sex.		

4.3.8 Red blood cell omega-3 fatty acids and systolic and diastolic blood pressure

4.3.8.1 High systolic and high diastolic blood pressure in males and females and in different treatment groups

Table 4-17 shows the numbers and percent of patients with NAFLD included in the INSYTE study with high systolic and diastolic blood pressure according to sex and whether or not the patient was treated with blood pressure lowering drugs. 65% of males had high SBP and 50% of the females had high SBP. 57% of those participants with high SBP were treated but 43% were not treated. Only 8% of males had high DBP and 20% of males were treated.

Table 4-17 The number and percent of NAFLD patients with high systolic and diastolic blood pressure

INSYTE study (n=63)				
Variable	Sex		Treated	
	Male (n= 37)	Female (n= 26)	Yes (n= 34)	No (n= 20)
	N (%)	N (%)	N (%)	N (%)
High SBP SBP≥130	24(65)	13 (50)	21 (57)	16(43)
High DBP DBP≥85	5 (8)	0	1 (20)	4 (80)
High SBP and DBP SBP≥130 and DBP≥85	5 (8)	0	1 (20)	4 (80)
Data describe the numbers and percent.				

4.3.8.2 Correlation between blood pressure and red blood cell omega-3 fatty acids

Correlation between systolic blood pressure and red blood cell omega-3 fatty acids

In 63 patients with NAFLD included in the INSYTE study,there was no significant correlation between SBP and EPA (r=-0.103, p= 0.423), DPA (r=0.116, p= 0.366), DHA (r= 0.037, p= 0.057) or omega-3 index (r=0.035, p= 0.783).

Correlation between diastolic blood pressure and red blood cell omega-3 fatty acids

In 63 patients with NAFLD included in the INSYTE study, there was no significant correlation between DBP and EPA (r=- .151, p= .236), DPA(r=-0.096, p= 0.454), DHA(r=0.078, p= 0.537) or omega-3 index (r=0.034, p= 0.793).

4.3.8.3 Relation of systolic and diastolic blood pressures with omega-3 fatty acids after adjustment for age and sex

Systolic blood pressure

Table 4-18 shows linear regressions between systolic blood pressure and omega-3 fatty acids after adjustment for age and sex for NAFLD patients in INSYTE and WELCOME studies. There were no statistically significant linear relationships between SBP and any individual fatty acids or omega-3 index. Thus, omega-3 fatty acids are not a significant predictor of systolic blood pressure in patients with NAFLD.

Table 4-18 Relationship of systolic and diastolic blood pressure with omega-3 fatty acids after adjustment for age and sex

Systolic blood pressure				
	INSYTE study (n= 63)		WELCOME study (n= 105)	
	Unstandardized B Coefficient	p-value	Unstandardized B Coefficient	p-value
RBC EPA	- 6.576	0.206	2.638	0.618
RBC DPA	- 4.239	0.414	3.572	0.420
RBC DHA	- 1.434	0.869	1.764	0.209
Omega-3 Index	- 4.239	0.621	6.540	0.372
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between systolic blood pressure and each fatty acid was performed adjusting for age and sex				

Diastolic blood pressure

Table 4-19 shows linear regressions between diastolic blood pressure and omega-3 fatty acids after adjustment for age and sex for NAFLD patients in INSYTE and WELCOME studies. There were no statistically significant linear relationships between DBP and any individual fatty acids or omega-3 index. Thus, omega-3 fatty acids are not a significant predictor of diastolic blood pressure in patients with NAFLD.

Table 4-19 Relationship of diastolic blood pressure with omega-3 fatty acids after adjustment for age and sex

Diastolic blood pressure				
	INSYTE study (n=63)		WELCOME study (n=105)	
	Unstandardized B Coefficients	p-value	Unstandardized B Coefficients	p-value
RBC EPA	- 3.430	0.218	1.544	0.668
RBC DPA	- 3.430	0.327	- 0.105	0.972
RBC DHA	- 3.430	0.514	1.822	0.055
Omega-3 Index	1.078	0.815	8.002	0.106
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between diastolic blood pressure and each fatty acid was performed adjusting for age and sex				

4.3.8.4 Relation of red blood cell omega-3 fatty acids with normal and high systolic blood pressure after adjustment for age and sex

Table 4-20 shows linear regressions between normotensive and hypertensive participants and omega-3 fatty acids after sort and split data of SBP to normal (SBP ≤ 130 mmHg) and high (SBP > 130 mmHg) and after adjustment for age and sex for NAFLD patients in INSYTE study. There were no statistically linear relationships between normotensive SBP and any individual fatty acids or omega-3 index. However, in hypertensive participants EPA, DHA and omega-3 index were all statistically significant predictors of SBP. There was a negative linear relationship (β= -9.79, p= 0.005) between SBP and EPA; the beta coefficient shows that for every percent increase in the EPA SBP will decrease by 9.8 mm Hg. There was also a negative linear relationship (β= -17.591.540, p= 0.009) between SBP and DHA; for every percent increase in the DHA the SBP will be decrease by 17.6 mm Hg. Finally, there was a negative linear relationship (β= -8.256, p= 0.004) between SBP and omega-3 index; for every percent increase in the omega-3 index the SBP will be decreased by 17.7 mm Hg. DPA showed no significant relationships with SBP in hypertensive participants.

Table 4-20 Relationship of systolic blood pressure with omega-3 fatty acids in normotensive and hypertensive participants after adjustment for age and sex in the INSYTE study

INSYTE study (n= 63)				
	Normal SBP		High SBP	
	Unstandardized coefficient	p-value	Unstandardized B coefficient	p-value
RBC EPA	- 6.609	0.413	- 9.793	0.005
RBC DPA	5.030	0.318	- 7.453	0.059
RBC DHA	- 5.849	0.46	- 17.591	0.009
Omega-3 Index	- 8.256	0.358	- 17.703	0.004
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between SBP and with the individual omega-3 fatty acids after sort and split SBP data to normal and high SBP. All data were adjusted for age and sex. Abbreviations: SBP, systolic blood pressure.				

Table 4-21 shows linear regressions between normotensive and hypertensive participants and omega-3 fatty acids after sort and split data of SBP to normal (SBP < 130 mmHg) and high (SBP > 130 mmHg) and after adjustment for age and sex for NAFLD patients the WELCOME study. There were no significant linear relationships between normotensive and hypertensive SBP and any individual fatty acid or omega-3 index.

Table 4-21 Relationship of systolic blood pressure with omega-3 fatty acids in normotensive and hypertensive participants after adjustment for age and sex in the WELCOME study

WELCOME study (n= 105)				
	Normal SBP		High SBP	
	Unstandardized B coefficient	p-value	Unstandardized B coefficient	p-value
RBC EPA	- 6.343	0.111	- 2.105	0.669
RBC DPA	- 2.669	0.251	2.814	0.545
RBC DHA	0.440	0.704	0.523	0.688
Omega-3 Index	- 0.072	0.950	282	0.803
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between SBP and with the individual omega-3 fatty acids after sort and split SBP data to normal and high SBP. All data were adjusted for age and sex. Abbreviations: SBP, systolic blood pressure.				

4.3.8.5 Relation between systolic blood pressure with red blood cell omega-3 fatty acids in treated and not treated participants after adjustment of age and sex

Table 4-22 shows the results of multiple logistic regression analysis between hypertension treatment categories and omega-3 fatty acids after adjustment for age and sex for NAFLD patients in INSYTE and WELCOME studies. There was a positive logistic regression relationship (β= 1.970, P=0.031) between hypertension treatment categories and EPA; EPA can predict for treated or not treated.

Table 4-22 Relationship between hypertension treatment categories and omega-3 fatty acids after adjusting for sex and age in the INSYTE study

Hypertension treatment (yes, no) INSYTE study (n= 63)		
	B Coefficient	p-value
RBC EPA	1.970	0.031
RBC DPA	0.375	0.630
RBC DHA	1.276	0.346
Omega-3 Index	1.941	0.168

Data that were non-normally distributed were log transformed before analysis. Multiple logistic regression analysis between treated and not treated participants with the individual omega-3 fatty acids. All data were adjusted for age and sex.

Table 4-23 shows the results of multiple logistic regression analysis between hypertension treatment categories and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the WELCOME study. There were no relationship between hypertension treatment categories and omega-3 fatty acids for treated or not treated.

Table 4-23 Relationship between hypertension treatment categories and omega-3 fatty acids after adjusting for sex and age in the WELCOME study

Hypertension treatment (yes, no) WELCOME study (n= 105)		
	B Coefficient	p-value
RBC EPA	0.155	0.828
RBC DPA	0.515	0.408
RBC DHA	- 0.128	0.501
Omega-3 Index	- 0.095	0.565
Data that were non-normally distributed were log transformed before analysis. Multiple logistic regression analysis between treated and not treated participants with the individual omega-3 fatty acids. All data were adjusted for age and sex.		

4.3.9 Red blood cell omega-3 fatty acids and cardiovascular 10-year risk prediction

4.3.9.1 Correlation between cardiovascular 10-year risk prediction with age

Figure 4-3 and 4-4 shows the correlation between age and CVD risk score for NAFLD patients in INSYTE and WELCOME studies. There was a significant positive correlation between CVD risk score and age in both INSYTE and WELCOME studies (r= 0.699, p<0.001 and r= 0.586, p<0.001 respectively).

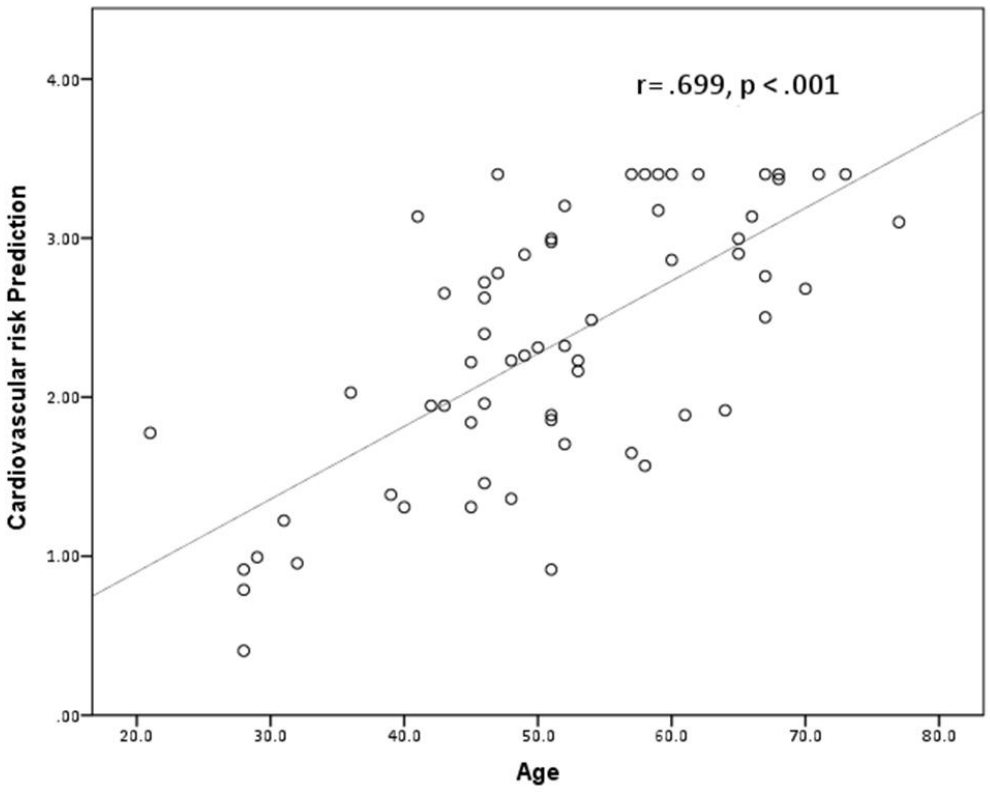


Figure 4-3 Correlation of age with cardiovascular 10-year risk prediction in 63 patients with NAFLD in the INSYTE study

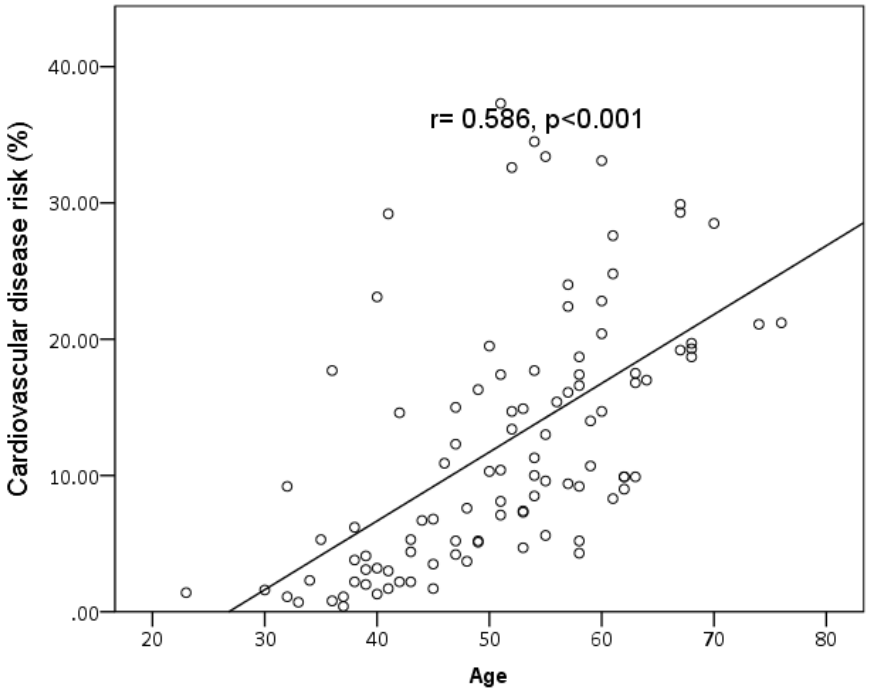


Figure 4-4 Correlation of age with cardiovascular 10-year risk prediction in 63 patients with NAFLD in the WELCOME study

4.3.9.2 Correlation between cardiovascular 10-year risk prediction and red blood cell omega-3 fatty acids

In patients with NAFLD included in the INSYTE study, there were no significant correlations between EPA and CVD risk score (r= 0.088, p= 0.493) or DPA and CVD risk score (r=-0.003, p= 0.979) or DHA and CVD risk score (r= 0.136, p= 0.289) or omega-3 index and CVD risk score (r= 0.145, p= 0.258).

4.3.9.3 Relationship between cardiovascular 10-year risk prediction and omega-3 fatty acids after adjustment of age and sex

Table 4-24 shows linear regressions between CVD risk score and omega-3 fatty acids after adjustment for age and sex for NAFLD patients in INSYTE and WELCOME studies. There were no statistically linear relationships between CVD risk score and any individual fatty acids or omega-3 index. Thus, omega-3 fatty acids are not a significant predictor of CVD risk score.

Table 4-24 Relationship between cardiovascular 10-year risk prediction and omega-3 fatty acids after adjustment of age and sex

CVD risk score				
	INSYTE study (n= 63)		WELCOME study (n= 105)	
	Unstandardized B Coefficient	p-value	Unstandardized B Coefficient	p-value
RBC EPA	- 0.081	0.675	- 3.116	0.225
RBC DPA	0.094	0.625	1.976	0.339
RBC DHA	- 0.406	0.203	- 0.535	0.807
Omega-3 Index	- 0.357	0.256	- 3.989	0.245
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between CVD risk with the individual blood lipids .All data were adjusted for age and sex				

4.3.9.3.1 Relationship between cardiovascular risk score (< 10 % and ≥ 10 %) and omega-3 fatty acids after adjustment of age and sex

Table 4-25 shows linear regressions between cardiovascular 10-year risk prediction and omega-3 fatty acids after sort and split CVD risk score data to CVD risk < 10 % and CVD risk ≥ 10 % and after adjustment for age and sex in patients with NAFLD included in the INSYTE study. There were no statistically linear relationships between CVD risk < 10 % and any individual fatty acid or omega-3 index, and there were no statistically linear relationships between CVD risk ≥ 10 % and any individual fatty acids or omega-3 index. Thus omega-3 fatty acids were not predictors of low or high CVD risk score.

Table 4-25 Relationship between cardiovascular risk and omega-3 fatty acids in each CVD risk score (CVD risk < 10 % and CVD risk ≥ 10 %) after adjustment of age and sex

CVD risk score categories				
INSYTE study (n= 63)				
	CVD risk score < 10 %		CVD risk score ≥ 10 %	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	- 0.390	0.173	- 0.125	0.364
RBC DPA	- 0.034	0.867	0.053	0.762
RBC DHA	- 0.663	0.097	0.172	0.522
Omega-3 Index	- 0.675	0.090	0.059	0.819
Data that were non-normally distributed were log transformed before analysis. Multiple linear regression analysis between CVD risk score with the individual omega-3 fatty acids after sort and split CVD risk score data to CVD risk score(< 10 % and ≥ 10 %). All data were adjusted for age and sex				

Table 4-26 shows logistic regressions between CVD risk prediction categories and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE study. Thus, omega-3 fatty acids are not a significant predictor of NAFLD participants to be in CVD risk categories.

Table 4-26 Relationship between cardiovascular risk categories and omega-3 fatty acids after adjustment of age and sex

CVD risk prediction categories		
INSYTE study (n= 63)		
	B Coefficient	p-value
RBC EPA	1.684	0.165
RBC DPA	1.132	0.302
RBC DHA	- 0.839	0.655
Omega-3 Index	0.074	0.969
Data that were non-normally distributed were log transformed before analysis. Multiple logistic regressions analysis between CVD risk categories with the individual omega-3 fatty acids .All data were adjusted for age and sex		

4.4 Discussion

This project focuses on the relation between red blood cell omega-3 fatty acids and cardiovascular risk factors in patients with NAFLD enrolled in the INSYTE and WELCOME studies. The overarching hypothesis is that higher omega-3 fatty acid status will be associated with lower cardiovascular risk in this group of individuals. The primary cohort is patients involved in the INSYTE study, but data available from patients involved in the WELCOME study are used to confirm findings from the INSYTE patients. This chapter describes the relationships between omega-3 fatty acids (EPA, DHA, DPA and omega-3 index) and cardiovascular risk factors (blood lipids, carotid intima-media thickness, blood pressure and cardiovascular risk score). Sex and age are considered to influence risk factors for CVD (Oyama et al., 2008, Jousilahti et al., 1999) and omega-3 fatty acid status (Bakewell et al., 2006, Childs et al., 2008, Walker et al., 2014, Rees et al., 2006); CVD risk is greater in men than women and increases with age. Therefore, data were adjusted for age and sex. In this chapter, correlations were made between omega-3 fatty acids and blood lipids, carotid intima-media thickness, blood pressure and cardiovascular risk score data in order to address the relationship between them. Additionally, multivariate analysis was performed to check the prediction relationship and to exclude the age and sex effects on these relationships.

The hypotheses of the work described in this chapter are that a) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and blood lipids, b) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and carotid intima media thickness, c) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and blood pressure, and d) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and cardiovascular 10-year risk score. These are

novel explorations of the link between omega-3 fatty acids and cardiovascular risk markers and risk score in patients with NAFLD.

In the current study effects of age and sex on blood lipids were assessed; age had a significant effect on TC: HDL ratio after controlling for sex; TC:HDL increased with age. Sex had a significant effect only on HDL concentration and only after adjustment for age; HDL was lower in men.

In this study the relationship between omega-3 fatty acid status and blood lipids was assessed. In general, the data described in this chapter are not in support of the first hypotheses, that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and blood lipids. This hypothesis must be rejected because only DPA had a negative relationship with TG and then only in patients in the WELCOME study. In patients with NAFLD included in the INSYTE cohort, omega-3 fatty acids (EPA, DHA, DPA and omega-3 index) had no significant association with blood lipids (total cholesterol, HDL, TC: HDL, LDL, and triglyceride). Also in patients with NAFLD included in the WELCOME cohort DHA had no significant association with blood lipids (total cholesterol, HDL, TC: HDL, LDL, and triglyceride). However, EPA in the WELCOME cohort had a positive association with total cholesterol, HDL, and LDL. As previously stated in the WELCOME cohort DPA had an inverse association with TGs.

Omega-3 fatty acids have been reported to have an effect on blood lipids especially TGs (see below), but this has been mainly explored in the context of supplemental omega-3 fatty acids. To my knowledge, no studies have been performed to study the relationship of hyperlipidaemia with omega-3 status in patients with NAFLD without increasing the oral intake of omega-3 fatty acids. It seems that supplementing omega-3 fatty acids might be required to provide the amounts of these fatty acids needed to influence blood lipids.

A consistently described therapeutic effect of omega-3 fatty acids is a reduction of plasma TG concentrations (Kris-Etherton et al., 2002). 4 g/day of Omacor (EPA plus DHA) has been shown to be effective in hypertriglyceridemia (Targher et al., 2010). A randomised placebo controlled trial (WELCOME) tested the effects of high dose purified omega-3 fatty acids (3.6 g / EPA plus DHA per day from Omacor, which is licensed for lowering plasma TG concentrations) for 15-18 months in NAFLD participants and there was a reduction in the TG concentration (Scorletti et al., 2014 b). This effect is achieved by a combination of a reduction in hepatic synthesis of triglycerides and an increased clearance of circulating triglycerides (Kris-Etherton et al., 2002). Doses of 2–4 g of omega-3 fatty acids are usually needed to see a therapeutic effect, and these doses will lower triglyceride concentrations by about 30 percent. Such an intake of omega-3 fatty acids cannot be easily obtained by diet alone and needs to be achieved from supplements (Saravanan et al., 2010). As described in chapter 3 the mean omega-3 index (EPA+DHA) for NAFLD patients in the INSYTE and the WELCOME studies was 5.5% and this may be too low to reduce TG levels.

In the current study some relationships between omega-3 status and blood lipids were different between the INSYTE and the WELCOME cohorts. EPA had a positive relation with total cholesterol, HDL, and LDL, and DPA had a negative relation with TGs in the WELCOME cohort, but there were no relations in the INSYTE cohort. The reason for this apparent discrepancy in findings between the INSYTE and the WELCOME cohorts is not immediately clear but may relate to sample size: the WELCOME cohort was larger. The findings of the current study may be compared with some intervention studies in the literature. (Mori et al., 2000) showed that 4 g/day EPA or DHA for 6 weeks in 56 overweight men aged 48.8 year with mild hyperlipidaemia, reduced TGs by 0.45 ± 0.15 mmol/L in the DHA group and by 0.37 ± 0.14 mmol/L in the EPA group. Neither EPA nor DHA had any effect on total cholesterol. LDL and HDL were not affected by EPA, while DHA increased LDL

cholesterol by 8%. (Balk et al., 2006) in review of 19 studies and (Hartweg et al., 2007) in review of 18 studies in diabetes participants conclude that total cholesterol was not effected by increased omega-3 supplementation but that LDL-cholesterol was increased by 3-6%.

In the current study, the relationships between omega-3 fatty acids with CIMT and the relationships between CIMT with blood lipids and age were assessed. Omega-3 fatty acids and omega-3 index had no significant association with CIMT in NAFLD participants in the INSYTE and WELCOME studies; furthermore they were associated with normal or high CIMT (< 0.8 mm or > 0.8 mm, respectively) after controlling for age and sex in the INSYTE study. The second hypothesis of the work described in this section is that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and carotid intima media thickness. This hypothesis must be rejected. (Monge et al., 2016) assessed the association between blood omega-3 fatty acids and CIMT in 1306 Mexican women free of disease. They did not observe a significant association between omega-3 fatty acids and CIMT in the overall study population, but they observed a strong statistically significant inverse association with CIMT in indigenous Mexican women, and they conclude that genetic markers may reflect differences in omega-3 fatty acid metabolism across populations that in turn affect atherosclerosis as assessed by CIMT. In the current study the absence of a relationship between omega-3 fatty acids and CIMT may be because the participants were almost all White or because sample size was small.

(Bhatia et al., 2016) investigated the effect of high dose (4 g/day) of omega-3 fatty acid for 15-18 months on NAFLD participants and evaluated if improvement in markers of NAFLD severity was associated with decreased CIMT progression over

time for 92 patients (age 51.5 ± 10.7 years). They found that reduction in NAFLD severity is associated with reduced CIMT progression.

Age did have a significant effect on CIMT independently of sex: CIMT increased with age. This finding supports that of (Ozturk et al., 2015); that study was performed on 61 adult men with biopsy-proven NAFLD, and the participant age range was 20 to 40 years, Theey found a positive correlation between CIMT and age in patients with NAFLD (r= 0.310, p= 0.002).

In this study, the relations between omega-3 fatty acids and systolic and diastolic blood were assessed. Overall the NAFLD participants of the INSYTE study had high SBP and 65% of the males and 50% of females had high SBP. However, only 8% of participants had high DBP. The third hypothesis of the work described in this section is that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and blood pressure. Some of the data are in support these hypotheses. Overall there were no relations between omega-3 fatty acids and systolic and diastolic blood pressure in the NAFLD participants; this lack of relationship was similar in the INSYTE and WELCOME cohorts. However, consistent with my hypothesis, EPA, DHA and omega-3 index had a negative relationship with SBP in hypertensive participants independently of sex and age in the INSYTE study participants. The third hypothesis can be accepted with the hypertensive participants in the INSYTE study only. This finding indicates that omega-3 fatty acids may have a role in controlling SBP of participants with high SBP. This finding is consistent with a meta-analysis of 31 placebo-controlled trials on 1356 subjects, which examined the effect of omega-3 fatty acids in fish oil on blood pressure (Morris et al., 1993). It was concluded that the hypotensive effect of omega-3 fatty acids may be strongest in hypertensive subjects. (Miller et al., 2014) conducted a meta-analysis of 36 randomised trials and found that fish-oil intake (median dose 3.7 g/day EPA plus DHA) reduced SBP by 2.1 mm Hg (p 0< 0.01) and DBP by 1.6 mm Hg (p < 0.01). Furthermore, treatment of patients with NAFLD with 3.6 g EPA plus

DHA per day for 15-18 months lowered both SBP and DBP (Scorletti et al., 2015). This effect could be related to at least two mechanisms. First, incorporation of EPA and DHA into membrane phospholipids could increase systemic arterial compliance (Scorletti et al., 2014b). Second, EPA and DHA could improve endothelial function (Kris-Etherton et al., 2002). This is consistent with the observation that the antihypertensive effect of fish oil may be greater in populations with arterial stiffness and/or microvascular dysfunction, i.e., populations with hypertension and older populations (Harris et al., 2008).

In the current study, cardiovascular risk score (prediction of coronary events: fatal/nonfatal myocardial infarction or sudden death) was assessed. As described earlier (section 3.3.1.7) CVD risk score was high in these NAFLD participants (10.2% and 11.75 % in the INSYTE and the WELCOME cohort, respectively); in this chapter, age had a very strong correlation with CVD risk score ($p < 0.001$). CVD risk score increased with age. Also CVD risk score was higher in males than females (14.2%, and 6.7% respectively, $p=0.035$). This finding supports work by (Treeprasertsuk et al., 2012) who assessed the Framingham risk score for 3554 people in total, 309 of whom had NAFLD, and they found that 10-year CHD risk was significantly higher in the NAFLD patients ($p=<0.0001$) and higher in men than women (12.6 ± 10.3% vs. 9.6 ± 8.1%, respectively, $P = 0.006$).

The relations between omega-3 fatty acids and cardiovascular risk score were assessed. The third hypothesis of the work described in this section is that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and cardiovascular risk score; however no significant relationships were observed. Thus, this hypothesis must be rejected. Independently of sex and age, in patients in the INSYTE study there was a negative relationship between CVD risk < 10% and DHA and omega-3 index, but this was not significant ($p= 0.097$, $p=0.090$

respectively). One reason for this may be because the value in CVD score in the Excel calculator was 30% for all the participants whose risk was higher than 30%. The other reason could be because the analysis was done independently of sex and age. McCormick et al. who investigated the effect of high dose of omega-3 fatty acid treatment (3.6 g/day DHA and EPA for 15-18 month), the mean of CVD risk score was 9.7%, and they concluded that high dose of omega-3 fatty acid treatment did not improve measures of microvascular function or vibration perception, but they didn't present the effect of omega-3 fatty acids on CVD risk score (McCormick et al., 2015).

The reason for the apparent discrepancies in some findings between the INSYTE and the WELCOME cohorts (e.g. for blood lipids and systolic blood pressure) may be related to the sample size. Also, many patients were using medications which may remove any relation between omega-3 and the risk factors being studied. For example, more than 30% of patients in INSYTE and 50% of those in WELCOME were using lipid lowering medications, more than 30% in INSYTE and more than 40% in WELCOME were using statins, and around half in INSYTE and WELCOME were using antihypertensive medications.

4.5 Summary and conclusion

Omega-3 fatty acids lower CVD risk by beneficially influencing the risk factor profile, but this is little explored in patients with NAFLD. In the INSYTE cohort, there was no significant relationship between omega-3 status and blood lipids. In the WELCOME cohort higher red blood cell DPA were associated with lower TGs. Omega-3 fatty acid status (EPA, DHA and DPA) is not associated with CIMT and cardiovascular 10-year risk score in patients with NAFLD. Increasing age was determinant of increasing CIMT and cardiovascular 10-year risk score in NAFLD patients. Increasing total cholesterol, LDL cholesterol and TC: HDL ratio are determinants of increasing CIMT in NAFLD patients. Overall there were no relations between omega-3 fatty acids and systolic and diastolic blood pressure in the NAFLD participants. In hypertensive patients a higher EPA, DHA and omega-3 index are each associated with lower systolic blood pressure.

To conclude, omega-3 fatty acids status (EPA, DHA and DPA) is not associated with blood lipids, diastolic blood pressure, CIMT, and cardiovascular 10-year risk score in patients with NAFLD.

It is included that differences in omega-3 fatty acid status have modest, if any, effects on blood lipids, blood pressure, CIMT and 10-year cardiovascular risk in patients with NAFLD. Any effects of omega-3 fatty acids may be masked by medication use to control risk factors or by the small sample size of both cohorts.

Chapter 5: **Relationship of red cell omega-3 fatty acids with body fatness, hyperglycaemia and insulin resistance, and inflammatory markers in patients with NAFLD**

5.1 Introduction, research questions and hypothesis

5.1.1 Introduction

Metabolic syndrome is a condition defined as a combination of visceral obesity with two or more of hypertension, hyperglycemia and dyslipidaemia (Ogurtsova et al., 2017). Visceral adipose tissue is positively associated with liver fat (Kotronen et al., 2007). This association helps to understand the strong relation between NAFLD and metabolic syndrome, because according to the International Diabetes Federation guidelines for diagnosing metabolic syndrome, the mandatory criteria is increased waist circumference. In addition, there is a well established independent link between increased CV morbidity and mortality and central obesity (Donahue et al., 1987). Adiposity itself could explain the raised CV risk seen with NAFLD instead of hepatic fat content per se (Bhatia et al., 2012). Adipose tissue, in addition to being a fat store, is metabolically active, secreting different adipokines, cytokines, and hormones that serve to regulate hepatic fat, inflammation, and insulin sensitivity and affect CV disease outcome (Bhatia et al., 2012), (Scorletti et al., 2011). In addition to the adverse effect of visceral fat, abnormal ectopic fat storage like increased myocardial and epicardial fat could be amongst the cumulative effects of insulin resistance (IR) and NAFLD with consequent adverse CV outcome (Bhatia et al., 2012). Even though no treatment has yet been approved for NAFLD, weight loss is one of the most important approaches to decrease liver fat (Pacifico et al., 2015, Ahmed et al., 2010).

Many studies of supplemental omega-3 fatty acids have described body weight and body mass index as a way of characterizing the study population; most of these studies describe no change in these measures with omega-3 fatty acids. Overall there are few reports of improved body fatness, waist circumference or waist-to-hip ratio with increased intake of omega-3 fatty acids. Nevertheless, some

studies do show greater beneficial effects when combining omega-3 fatty acids with exercise or energy restriction than the effects seen with exercise or energy restriction alone (Calder, 2015a). For example, Thorsdottir at al. investigated the effect of increasing omega-3 intake from supplements (or with fatty or lean fish consumption) combined with energy restriction for 8 weeks in obese and overweight males and females. Males who had consumed supplemental omega-3 fatty acids had a reduction in waist circumference (Thorsdottir et al., 2007). Also, (Kunesova et al., 2006) reported that omega-3 fatty acids enhance weight loss in obese females treated by very low calorie diet. However, a number of studies do not report body weight or fat reduction with omega-3 fatty acids alone; they also do not report any relation between omega-3 fatty acids with body fatness before the interventions (Munro and Garg, 2013).

A recent study undertaken across 19 European centres showed a strong association between increased insulin resistance (IR) and the excess incidence of cardiovascular disease (CVD) in patients with NAFLD (Byrne, 2012). IR in the liver leads to impaired shutdown of glucose production by insulin in liver cells leading to hyperglycaemia. One important and initial complication of IR in the liver is the induction of hepatic very low density lipoprotein (VLDL) production, through de novo lipogenesis, or through increased free fatty acid flux from adipocytes into the liver. In addition, IR encourages the production of C-reactive protein (CRP) and plasminogen activator inhibitor-1 (PAI-1), which are markers of the inflammatory state. All these abnormalities are subsequently metabolically related to hepatic IR and have been shown to promote atherosclerosis directly or indirectly (Tarantino and Caputi, 2011). An increasingly insulin resistant individual must secrete more and more insulin in order to prevent the consequences of IR. However, the combination of IR with compensatory hyperinsulinaemia increases the possibility that an individual will be hypertensive, and have a dyslipidaemia. These changes

have been shown to be associated with increased CVD risk (Tarantino and Caputi, 2011, Reaven, 2011).

Omega-3 fatty acids induce improvements in inflammation and have effects on insulin signaling pathways in adipose tissue and skeletal muscle to improve insulin sensitivity, so promoting more efficient glucose removal from the circulation. In addition, lower blood non-esterified fatty acid concentrations that result from improved insulin sensitivity permit more efficient glucose utilization. Consequently, omega-3 fatty acids would be expected to result in a lower fasting blood glucose concentration. A large number of studies have examined the effect of supplemental omega-3 fatty acids on fasting blood glucose concentrations. These studies have been performed in healthy subjects, type 2 diabetics, hyperlipidemics, and patients with cardiovascular disease. Several meta-analyses of the effects of supplemental omega-3 fatty acids on fasting blood glucose levels have been conducted (Montori et al., 2000, Balk et al., 2006, Hartweg et al., 2007) including one that included 18 studies (total of 823 subjects) of patients with type 2 diabetes (Montori et al., 2000). None of these meta-analyses determined a significant effect of omega-3 fatty acids on fasting blood glucose concentration. However, some individual studies report a significant elevation in blood glucose, perhaps related to the high doses of omega-3 fatty acids used (Calder, 2015a). This could be interpreted to be an adverse effect of omega-3 fatty acids, and may suggest development of IR.

Non-alcoholic steatohepatitis (NASH) is the second stage of non-alcoholic fatty liver disease (NAFLD); it is characterized by hepatocyte injury and inflammation in addition to the fat accumulation. Consequently, in patients with NASH there is an elevated level of inflammatory markers, pro-coagulant factors and oxidative stress markers. Excessive storage of fatty acids in hepatocytes and insulin resistance may increase mitochondrial β-oxidation promoting free-radical and reactive oxygen

species production. In turn reactive oxygen species may induce nuclear factor kappa-B (NF-κB) activity, that activates the transcription of several pro-inflammatory genes, leading to a production of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin-8 (IL-8) by hepatocytes, Kupffer cells and hepatic stellate cells (Scorletti et al., 2011). In a South Korean community-based healthy non-obese cohort study (n=30,172), researchers identified increased high-sensitivity C-reactive protein (CRP) in patients with NAFLD (Sung et al., 2009). Inflammation plays a central role in the development of atherosclerosis, and elevated plasma CRP levels are an important risk factor for CVD and type 2 diabetes. Similarly, plasma levels of TNF-α and IL-6 are also predictive of CVD (Calder, 2015b). CRP is a strong predictive biomarker of cardiovascular events; also CRP is a direct participant in atherosclerosis. CRP can accelerate atherosclerosis by increasing the expression of plasminogen activator inhibitor type 1 (PAI-1) and adhesion molecules in endothelial cells, inhibiting nitric oxide formation and increasing LDL uptake into macrophages (Chiang et al., 2010). According to Myhrstad et al. (2011) only CRP has emerged as a biomarker of inflammation for clinical application; other markers with appropriate robustness, sensitivity and specificity have not yet emerged. Based on the fact that endothelial dysfunction is the earliest manifestation of atherosclerosis, along with the involvement of oxidative stress and inflammation at all stages of coronary atherosclerosis development, biomarkers such as intracellular adhesion molecule (ICAM-1), vascular adhesion molecule (VCAM-1), soluble E-selectin (for endothelial activation), IL-6 (transcriptional driver of CRP) and monocyte chemoattractant protein (MCP-1) (produced by activated vasculature) are of particular interest (Myhrstad et al., 2011). Obese people have higher blood concentrations of several proinflammatory mediators than seen in normal weight people. Among the inflammatory markers with elevated concentrations in obesity are TNF-α, IL-6, MCP-1, IL-18, and CRP. Adipose tissue itself produces and secretes a broad range of inflammatory mediators including cytokines, chemokines, and acute-phase proteins. During weight loss programs and with bariatric surgery, a decrease in

concentrations of numerous inflammatory molecules has been observed, clearly demonstrating the relation between excess adipose tissue and inflammation (Calder, 2015a).

Omega-3 fatty acids have an anti-inflammatory effect on the vascular endothelium. A key aspect of this anti-inflammatory action is the reduced production of eicosanoids from arachidonic acid and the increased production of proresolving mediators from EPA and DHA. Many cohort studies report inverse associations between dietary intake of EPA + DHA or blood concentrations of EPA and DHA and the concentrations of a range of inflammatory markers, including cytokines, adhesion molecules, and acute phase proteins (Calder, 2015b). DHA also limited cytokine-stimulated endothelial cell expression of E-selectin and intercellular adhesion molecule 1 and the secretion of IL-6 and IL-8 by cultured endothelial cells. DHA, but not EPA, decreased in a dose- and time-dependent fashion the expression of VCAM-1 induced by interleukin-1, TNF, interleukin-4, or bacterial lipopolysaccharide (De Caterina et al., 1994). In dysfunctional or activated endothelium, an early stage in atherosclerosis, the endothelial cells express various adhesion molecules (CAMs) like ICAM-1, VCAM-1 and E-selectin, responsible for the adhesion of leukocytes to the endothelium. These molecules may be looked upon as markers of endothelial dysfunction as they are related to the development of atherosclerosis (Yli-Jama et al., 2002). However, Myhrstad et al. performed a systematic review of thirty-three articles reporting findings of intervention studies of the effects of marine omega-3 fatty acids (either fish or fish oil) on circulating inflammatory markers in healthy subjects, subjects with high risk of CVD and patients with CVD. They conclude that no definite conclusion can be drawn about the effect of marine n-3 fatty acids on circulating inflammatory markers in healthy individuals, individuals with high risk of developing CVD or individuals with CVD related diseases (Myhrstad et al., 2011).

This research focused on the relation between the status of red blood cell omega-3 fatty acids and cardiovascular risk factors in patients with NAFLD. This chapter describes the relationship between body fatness, insulin resistance, and inflammation markers as cardiovascular risk factors and omega-3 fatty acids status. To my knowledge, no studies have been done to study the relation of omega-3 fatty acids with body fatness, insulin resistance, and vascular inflammation markers in NAFLD patients without increasing the oral intake of omega-3 fatty acids. The majority of the studies in the research field investigate the effect of increase omega-3 intake with different dose and duration of intervention on the different aspects of health; however, in these studies, the investigators did not study the relationship between omega-3 status and different aspects of health in the baseline and before the intervention. In this study, I investigated relationship between CVD risk factors with the actual state of omega-3 fatty acids in patients with NAFLD without any intervention.

5.1.2 Research questions

What is the relationship between red blood cell omega-3 fatty acids and body fatness in patients with NAFLD?

What is the relationship between red blood cell omega-3 fatty acids and hyperglycaemia and insulin resistance in patients with NAFLD?

What is the relationship between red blood cell omega-3 fatty acids and inflammatory markers in patients with NAFLD?

5.1.3 Hypotheses

The hypotheses of the work described in this chapter are:

a) That patients with NAFLD will have an inverse relationship between omega-3 fatty acid status and body mass index and waist circumference.

b) That patients with NAFLD will have an inverse relationship between omega-3 fatty acid status with hyperglycaemia and insulin resistance.

c) That patients with NAFLD will have an inverse relationship between omega-3 fatty acid status and inflammatory markers.

5.2 Methods

5.2.1 Study design

This research is a sub-project of the INSYTE study which is a randomised, controlled, double-blind clinical trial of a synbiotic in patients with NAFLD. The focus of this sub-project is to study the relation between cardiovascular risk factors (and cardiovascular risk) and the omega-3 fatty acids status at baseline in 63 patients enrolled in the INSTYE study. Participants attended a clinic visit at the NIHR Wellcome Trust Clinical Research Facility, Southampton General Hospital, at INSYTE study entry for baseline measurements (as described in section 2.1). To confirm findings made with the INSYTE patients, this research also used the baseline data set of the WELCOME study. The WELCOME study was a randomised double blind placebo controlled trial of omega-3 fatty acids in 105 patients with NAFLD (Scorletti et al., 2014a). The WELCOME study was approved by the local ethics committee (REC: 08/H0502/165). The patient inclusion criteria and the methods of analysis were identical in the WELCOME study as in the INSYTE study.

5.2.2 Analyses performed:

The status of omega-3 fatty acids was determined by analysing red blood cell fatty acid composition using gas chromatography (as described in section 2.4). The following are presented here: EPA content, DHA content, DPA content, and Omega-3 index (the sum of EPA+DHA).

Cardiovascular risks were determined using the following:

Body fatness (body weight, body mass index and waist circumference) as determined using anthropometric measurements (as described in section 2.5).

Fasting blood glucose and insulin concentrations were analysed using enzymatic methods (as described in sections 2.11 and 2.12), and insulin resistance estimated by the homeostatic model assessment (as described in section 2.13).

Fasting blood concentrations of inflammatory markers (TNF-α, IL-8, IL-6, IL-10, MCP-1, VCAM-1, ICAM-1, P-Selectin, E-Selectin, and MMP-9) were measured using a Luminex Assay (as described in section 2.14.2) and CRP was measured using an Immuno-turbidimetric assay (as described in section 2.14.1).

5.2.3 Statistical analysis

The chapter addresses the hypotheses: a) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and body mass index and waist circumference, b) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and insulin resistance, c) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and inflammation markers. All data were entered into an Excel database and transferred to SPSS for analysis. Non-normally distributed variables were log base 10 transformed to obtain a symmetrical data distribution before proceeding with data analysis. Univariate correlations were employed to study the relationships between red cell omega-3 fatty acids and CVD risk factors (body mass index, waist circumference, insulin resistance and inflammatory markers) as Pearson's or Spearman correlations. Subsequently, multivariate analysis was performed using each CVD risk factor (body mass index, waist circumference, insulin resistance and inflammatory markers) as the outcome and fatty acids and other variables (age, sex) as confounding factors. Significance was considered when the p-value was below 0·05 for all analyses. All statistical analyses were performed using IBM SPSS Statistics for Windows (version 21.0; SPSS, Inc., Chicago, IL).

5.3 Results

5.3.1 Correlation of inflammatory markers with waist circumference, body mass index and insulin resistance

Table 5-1 shows correlations between the inflammatory markers and waist circumference, body mass index and insulin resistance. Waist circumference had significant positive relationships with TNF-α, IL-6, IL-8, RANTES and P-Selectin ((r=0.301, p<0.05), (r= 0.283, p<0.05), (r=0.260, p<0.05), (r=0.260, p<0.05), (r=0.316, p<0.05) and (r=0.331, p<0.01) respectively). Body mass index had significant positive relationships with TNF-α, IL-6, RANTES and P-Selectin ((r=0.293, p<0.05), (r= 0.324, p<0.05), (r= 0.338, p<0.01) and (r= 0.258, p<0.05) respectively). Insulin resistance had significant positive relationships with TNF-α, IL-8 and P-Selectin ((r= 0.282, p <0.05), (r= 0.431, p <0.01), and (r= 0.396, p <0.01)). However, CRP, IL-10, MCP-1, VCAM-1, ICAM-1 and E-Selectin had no relationships with waist circumference, body mass index or insulin resistance.

Table 5-1 Correlations between inflammatory markers and waist circumference, body mass index and insulin resistance in 63 patients with NAFLD in INSYTE study

Variables	Waist circumference	Body mass index	Insulin resistance
Waist circumference	1	0.860***	0.321*
Body mass index	0.860***	1	0.296*
Insulin resistance	0.321*	0.296*	1
CRP	0.077	0.160	0.084
TNF-α	0.301*	0.293*	0.282*
IL-6	0.283*	0.324*	0.207
IL-8	0.260*	0.232	0.431**
IL10	0.063	0.011	0.213
MCP-1	-0.015	0.089	0.077
RANTES	0.316*	0.388**	0.046
VCAM-1	0.061	0.006	-0.125
ICAM-1	0.069	0.071	0.061
P-Selectin	0.331**	0.258*	0.396**
E-Selectin	0.157	0.153	-0.112

Data are B coefficients of the liner correlation with each inflammation marker. Not normally distributed data were log transformed before analysis.* p-value <0.05, **p-value <0.01, *** p-value <0.001. Abbreviations: CRP, C-reactive protein; TNF-α, tumour necrosis factor alpha; IL-6, Interleukin 6; IL-8, Interleukin-8; IL-10, Interleukin-10; MCP-1,monocyte chemoattractant protein1 ; VCAM-1, vascular adhesion molecule1; ICAM-1, intracellular adhesion molecule1.

5.3.2 Red blood cell omega-3 fatty acids and body mass index

5.3.2.1 Correlation between body mass index (BMI) and red blood cell omega-3 fatty acids

The correlation between body mass index (BMI) and red blood cell omega-3 fatty acids were assessed. As shown in Figure 5-1 and 5-2 there was a strong negative correlation between DHA and BMI (r=-0.258, p= 0.043) and omega-3 index and BMI (r= - 0.251, p= 0.048) in patients with NAFLD included in the INSYTE study. Furthermore, as shown in Figure 5-3 and 5-4 there were a similar significant negative correlations between DHA and BMI (r=-0.238, p= 0.015) and omega-3 index and BMI (r= - 0.250, p= 0.011) in the 105 patients with NAFLD in the WELCOME study. However, there were no significant correlations between EPA and BMI (r=- 0.15, p= 0.250) or between DPA and BMI (r=0.50, p= 0.700) in patients with NAFLD included in the INSYTE study. Similarly there were no significant correlations between EPA and BMI (r= 0.144, p= 0.144) or between DPA and BMI (r=0.155, p= 0.116) in the 105 patients with NAFLD in the WELCOME study.

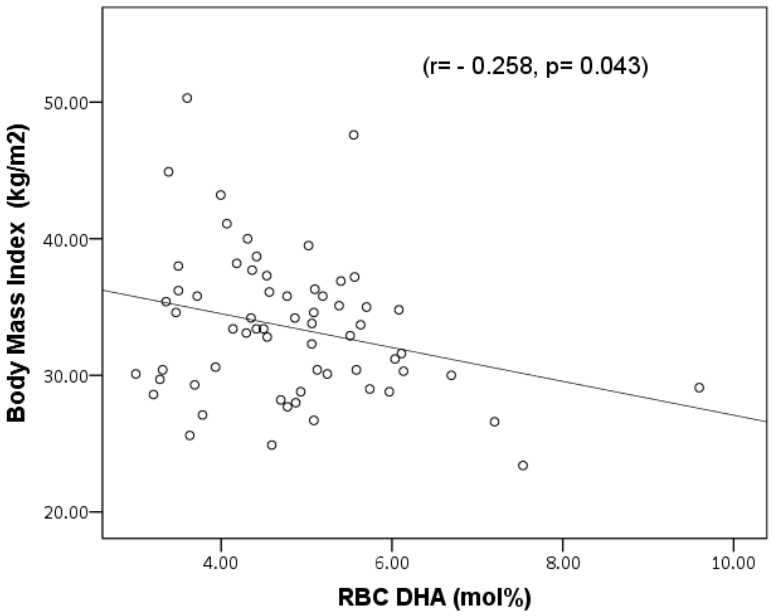


Figure 5-1 Correlation between body mass index and red blood cell DHA in 63 patients with NAFLD in the INSYTE study.

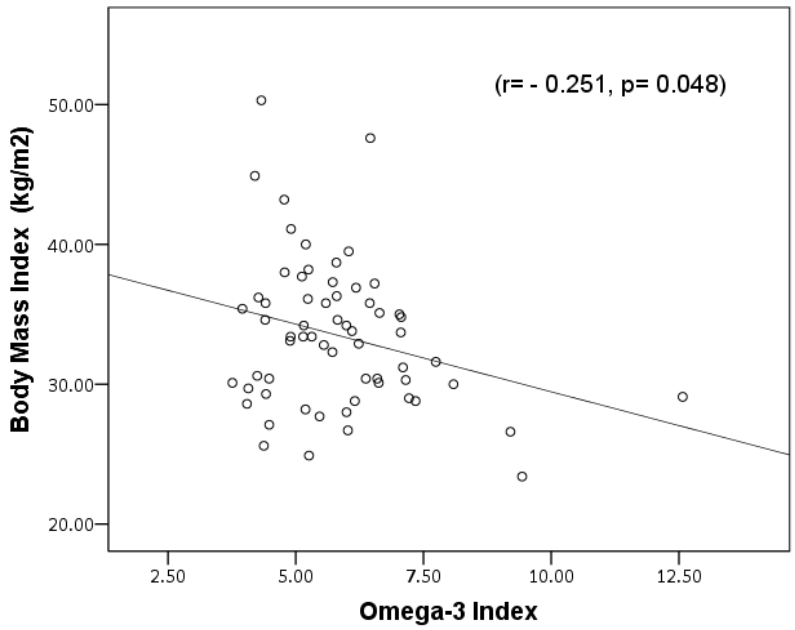


Figure 5-2 Correlation between body mass index and red blood cell omega-3 index in 63 patients with NAFLD in the INSYTE study.

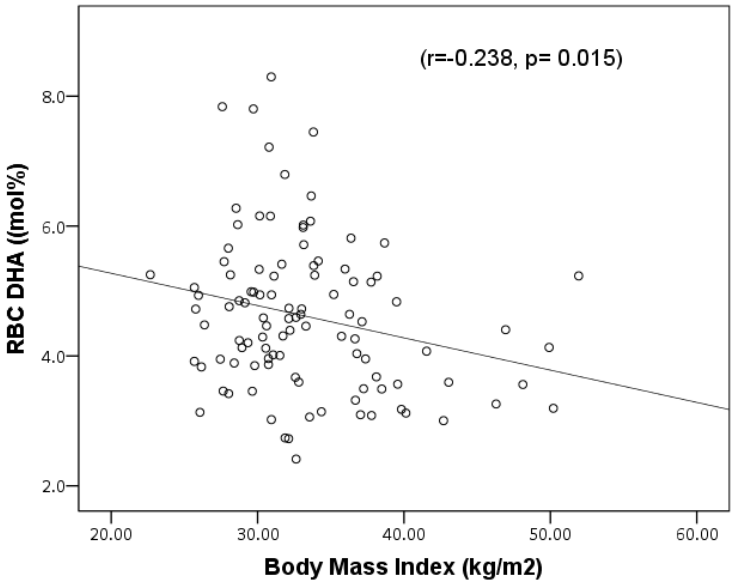


Figure 5-3 Correlation between body mass index and red blood cell DHA in 105 patients with NAFLD in the WELCOME study.

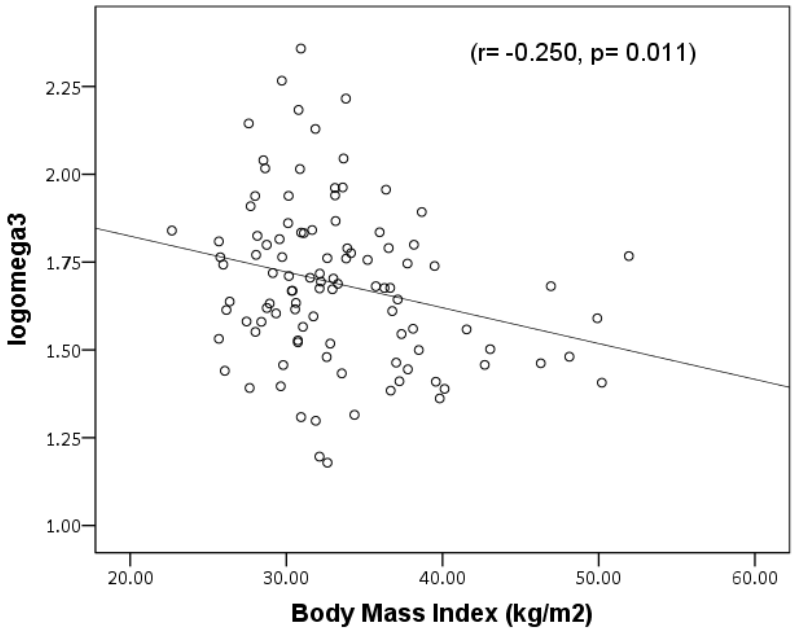


Figure 5-4 Correlation between body mass index and red blood cell omega-3 index in 105 patients with NAFLD in the WELCOME study

5.3.2.2 Relationship between body mass index and omega-3 fatty acids after adjusting for sex and age

Table 5-2 shows the results of multiple linear regressions between BMI and red blood cell omega-3 fatty acids after adjustment for age and sex. DHA and omega-3 index were statistically significant predictors of BMI. In 63 patients with NAFLD included in the INSYTE study, there was a negative linear relationship (β= -6.420, p= 0.042) between BMI and DHA; the beta coefficient shows that every percent increase in DHA will decrease the BMI by 6.42 kg/m². Also, there was a negative linear relationship (β= -6.374, p= 0.040) between BMI and omega-3 index; every percent increase in the omega-3 index the BMI will decrease by 6.374 kg/m². EPA and DPA showed no significant relationships with BMI. In the 105 patients with NAFLD in the WELCOME study, there was a positive linear relationship between BMI and DPA (β= 0.014, p= 0.026). However, the negative relationship between BMI with DHA and omega-3 index (p=0.073, p=0.067 respectively) were not quite significant. Again there was no significant relationship between BMI and EPA.

Table 5-2 Relation of body mass index with red blood cell omega-3 fatty acids after adjustment for age and sex in patients with NAFLD

	INSYTE study (n= 63)		WELCOME study (n= 104)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-2.467	0.172	-0.006	0.262
RBC DPA	0.853	0.656	0.014	0.026
RBC DHA	-6.420	0.042	-0.037	0.073
Omega-3 Index	-6.374	0.040	-0.042	0.067
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between BMI and each fatty acid was performed adjusting for age and sex				

5.3.3 Red blood cell omega-3 fatty acids and waist circumference

5.3.3.1 Correlations between waist circumference and red blood cell omega-3 fatty acids

As shown in Figure 5-5 and 5-6 there was a strong negative correlation between DHA and waist circumference (WC) (r=-0.333, p= 0.011) and between omega-3 index and WC (r= - 0.290, p= 0.021) in patients with NAFLD included in the INSYTE study. Furthermore, as shown in Figure 5-7 and 5-8 there were similar significant negative correlations between DHA and WC (r=-0.194, p= 0.049) and omega-3 index and WC (r= - 0.198, p= 0.044) in the 105 patients with NAFLD in the WELCOME study. However, there were no significant correlations between EPA and WC (r=- 0.221, p= 0.090) or between DPA and WC (r=0.132, p= 0.321) in patients with NAFLD included in the INSYTE study. Similarly there were no significant correlations between EPA and WC (r=- 0.114, p= 0.250) or between DPA and WC (r=0.149, p= 0.131) in the 105 patients with NAFLD in the WELCOME study.

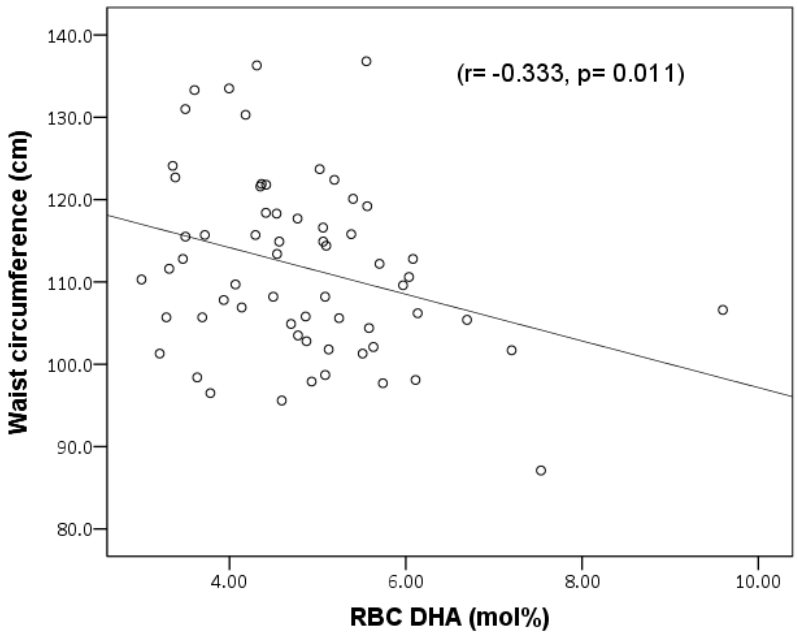


Figure 5-5 Correlations between waist circumference with red blood cell DHA in 63 patients with NAFLD in INSYTE study.

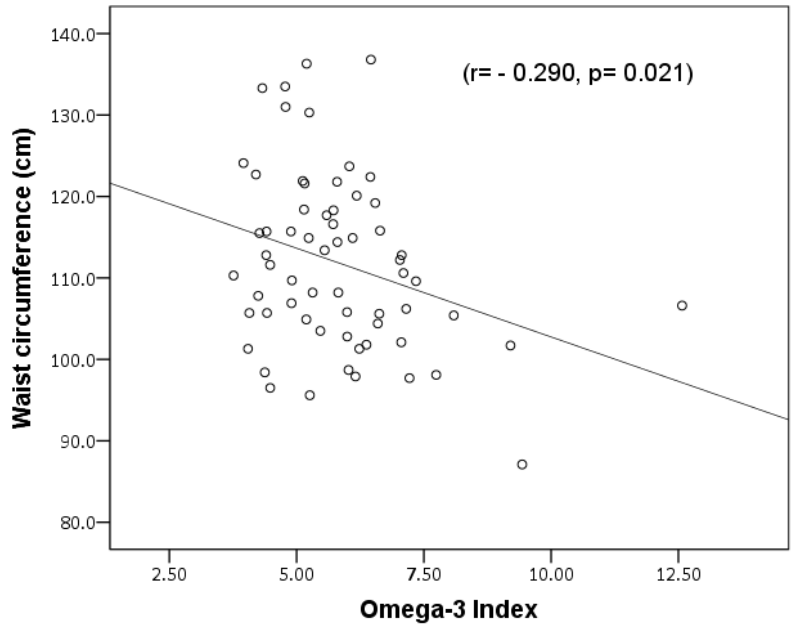


Figure 5-6 Correlations between waist circumference with red blood cell omega-3 index in 63 patients with NAFLD in INSYTE study.

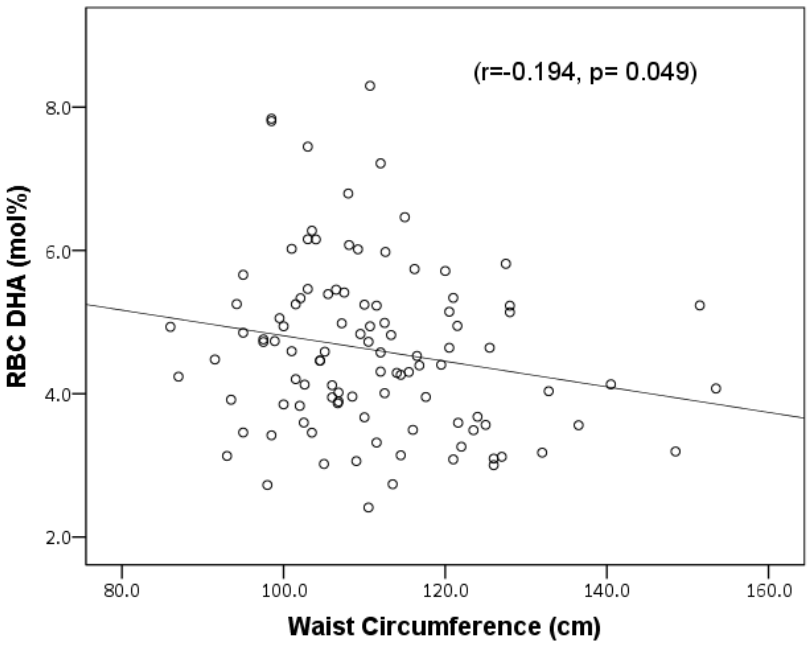


Figure 5-7 Correlations between waist circumference with red blood cell DHA in 105 patients with NAFLD in WELCOME study.

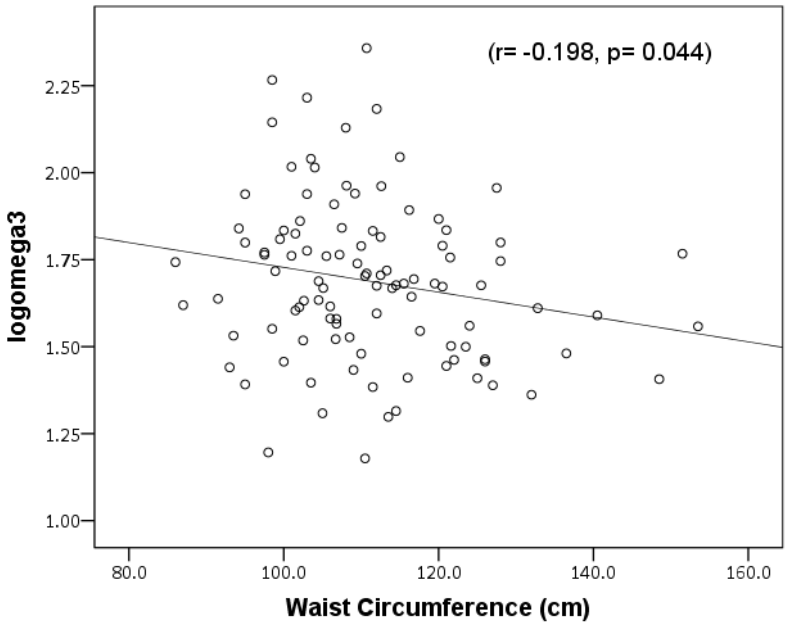


Figure 5-8 Correlations between waist circumference with red blood cell omega-3 index in 105 patients with NAFLD in WELCOME study.

5.3.3.2 Relationship between waist circumference and omega-3 fatty acids after adjusting for sex and age

Table 5-3 shows the results of multiple linear regression analysis between waist circumference and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. There were no statistically significant linear relationships between waist circumference and EPA, DPA, DHA and omega-3 index. Thus omega-3 fatty acids were not predictors of waist circumference.

Table 5-3 Relations of waist circumference and red blood cell omega-3 fatty acids after adjustment for age and sex

Waist circumference				
	INSYTE study (n= 63)		WELCOME study (n= 104)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.007	0.153	-0.002	0.481
RBC DPA	0.004	0.408	0.005	0.059
RBC DHA	-0025	0.053	-0.011	0.212
Omega-3 Index	-0.032	0.056	-0.013	0.213
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between waist circumference and each fatty acid was performed adjusting for age and sex				

5.3.4 Red blood cell omega-3 fatty acids and glucose

5.3.4.1 Correlations between glucose and red blood cell omega-3 fatty acids.

In the INSYTE study, there were no significant correlations between EPA and glucose (r= 0.061, p=0.643), DPA and glucose (r=-0.019, p=0.887), DHA and glucose (r= 0.026, p=0.845) or omega-3 index and glucose (r= 0.022, p=0.866). Also, in the WELCOME study there were no significant correlations between EPA and glucose (r=--0.005, p=0.957), DPA and glucose (r=0.156, p=0.113), DHA and glucose (r=0.105, p=0.289) or omega-3 index and glucose (r= 0.099, p=0.317).

5.3.4.2 Relationship between glucose and omega-3 fatty acids after adjusting for sex and age

Table 5-4 shows the results of multiple linear regression analysis between glucose and omega-3 fatty acids after adjustment for age and sex. In patients with NAFLD included in the INSYTE and the WELCOME studies, there were no statistically significant linear relationships between glucose and EPA, DPA, DHA and omega-3 index. Thus omega-3 fatty acids were not predictors of glucose in patients with NAFLD.

Table 5-4 Relationship between glucose and omega-3 fatty acids after adjusting for sex and age in patients with NAFLD

Glucose				
	INSYTE study (n= 63)		WELCOME study (n= 105)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.028	0.983	-0.023	0.861
RBC DPA	0.006	0.952	0.225	0.142
RBC DHA	0.034	0.855	0.596	0.216
Omega-3 Index	0.029	0.874	0.090	0.332
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between fasting blood glucose and each fatty acid was performed adjusting for age and sex				

5.3.4.3 Relationship between omega-3 fatty acids with normal and high glucose levels after adjusting for sex and age

Table 5-5 shows linear regressions between glucose and omega-3 fatty acids after splitting data for glucose into normal (glucose < 5.6 mmol/L) and high (glucose ≥ 5.6 mmol/L) and after adjustment for age and sex. In patients with NAFLD included in the INSYTE study, there were no significant linear relationships between glucose and any individual fatty acid or omega-3 index in either the normal or high glucose categories. Thus, omega-3 fatty acids are not a significant predictor of fasting blood glucose in patients with normal or high glucose.

Table 5-5 Relationship between glucose and omega-3 fatty acids in normal and high glucose categories after adjusting for sex and age

Fasting blood Glucose				
INSYTE study (n= 63)				
	Normal (glucose < 5.6 mmol/L)		High (glucose ≥ 5.6 mmol/L	
	Unstandardized Coefficients B	p-value	Unstandardized Coefficients B	p-value
RBC EPA	-0.017	0.782	-0.007	0.954
RBC DPA	0.022	0.612	0.019	0.893
RBC DHA	-0.114	0.242	0.179	0.411
Omega-3 Index	-0.098	0.301	0.145	0.505
Data that were non-normally distributed were log transformed before analysis. After splitting data into normal (glucose < 5.6 mmol/L) and high (glucose ≥ 5.6 mmol/L) category, multiple linear regression analysis was performed between glucose with each individual omega-3 fatty acids. All data were adjusted for age and sex				

5.3.5 Red blood cell omega-3 fatty acids and insulin

5.3.5.1 Correlations between insulin and red blood cell omega-3 fatty acids.

In the 63 patients included in the INSYTE study, there were no significant correlations between EPA and insulin (r= -0.060, p=0.638), DPA and insulin (r=-0.141, p= 0.269), DHA and insulin (r= -0.049, p=0.702) or omega-3 index and insulin (r= -0.059, p=0.646). Also, in the 105 patients with NAFLD in the WELCOME study, there were no significant correlations between DPA and insulin (r=-0.097, p= 0.327), DHA and insulin (r= -0.078, p=0.431) or omega-3 index and insulin (r= -0.132, p=0.183). However, there was a strong negative correlation between EPA and insulin (r= -0.297, p=0.002) in patients in the WELCOME study as shown in Figure 5-9.

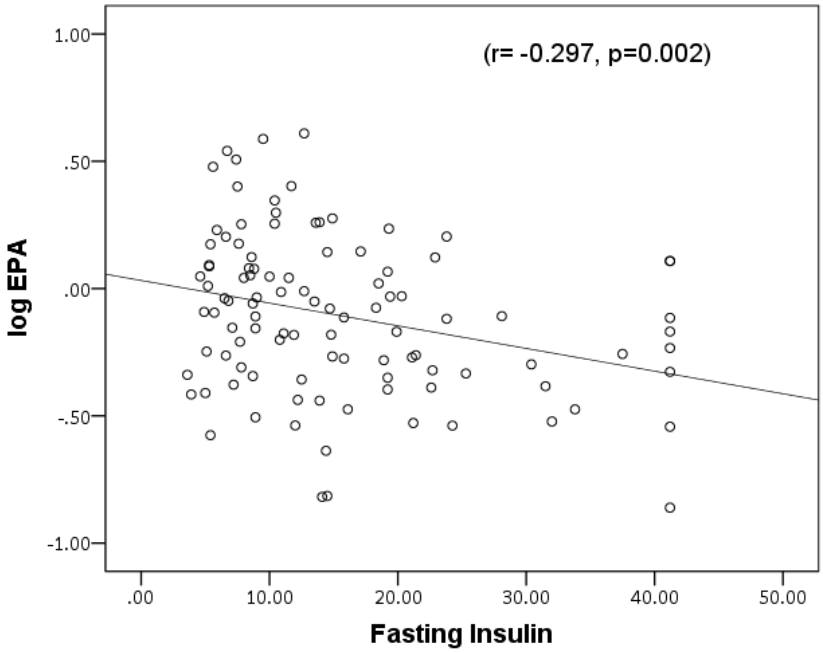


Figure 5-9 Correlations between insulin and red blood cell EPA in 105 patients with NAFLD in the WELCOME study.

5.3.5.2 Relationship between insulin and omega-3 fatty acids after adjusting for sex and age

Table 5-6 shows the results of multiple linear regression analysis between insulin and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. There were no statistically significant linear relationships between insulin and EPA, DPA, DHA and omega-3 index. Thus omega-3 fatty acids were not predictors of insulin.

Table 5-6 Relationship between insulin and omega-3 fatty acids after adjusting for sex and age

Insulin				
	INSYTE study (n= 63)		WELCOME study (n= 104)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.084	0.716	-0.001	0.110
RBC DPA	-0.282	0.215	-0.003	0.369
RBC DHA	-0.082	0.831	-0.005	0.617
Omega-3 Index	-0.081	0.830	-0.002	0.294
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between insulin and each fatty acid was performed adjusting for age and sex				

5.3.6 Red blood cell omega-3 fatty acids and insulin resistance

5.3.6.1 Correlations between insulin resistance and red blood cell omega-3 fatty acids.

In the 63 patients included in the INSYTE study, there were no significant correlations between EPA and insulin resistance (r= -0.042, p=0.749), DPA and insulin resistance (r=-0.159, p= 0.222), DHA and insulin resistance (r= -0.021, p=0.872) or omega-3 index and insulin resistance (r= -0.028, p=0.829). Also, in the 105 patients with NAFLD in the WELCOME study, there were no significant correlations between insulin resistance and DPA (r=-0.030, p= 0.768) or DHA (r= -0.044, p=0.673) or omega-3 index (r= -0.080, p=0.436). However, there was a strong negative correlation between EPA and insulin resistance (r= -0.242, p=0.017) in 105 patients in the WELCOME study as shown in Figure 5-10.

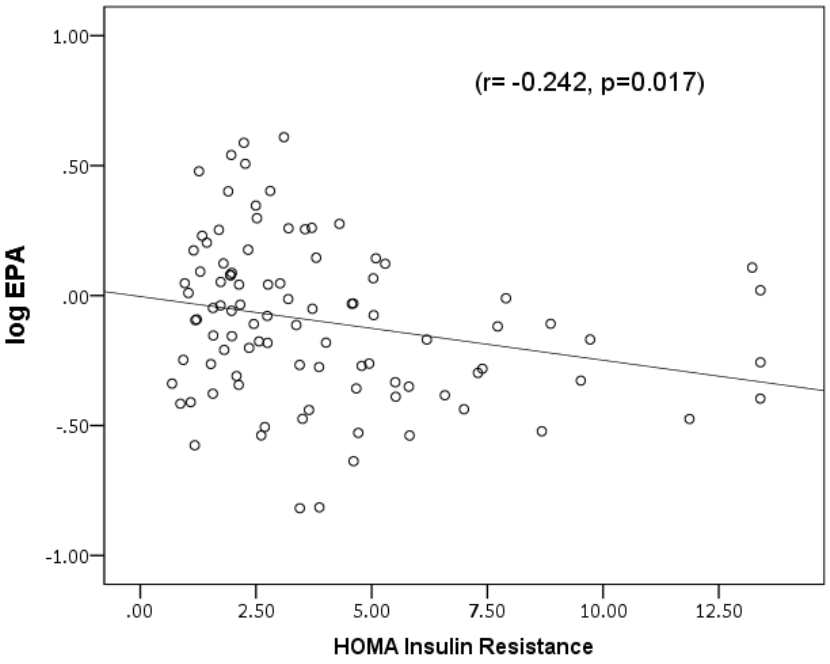


Figure 5-10 Correlation between insulin resistance and red blood cell EPA in 105 patients with NAFLD in the WELCOME study

5.3.6.2 Relationship between insulin resistance and omega-3 fatty acids after adjusting for sex and age

Table 5-7 shows the results of multiple linear regression analysis between insulin resistance and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. There were no statistically significant linear relationships between insulin resistance and EPA, DPA, DHA and omega-3 index. Thus omega-3 fatty acids were not predictors of insulin resistance.

Table 5-7 Relationship between insulin resistance and omega-3 fatty acids after adjusting for sex and age

Insulin resistance				
	INSYTE study (n= 63)		WELCOME study (n= 104)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.094	0.732	-0.082	0.181
RBC DPA	-0.273	0.313	-0.002	0.909
RBC DHA	-0.037	0.937	-0.125	0.588
Omega-3 Index	-0.047	0.920	-0.021	0.619
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between insulin resistance and each fatty acid was performed adjusting for age and sex				

5.3.6.2.1 Relationship between omega-3 fatty acids with normal and high level of insulin resistance

Table 5-8 shows the results of multiple logistic regressions between insulin resistance categories (normal < 2.41 and high ≥ 2.41) and red blood cell omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE study. There was a negative relationship (β= -2.331, p= 0.045) between insulin resistance categories and DPA; thus DPA can predict the insulin resistance level. The logistic regression showed the higher the DPA the lower the insulin resistance. EPA and DHA and omega-3 showed no significant relationships with insulin resistance categories.

Table 5-8 Relation of insulin resistance categories (normal and high cut-off values) with red blood cell omega-3 fatty acids after adjustment for age and sex

Insulin resistance normal and high level categories		
INSYTE study (n= 63)		
	B Coefficient	p-value
RBC EPA	-0.533	0.574
RBC DPA	-2.331	0.045
RBC DHA	-1.468	0.358
Omega-3 Index	-1.377	0.370
Data that were non-normally distributed were log transformed before analysis. Multiple logistic regressions analysis between insulin resistance (normal < 2.41 and high ≥ 2.41) and each fatty acids. All data were adjusted for age and sex.		

5.3.6.3 Red blood cell omega-3 fatty acids and inflammatory markers

5.3.6.4 Correlations among inflammatory markers

Table 5-9 shows correlations among the different inflammatory markers in patients with NAFLD included in the INSYTE study. There was no significant negative relationship between any markers. However, there were many significant positive relationships between markers. Each inflammatory marker had at least four significant positive correlations with other markers. Only VCAM-1 and ICAM-1 shared all the same significant correlations with other markers, but they had different strengths of correlation. CRP had a very strong positive correlation with IL-6 (r= 0.516, P<0.001) and RANTES (r= 0.484, p<0.001), a strong positive correlation with VCAM-1 (r= 0.356, p<0.01) and a positive correlation with ICAM-1 (r= 0.259, p<0.05).

Table 5-9 Correlations among the inflammatory markers in patients with NAFLD

Inflammatory markers INSYTE study (n= 63)											
	CRP	*TNF-α*	*IL-6*	*IL-8*	*IL-10*	*MCP-1*	*RANTES*	*VCAM-1*	*ICAM-1*	*E-Selectin*	*P-Selectin*
CRP	**1**	**.145**	**.516*** **	**.087**	**.211**	**.099**	**.484*** **	**.365** **	**.259***	**.231**	**.006**
TNF-α	**.145**	**1**	**.290***	**.252***	**.202**	**.410** **	**.258***	**.171**	**.212**	**.119**	**-.045**
IL-6	**.516*** **	**.290***	**1**	**.244**	**.286***	**.228**	**.499*** **	**.382** **	**.461*** **	**.270***	**.126**
IL-8	**.087**	**.252***	**.244**	**1**	**.300***	**.336** **	**.163**	**.027**	**.142**	**.289***	**.175**
IL-10	**.211**	**.202**	**.286***	**.300***	**1**	**.303***	**.091**	**.281***	**.365** **	**.396** **	**.108**
MCP-1	**.099**	**.410** **	**.228**	**.336** **	**.303***	**1**	**.226**	**.250***	**.355** **	**.158**	**.275***
RANTES	**.484*** **	**.258***	**.499*** **	**.163**	**.091**	**.226**	**1**	**.285***	**.327** **	**.191**	**.132**
VCAM-1	**.365** **	**.171**	**.382** **	**.027**	**.281***	**.250***	**.285***	**1**	**.597*** **	**.354** **	**.276***
ICAM-1	**.259***	**.212**	**.461*** **	**.142**	**.365** **	**.355** **	**.327** **	**.597*** **	**1**	**.656*** **	**.354** **
E-Selectin	**.231**	**.119**	**.270***	**.289***	**.396** **	**.158**	**.191**	**.354** **	**.656*** **	**1**	**.305***
P-Selectin	**.006**	**-.045**	**.126**	**.175**	**.108**	**.275***	**.132**	**.276***	**.354** **	**.305***	**1**

Data are β coefficients of the correlation between each pair of inflammatory markers. Not normally distributed data were log transformed for each variables before analysis. Abbreviations: CRP, C-reactive protein; TNF-α, tumour necrosis factor alpha; IL-6, Interleukin 6; IL-8, Interleukin-8; IL-10, Interleukin-10; MCP-1,monocyte chemoattractant protein1 ; VCAM-1, vascular adhesion molecule1; ICAM-1, intracellular adhesion molecule1; N, number., * p-value <0.05, **p-value <0.01, *** p-value <0.001.

5.3.7 Red blood cell omega-3 fatty acids and C-reactive protein (CRP)

5.3.7.1 Correlation between CRP and red blood cell omega-3 fatty acids

In the 63 patients with NAFLD included in the INSYTE study, there was a significant positive correlation between DPA and CRP (r=0.302, p=0.016) as shown in Figure 5 11; however there were no significant correlations between EPA and CRP (r=0.126, p=0.126), DHA and CRP (r=-0.011, p=0.933) or omega-3 index and CRP (r= 0.017, p=0.894). In the 105 patients with NAFLD in the WELCOME study, there were no significant correlations between EPA and CRP (r=-0.060, p=0.544), DPA and CRP (r=-0.053, p=0.590), DHA and CRP (r=-0.079, p=0.427) or omega-3 index and CRP (r= -0.086, p=0.387).

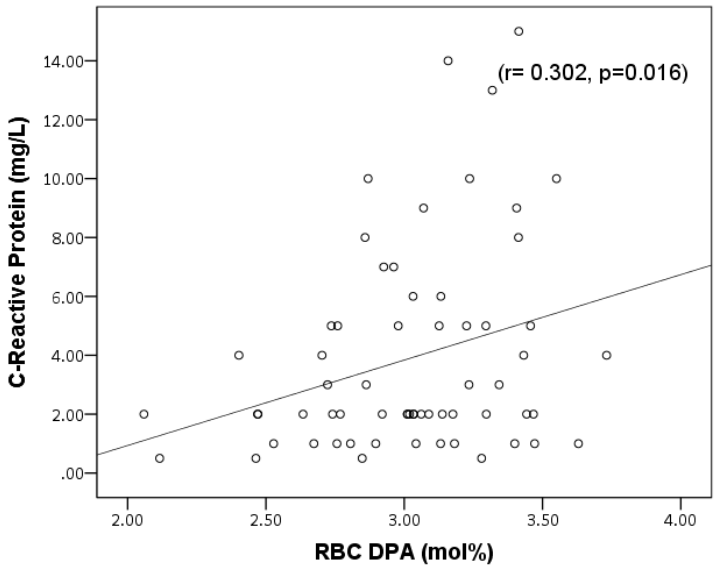


Figure 5-11 Correlations between CRP and red blood cell DPA in 63 patients with NAFLD included in the INSYTE study.

5.3.7.2 Relationship of red blood cell omega-3 fatty acids with CRP after adjusting for sex and age

Table 5-10 shows the results of multiple linear regressions analysis between CRP and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. In the INSYTE study, there was a positive linear relationship (β 0.034, p=0.011) between CRP and DPA; the beta coefficient shows that for every percent increase in DPA, CRP will increase by 0.034 mg/L; However, there were no statistically significant linear relationships between CRP and EPA or DHA or omega-3 index. In the WELCOME study, there ware no significant relationships between CRP and EPA , DPA, DHA and omega-3 index.

Table 5-10 Relationship of red blood cell omega-3 fatty acids with CRP after adjusting for sex and sex

C-reactive protein (CRP)				
	INSYTE study (n= 63)		WELCOME study (n= 104)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.006	0.707	-0.007	-0.471
RBC DPA	0.034	0.011	-0.001	0.945
RBC DHA	-0.038	0.361	-0.006	0.858
Omega-3 Index	-0.034	0.415	-0.013	0.753
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between CRP and each fatty acid was performed adjusting for age and sex				

5.3.7.3 Relationship between omega-3 fatty acids with normal and high level of CRP

Table 5-11 shows the results of multiple logistic regressions between CRP categories (normal < 3.0 mg/L and high ≥ 3.0 mg/L) and red blood cell omega-3 fatty acids after adjustment for age and sex in the 63 patients with NAFLD included in the INSYTE study. There was a posetive logistic regression relationship (β= 1.884, p=0.044) between CRP categories and DPA, suggesting that DPA can predict the CRP level. The logistic regression showed the higher the DPA level in red blood cells the higher the CRP. However, DHA showed a significant negative relationship (β= -2.892, p=0.038) with CRP categories. The logistic regression showed the higher the DHA the lower the CRP. Also omega-3 index had a negative relationship with CRP but this did not quite reach significance (β=-2.892, p=0.055). DHA and omega-3 index have an effect that is opposite to that of DPA.

Table 5-11 Relationship of red blood cell omega-3 fatty acids with CRP categories (normal and high cut-off values) after adjusting for age and sex

CRP level categories		
INSYTE study (n=63)		
	B Coefficient	*P-value*
RBC EPA	-0.373	0.649
RBC DPA	1.884	0.044
RBC DHA	-3.275	0.038
Omega-3 Index	-2.892	0.055
Data that were non-normally distributed were log transformed before analysis. Multiple logistic regressions analysis between CRP categories (normal < 3.0 mg/L and high ≥ 3.0 mg/L) and each fatty acid was performed adjusting for age and sex		

5.3.8 Red blood cell omega-3 fatty acids and tumour necrosis factor alpha (TNF-α)

5.3.8.1 Correlation between TNF-α and red blood cell omega-3 fatty acids

In the 63 patients with NAFLD included in the INSYTE study, there was no significant correlation between EPA and TNF-α (r=- 0.073, p=0.567), DPA and TNF-α (r=-0.084, p=0.376), DHA and TNF-α (r=-0.084, p=0.511) or omega-3 index and TNF-α (r=- 0.087, p=0.500). In the 105 patients with NAFLD in the WELCOME study, there were no significant correlations between EPA and TNF-α (r=-0.159, p=0.108) or DPA and TNF-α (r=-0.066, p=0.506) or DHA and TNF-α (r=-0.084, p=0.397) or omega-3 index and TNF-α (r= -0.099, p=0.318).

5.3.8.2 Relationship of red blood cell omega-3 fatty acids with TNF-α after adjusting for sex and age

Table 5-12 shows the results of multiple linear regression analysis between TNF-α and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. There were no statistically significant linear relationships between TNF-α and EPA, DPA, DHA and omega-3 index. Thus, omega-3 fatty acids were not predictors of TNF-α.

Table 5-12 Relationship of red blood cell omega-3 fatty acids with TNF-α after adjusting for sex and age

Tumour necrosis factor alpha (TNF-α)				
	INSYTE study (n=63)		WELCOME study (n=104)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.006	0.499	-0.024	0.053
RBC DPA	-0.002	0.796	-0.013	0.411
RBC DHA	-0.008	0.743	-0.057	0.251
Omega-3 Index	-0.015	0.655	-0.081	0.149
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between TNF-α and each fatty acid was performed adjusting for age and sex				

5.3.9 Red blood cell omega-3 fatty acids and interleukin 6 (IL-6)

5.3.9.1 Correlation between IL-6 and red blood cell omega-3 fatty acids

In the 63 patients included in the INSYTE study, there were no significant correlations between EPA and IL-6 (r= -0.061, p=0.634), DPA and IL-6 (r=0.009, p=0.946), DHA and IL-6 (r=- 0.011, p=0.934) or omega-3 index and IL-6 (r=- 0.031, p=0.812). In the 105 patients in the WELCOME study, there were no significant correlations between EPA and IL-6 (r=-0.015, p=0.916) or DPA and IL-6 (r=-0.127, p=0.364) or DHA and IL-6 (r=-0.086, p=0.541) or omega-3 index and IL-6 (r= -0.078, p=0.577).

5.3.9.2 Relationship of red blood cell omega-3 fatty acids with IL-6 after adjusting for age and sex

Table 5-13 shows the results of multiple linear regression analysis between IL-6 and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. There were no statistically significant linear relationships between IL-6 and EPA, DPA, DHA and omega-3 index. Thus omega-3 fatty acids were not predictors of IL-6.

Table 5-13 Relationship of red blood cell omega-3 fatty acids with IL-6 after adjusting for sex

Interleukin 6 (IL 6)				
	INSYTE study (n= 63)		WELCOME study (n= 53)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.020	0.474	0.013	0.762
RBC DPA	0.014	0.564	0.079	0.144
RBC DHA	-0.009	0.902	-0.060	0.741
Omega-3 Index	-0.029	0.766	-0.045	0.829
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between IL 6 and each fatty acid was performed adjusting for age and sex				

5.3.10 Red blood cell omega-3 fatty acids and interleukin-8 (IL-8)

5.3.10.1 Correlation between IL-8 and red blood cell omega-3 fatty acids

In the 63 patients included in the INSYTE study, there was a negative correlation between IL-8 and red blood cell DPA (r= -0.306, p=0.015) as shown in Figure 5-12; However, there were no significant correlations between EPA and IL-8 (r= -0.143, p=0.265), DHA and IL-8 (r=-0.045, p= 0.724) or omega-3 index and IL-8 (r= 0.006, p=0.964). In the 105 patients with NAFLD in the WELCOME study, there were no significant correlations between EPA and IL-8 (r=-0.046, p=0.646) or DPA and IL-8

(r=-0.007, p=0.941) or DHA and IL-8 (r=-0.053, p=0.596) or omega-3 index and IL-8 (r= -0.060, p=0.546).

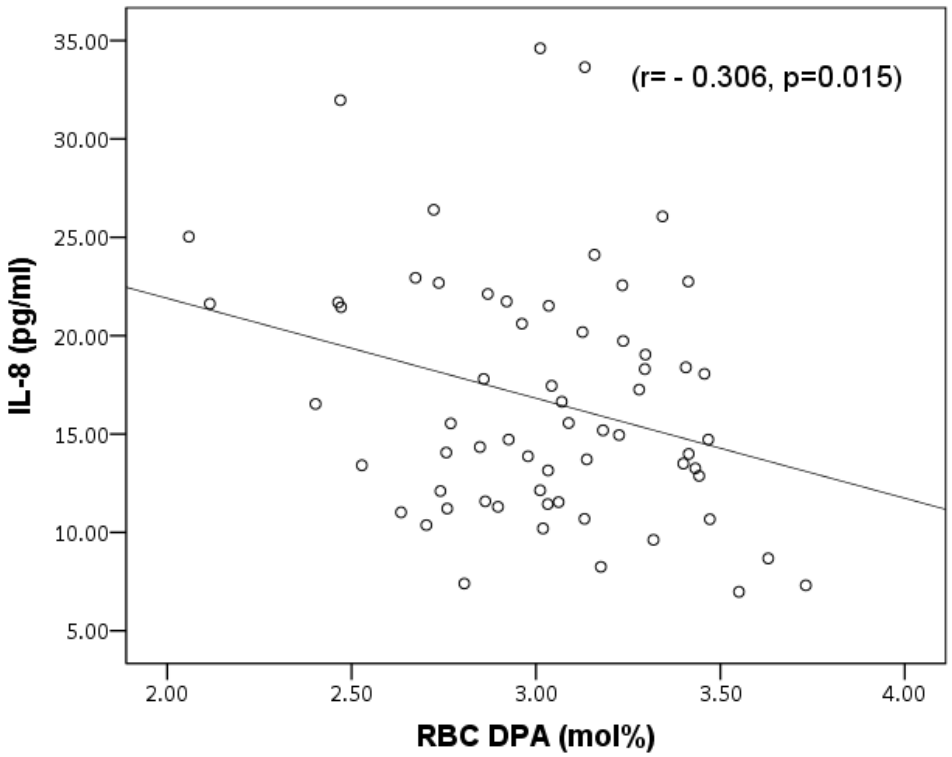


Figure 5-12 Correlations between IL-8 and red blood cell DPA in the 63 patients with NAFLD included in the INSYTE study.

5.3.10.2 Relationship of red blood cell omega-3 fatty acids with IL-8 after adjusting for sex and age

Table 5 14 shows linear regressions between IL-8 and omega-3 fatty acids and omega-3 index after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. In the 63 patients included in the INSYTE study, DPA was a statistically significant predictor of IL-8; there was a negative linear relationship (β= -0.016, P= 0.019) between IL-8 and DPA; the beta coefficient shows that for every percent increase in DPA in red blood cells, IL-8 will decrease by 0.016 (pg/ml). However, EPA, DHA and omega-3 index showed no significant relationships with IL-8. In the 105 patients with NAFLD in the WELCOME study there were no significant relationships between IL-8 and EPA, DPA, DHA and omega-3 index.

Table 5-14 Relationship of red blood cell omega-3 fatty acids with IL-8 after adjusting for sex and age

Interleukin-8 (IL-8)				
	INSYTE study (n= 63)		WELCOME study (n= 104)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.006	0.464	0.002	0.292
RBC DPA	-0.016	0.030	0.002	0.555
RBC DHA	0.012	0.588	0.010	0.088
Omega-3 Index	-0.006	0.832	0.016	0.089
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between IL-8 and each fatty acid was performed adjusting for age and sex				

5.3.11 Red blood cell omega-3 fatty acids and interleukin-10 (IL-10)

5.3.11.1 Correlation between IL-10 and red blood cell omega-3 fatty acids

In the 63 patients included in the INSYTE study, there was a negative correlation between DPA and IL-10 but this did not quite reach significance (r=- 0.230, p= 0.070). There were no significant correlations between IL-10 and EPA (r=-0.157, p=0.220), IL-10 and DHA (r=0.077, p= 0.546) and IL-10 and omega-3 index (r= 0.022, p=0.862). In the 105 patients with NAFLD in the WELCOME study, there were no significant correlations between EPA and IL-10 (r=-0.100, p=0.315) or DPA and IL-10 (r=-0.104, p=0.292) or DHA and IL-10 (r=-0.029, p=0.773) or omega-3 index and IL-10 (r= -0.042, p=0.669).

5.3.11.2 Relationship of red blood cell omega-3 fatty acids with IL-10 after adjusting for sex and age

Table 5 15 shows linear regressions between IL-10 and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. In the 63 patients included in the INSYTE study, there were no statistically linear relationships between IL-10 and any individual fatty acids or omega-3 index. In the 105 patients with NAFLD in the WELCOME study, there were negative correlations between IL-10 with DHA and omega-3 index but these did not quite reach significance (p= 0.077, p= 0.079 respectively); there were no statistically linear relationships between IL-10 with EPA and DPA. Thus, omega-3 fatty acids are not a significant predictor of IL-10.

Table 5-15 Relationship of red blood cell omega-3 fatty acids with IL-10 after adjusting for sex and age

Interleukin-10 (IL 10)				
	INSYTE study (n= 63)		WELCOME study (n= 104)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.089	0.173	-0.003	0.533
RBC DPA	-0.065	0.267	-0.001	0.309
RBC DHA	0.031	0.862	-0.003	0.077
Omega-3 Index	0.058	0.804	-0.003	0.079
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between IL 10 and each fatty acid was performed adjusting for age and sex				

5.3.12 Red blood cell omega-3 fatty acids and monocyte chemoattractant protein1 (MCP-1)

5.3.12.1 Correlation between MCP-1 and red blood cell omega-3 fatty acid

In the 63 patients with NAFLD included in the INSYTE study, MCP-1 was significantly negatively related to DPA (r=- 0.349, p= 0.005) as shown in Figure 5 13. However, there was no significant correlation between MCP-1 and red blood cell EPA (r=0.167, p= 0.191), MCP-1 and red blood cell DHA (r=- 0.192, p=0.131) or MCP-1 and omega-3 index (r=- 0.199, p= 0.118). In the 105 patients in the WELCOME study, there were no significant correlations between EPA and MCP-1 (r=-0.068, p=0.494) or DPA and MCP-1 (r=-0.006, p=0.955) or DHA and MCP-1 (r=-0.042, p=0.672) or omega-3 index and MCP-1 (r= -0.009, p=0.931).

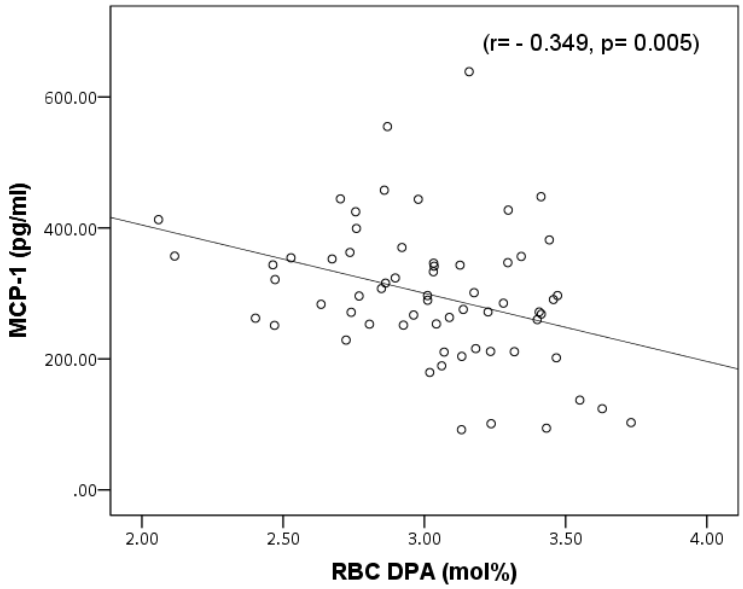


Figure 5-13 Correlations between MCP-1 and red blood cell omega-3 fatty acids in the 63 patients with NAFLD included in the INSYTE study.

5.3.12.2 Relationship of red blood cell omega-3 fatty acids with MCP-1 after adjusting for age and sex

Table 5 16 shows linear regressions between MCP-1 and red blood cell omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. DPA was a statistically significant predictor of MCP-1 in the 63 patients included in the INSYTE study; there was a negative linear relationship (β= -0.345, p= 0.007) between MCP-1 and DPA. The beta coefficient shows that for every percent increase in DPA MCP-1 will decrease by 0.345 (pg/ml). EPA, DHA and omega-3 index showed no significant relationships with MCP-1. In patients with NAFLD included in the INSYTE and the WELCOME studies, there were no significant relationships between EPA, DPA, DHA and omega-3 index.

Table 5-16 Relationship of red blood cell omega-3 fatty acids with MCP-1 after adjusting for age and sex

Monocyte chemoattractant protein1 (MCP-1)				
	INSYTE study (n= 63)		WELCOME study (n= 104)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.001	0.268	0.001	0.367
RBC DPA	-0.001	0.007	0.001	0.412
RBC DHA	-0.002	0.148	0.001	0.698
Omega-3 Index	-0.002	0.151	0.002	0.946
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between MCP-1 and each fatty acid was performed adjusting for age and sex				

5.3.13 Red blood cell omega-3 fatty acids and RANTES

5.3.13.1 Correlation between RANTES and red blood cell omega-3 fatty acids

There was no significant correlation between EPA and RANTES (r=- 0.150, p= 0.242), DPA and RANTES (r=0.102, p= 0.424), DHA and RANTES (r=-0.200, p =0.116) and RANTES and omega-3 index (r= - 0.208, p= 0.102) in patients in the INSYTE study.

5.3.13.2 Relationship of red blood cell omega-3 fatty acids with RANTES after adjusting for sex and age

Table 5-17 shows linear regressions between RANTES and red blood cell omega-3 fatty acids after adjustment for age and sex in the 63 patients with NAFLD included in the INSYTE study. There were no significant linear relationships between RANTES and any individual fatty acids or omega-3 index. There were negative relationships between DHA and RANTES (β= -0.228, p=0.079) and omega-3 index with RANTES (β= -0.238, p=0.067), but these were not quite significant. Thus, omega-3 fatty acids are not a significant predictor of RANTES.

Table 5-17 Relationship of red blood cell omega-3 fatty acids with RANTES after adjusting for sex and age

RANTES		
	Unstandardized B coefficients	*P-value*
RBC EPA	-0.004	0.091
RBC DPA	0.002	0.316
RBC DHA	-0.012	0.087
Omega-3 Index	-0.016	0.070
Data that are non-normal distributed were log transformed before analysis. Multiple linear regressions analysis between RANTES and each fatty acid was performed adjusting for age and sex		

5.3.14 Red blood cell omega-3 fatty acids and vascular adhesion molecule1 (VCAM-1)

5.3.14.1 Correlation between VCAM-1 and red blood cell omega-3 fatty acids

In the 63 patients included in the INSYTE study, there were no significant correlations between EPA and VCAM-1 (r=- 0.043, p= 0.739), DPA and VCAM-1 (r=-0.021, p= 0.871), DHA and VCAM-1 (r=0.015, p= 0.909) or omega-3 index and VCAM-1 (r=0.001, p= 0.992). In the 105 patients in the WELCOME study, there were no significant correlations between EPA and VCAM-1 (r=-0.181, p=0.066), DPA and VCAM-1 (r=0.002, p=0.982), DHA and VCAM-1 (r=0.102, p=0.302) or omega-3 index and VCAM-1 (r= 0.056, p=0.574).

5.3.14.2 Relationship of red blood cell omega-3 fatty acids with VCAM-1 after adjusting for sex and age

Table 5-18 shows linear regressions between VCAM-1 and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. There were no significant linear relationships between VCAM-1 and any individual omega-3 fatty acid or omega-3 index. Thus, omega-3 fatty acids are not a significant predictor of VCAM-1.

Table 5-18 Relationship of red blood cell omega-3 fatty acids with VCAM-1 after adjusting for sex and age

Vascular adhesion molecule1 (VCAM-1)				
	INSYTE study (n= 63)		WELCOME study (n= 104)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.008	0.941	-0.082	0.080
RBC DPA	0.011	0.920	0.004	0.946
RBC DHA	-0.010	0.977	0.226	0.202
Omega-3 Index	-0.010	0.999	0.028	0.417
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between VCAM-1 and each fatty acid was performed adjusting for age and sex				

5.3.15 Red blood cell omega-3 fatty acids and intracellular adhesion molecule1 (ICAM-1)

5.3.15.1 Correlation between ICAM-1 and red blood cell omega-3 fatty acids

There were no significant correlations between EPA and ICAM-1 (r=- 0.009, p= 0.945), DPA and ICAM-1 (r=-0.104, p= 0.415), DHA and ICAM-1 (r=0.135, p= 0.290) or omega-3 index and ICAM-1 (r=-0.112, p= 0.381) in patients in the INSYTE study.

5.3.15.2 Relationship of red blood cell omega-3 fatty acids with ICAM-1 after adjusting for sex and age

Table 5-19 shows linear regressions between ICAM-1 and omega-3 fatty acids after adjustment for age and sex in the 63 patients with NAFLD included in the INSYTE study. There were no significant linear relationships between ICAM-1 and any individual fatty acids or omega-3 index. Thus, omega-3 fatty acids are not a significant predictor of ICAM-1.

Table 5-19 Relationship of red blood cell omega-3 fatty acids with ICAM-1 after adjusting for sex and age

intracellular adhesion molecule1 (ICAM-1)		
	Unstandardized B coefficients	p-value
RBC EPA	0.032	0.585
RBC DPA	0.088	0.489
RBC DHA	-0.002	0.186
Omega-3 Index	-0.002	0.238
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between ICAM-1 and each fatty acid was performed adjusting for age and sex		

5.3.16 Red blood cell omega-3 fatty acids and E-selectin

5.3.16.1 Correlation between E-selectin and red blood cell omega-3 fatty acids

In the 63 patients included in the INSYTE study, there was no significant correlation between EPA and E-selectin (r=- 0.031, p= 0.808), DPA and E-selectin (r=0.010, p= 0.939), DHA and E-selectin (r=- 0.176, p= 0.168) or omega-3 index and E-selectin (r=-0.148, p= 0.247). In the 105 patients with NAFLD in the WELCOME study, there were no significant correlations between EPA and E-selectin (r=-0.161, p=0.105) or DPA and E-selectin (r=- 0.106, p=0.288) or DHA and E-selectin (r=- 0.017, p=0.863) or omega-3 index and E-selectin (r=- 0.049, p=0.620).

5.3.16.2 Relationship of red blood cell omega-3 fatty acids with E-Selectin after adjusting for sex and age

Table 5-20 shows linear regressions between E-Selectin and omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. There were no significant linear relationships between E-Selectin and any individual fatty acids or omega-3 index. Thus, omega-3 fatty acids are not a significant predictor of E-Selectin.

Table 5-20 Relationship of red blood cell omega-3 fatty acids with E-Selectin after adjusting for sex and age

E-Selectin				
	INSYTE study (n= 63)		WELCOME study (n= 103)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	0.007	0.821	-0.003	0.276
RBC DPA	-0.006	0.796	-0.001	0.444
RBC DHA	-0.002	0.555	0.003	0.580
Omega-3 Index	-0.002	0.602	-0.003	0.714
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between E-Selectin and each fatty acid was performed adjusting for age and sex				

5.3.17 Red blood cell omega-3 fatty acids and P-selectin

5.3.17.1 Correlation between P-selectin and red blood cell omega-3 fatty acids

In the 63 patients included in the INSYTE study, P-selectin was significantly negatively related to DHA (r=- 0.289, p= 0.018) and omega-3 index (r= -0.319, p= 0.011) as shown in Figure 5 14 and 5-15 respectively. However, there were no significant correlation between P-selectin and red blood cell EPA (r=-0.212, p= 0.095) or between P-selectin and red blood cell DPA (r=-0.220, p= 0.083). In the 105 patients with NAFLD in the WELCOME study, there were no significant correlations between EPA and P-selectin (r=-0.118, p=0.235) or DPA and P-selectin (r=- 0.133, p=0.178) or DHA and P-selectin (r=- 0.173, p=0.079) or omega-3 index and E-selectin (r=- 0.182, p=0.065).

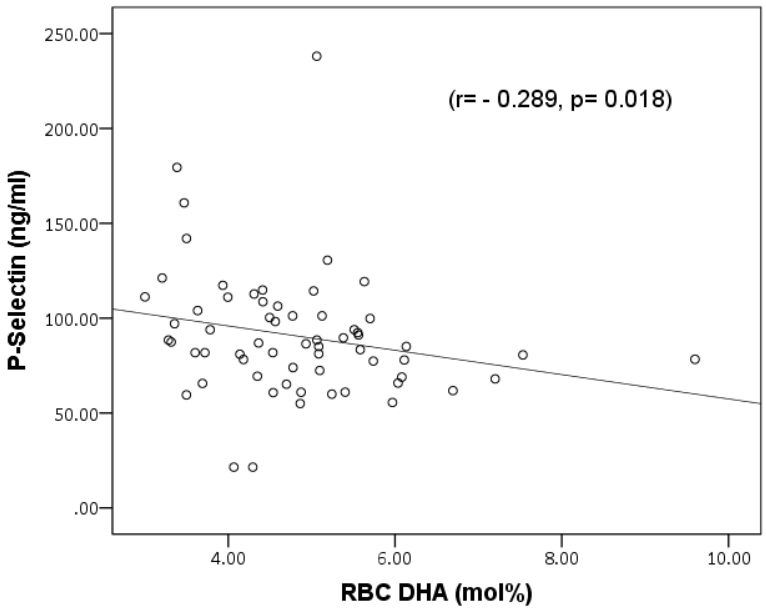


Figure 5-14 Correlation between P-selectin and red blood cell DHA in the 63 patients with NAFLD included in the INSYTE study.

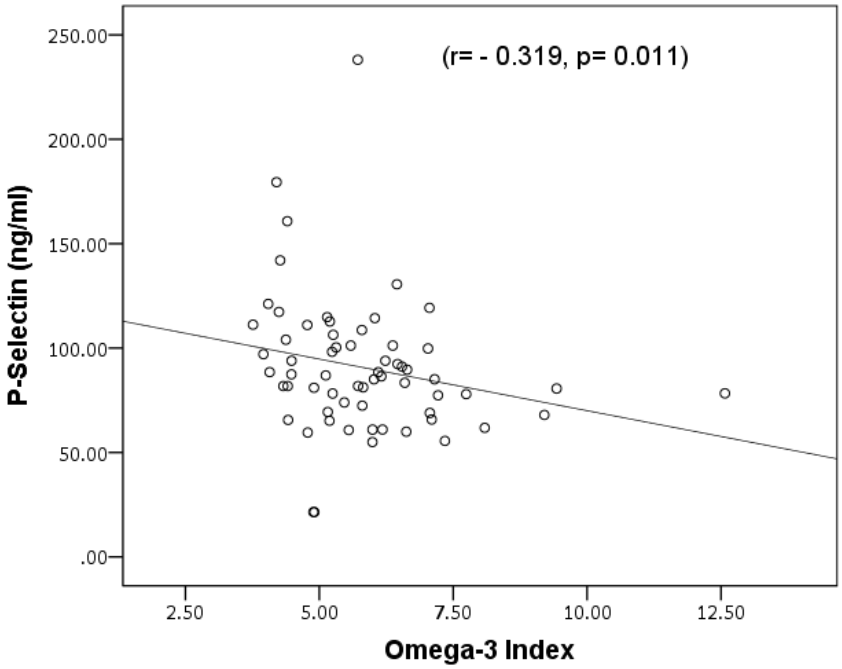


Figure 5-15 Correlation between P-selectin and red blood cell omega-3 index in the 63 patients with NAFLD included in the INSYTE study.

5.3.17.2 Relationship of red blood cell omega-3 fatty acids with P-Selectin after adjusting for sex and age

Table 5-21 shows linear regressions between P-Selectin and red blood cell omega-3 fatty acids after adjustment for age and sex in patients with NAFLD included in the INSYTE and the WELCOME studies. In the 63 patients included in the INSYTE study, there were no significant linear relationships between P-Selectin and any individual fatty acids or omega-3 index. There was a negative relationship between P-Selectin and DHA and omega-3 index (p= 0.068, p= 0.053), but this was not quit significant. Thus, omega-3 fatty acids are not a significant predictor of P-Selectin.

Table 5-21 Relationship of red blood cell omega-3 fatty acids with P-Selectin after adjusting for sex and age

P-Selectin				
	INSYTE study (n=63)		WELCOME study (n=104)	
	Unstandardized β coefficients	p-value	Unstandardized β coefficients	p-value
RBC EPA	-0.001	0.434	-0.001	0.162
RBC DPA	-0.001	0.246	-0.001	0.153
RBC DHA	-0.004	0.308	-0.006	0.068
Omega-3 Index	-0.006	0.311	-0.007	0.053
Data that were non-normally distributed were log transformed before analysis. Multiple linear regressions analysis between P-Selectin and each fatty acid was performed adjusting for age and sex				

5.4 Discussion

This project focuses on the relation between red blood cell omega-3 fatty acids and some cardiovascular risk factors in patients with NAFLD, using data from patients involved in two different studies. This chapter describes the relationship between red blood cells omega-3 fatty acids, as a measure of status of these fatty acids, and CVD risk factors (body fatness, hyperglycaemia and insulin resistance, and inflammatory markers). The red blood cell omega-3 fatty acids that were investigated were eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA) and omega-3 index (EPA+DHA). The primary cohort is patients involved in the INSYTE study, but data available from patients involved in the WELCOME study were used to confirm findings from the INSYTE patients where possible. In this chapter correlations were made between omega-3 fatty acids and body fatness, hyperglycaemia, insulin resistance, and inflammatory markers data in order to address the relationship between them. Additionally, multivariate analysis was performed to exclude any age and sex effects on the association between omega-3 fatty acids and CVD risk factors. Sex and age are considered to influence risk factors for CVD (Oyama et al., 2008, Jousilahti et al., 1999) and omega-3 fatty acid status (Bakewell et al., 2006, Childs et al., 2008, Walker et al., 2014, Rees et al., 2006); Therefore, data were adjusted for age and sex. The hypotheses of the work described in this chapter are that a) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and body mass index and waist circumference, b) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and hyperglycaemia and insulin resistance, c) that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and inflammation markers.

In this study correlations were made between inflammatory markers and BMI, waist circumference, and insulin resistance in the patients in INSYTE study. As a novel finding in NAFLD, BMI had a significant positive relationship with TNF-α, IL-6, RANTES and P-Selectin. Also waist circumference had positive significant relationships with TNF-α, IL-6, IL-8, RANTES and P-Selectin and insulin resistance had positive significant relationships with TNF-α, IL-8 and P-Selectin.

In this study the correlation between omega-3 fatty acids and BMI was assessed. There was a negative correlation between DHA and omega-3 index with BMI in the patients in INSYTE study; after adjusting for age and sex, DHA and omega-3 index were statistically significant predictors of BMI. Furthermore, this relationship was also seen in the WELCOME study cohort, confirming the findings from the INSYTE patients, although the effect was weaker and not quite significant.

Also, in this study the correlation between omega-3 fatty acids and waist circumference was assessed. There was a negative correlation between DHA and omega-3 index and waist circumference in the patients in the INSYTE study, and after adjusting for age and sex, DHA and omega-3 index were statistically significant predictors of waist circumference. In the WELCOME cohort, the crude correlation was also seen but the significance was lost after adjusting for age and sex.

Thus, the first hypothesis that omega-3 fatty acid status will have a negative relationship with body mass index and waist circumference in patients with NAFLD can be accepted in part. This is a novel finding in NAFLD patients.

The reasons behind the negative correlations between adiposity with DHA and omega-3 index are not entirely clear. One possibility is that DHA has biological effects that limit body fatness. There are two possible mechanisms for this. First, DHA is known to increase fatty acid oxidation in liver, adipose tissue, and small intestine. Second, DHA is known to inhibit hepatic lipogenesis. Both these processes shift the balance of fatty acid metabolism toward oxidation rather than storage. Further, DHA is also known to induce mitochondrial biogenesis (Kalupahana et al., 2011) which would promote oxidative processes.

In this study, the relationships between omega-3 fatty acids and omega-3 index with glucose, insulin, and insulin resistance were assessed. A variety of mechanisms could link higher omega-3 fatty acid status to less insulin resistance that would, in turn, be seen by improvements in blood glucose and insulin concentrations. Omega-3 fatty acids decrease inflammation, and inflammation induces insulin resistance. Alternatively omega-3 fatty acids could have beneficial effects on adipokine secretion from adipose tissue (Kim and Shin, 2014) (Kalupahana et al., 2011). For example, omega-3 fatty acids increase plasma adiponectin levels in obese humans (Flachs et al., 2006) , which could be a potential mechanism by which EPA and DHA improve insulin sensitivity (Flachs et al., 2006). They also induce leptin and visfatin secretion and reduce the expression of several proinflammatory cytokines from the adipose tissue, including TNF-α, IL-6, MCP-1, and PAI-1. Current evidence suggests that these anti-inflammatory actions of EPA and DHA play a major role in their insulin-sensitizing effects (Kalupahana et al., 2011).

In the current study, there were no significant correlations or linear relationships between glucose with any individual fatty acids or omega-3 index in the patients in the INSYTE or WELCOME studies. Furthermore, there were no statistically correlations between insulin with any individual fatty acids or omega-3 index in

patients with NAFLD included in the INSYTE study. However, in the WELCOME study there was a strong negative correlation between EPA and insulin (r= - 0.297, p=0.002), although after adjusting for age and sex, this negative association was lost.

There was a significant negative logistic relationship between DPA and insulin resistance in patients in the INSYTE study (p=0.045): the higher the DPA the less the insulin resistance. This is a novel finding. In the WELCOME study data there was a strong negative correlation between EPA, but not DPA, and insulin resistance (r= -0.242, p=0.017). However, after adjusting for age and sex, this negative association was lost.

Any relationship between DPA and insulin resistance has not previously been reported. However the absence of improvement in blood glucose or insulin resistance with EPA or DHA has been reported by many studies. For example, in the WELCOME randomised placebo controlled trial high dose purified omega-3 fatty acids (3.6 g / EPA plus DHA per day day) for 15-18 months did not improve IR (McCormick et al., 2015). Also, some studies reported increased glucose or insulin after intervention with EPA and DHA; (Mori et al., 2000) studied the effect of 4 g/day EPA or DHA for 6 weeks in overweight men aged 48.8 years and with mild hyperlipidaemia. Both EPA and DHA increased fasting insulin significantly and EPA, but not DHA, tended to increase fasting glucose. Also, Woodman et al. (2002) reported elevations of fasting glucose with 4 g/day EPA or DHA in type 2 diabetics (increases of 1.40 and 0.98 mmol/L, respectively). Also, in a clinical trial (Godeny et al., 2010) investigating the effect of 3 g/day of EPA and DHA in 40 patients with the metabolic syndrome (20 controls and 20 patients) insulin resistance was increased.

Thus, the data obtained from INSYTE and WELCOME do not support the second hypothesis, that there is negative relationship between omega-3 status and insulin resistance in patients with NAFLD. Moreover, the data do not support that there is relationship between omega-3 status and hyperglycaemia. Thus, this hypothesis must be rejected.

In this chapter correlations were made between omega-3 fatty acids and a number of inflammatory markers (CRP, TNF-α, IL-6, IL-8, IL-10, MCP-1, RANTES, VCAM-1, ICAM-1, E-Selectin, and P-selectin). According to (Kalupahana et al., 2011) omega-3 fatty acids have anti-inflammatory effects. Production of proinflammatory cytokines by macrophages is dependent on activation of the NF-κB and JNK pathways. EPA and DHA bind to G protein-coupled receptor GPR 120, and inhibit NF-κB and JNK, reducing this response (Talukdar et al., 2010). Moreover, resolution of acute inflammation is an active process essential for appropriate host responses, tissue protection and the return to homeostasis. Recent evidence has highlighted the role of EPA and DHA in this process, through mechanisms involving EPA-derived resolvin E1 and DHA-derived protectin D1 (Schwab et al., 2007). In addition, transgenic restoration of omega-3 fatty acids and protectin D1 prevents the high-fat-diet–induced insulin resistance, highlighting the important role of this DHA derivative (White et al., 2010). It is possible that EPA and DHA supplementation reduces production of arachidonic acid derived eicosanoids that are involved in inflammatory processes (Kalupahana et al., 2011).

In the patients in INSYTE study, there was a negative significant relationship ($p=0.038$) between DHA and the high level (≥ 5 mg/L) of CRP independently of sex and age. In contrast to the negative relationship between DHA and CRP; DPA had a positive correlation with CRP. This significant positive relationship got stronger after multivariate linear regression analysis adjusted for age and sex. The beta coefficient shows that for every percent increase in DPA, CRP will increase by 0.331 mg/L. Furthermore, after multivariate logistic regression DPA had a positive

correlation with the high level (≥ 5 mg/L) of CRP. However, these interesting observations could not be confirmed using data from the WELCOME study. Nevertheless, the findings from INSYTE suggest that DPA and DHA may have different effects on inflammation. This deserves further research. Indeed, according to (Dyall, 2015) there has been a lack of discrimination between the different omega-3 fatty acids and effects have been broadly accredited to the series as a whole. The differences in the effect between DPA compared to DHA in NAFLD patients in INSYTE is a novel finding. However since it could not be confirmed in WELCOME dataset the finding may not be robust. Certainly it is worth further investigation. One study conducted in healthy individuals: (Skulas-Ray et al., 2015) examined associations between pre-supplementation RBC omega-3 DPA content and markers of inflammation (IL-6, TNF-α, and CRP) in healthy young adults (n= 115) and in individuals with elevated triglycerides (n= 28). They found an inverse correlation between baseline CRP concentrations and DPA only in the population of healthy adults.

In the current study there were no significant relationships between any individual omega-3 fatty acids with IL-6, IL-10, and E-Selectin in either the INSYTE or the WELCOME cohorts. Also, there were no significant relationships with ICAM-1 in the INSYTE cohort; in the WELCOME study there were no data for ICAM-1.

Also in the patients with NAFLD included in the INSYTE study, DPA had a negative correlation with IL-8 and MCP-1, which remained significant in multivariate analysis independently of sex and age. However, these relationships were absent in the WELCOME study data. In addition, EPA and DHA had negative relationships with TNF-α, RANTES, P-selectin and VCAM-1, although these relationships did not reach the significance in both the INSYTE and the WELCOME cohorts.

Nevertheless, this inverse association between omega-3 fatty acids and some inflammatory markers is supported by many cohort studies that report inverse associations between dietary intake of EPA + DHA or blood concentrations of EPA and DHA and the concentrations of a range of inflammatory markers, including cytokines, adhesion molecules, and acute phase proteins (Calder, 2015b). In 152 elderly men with high risk for coronary heart disease Yli-Jama et al. investigated the relation between EPA and DHA in the NEFA fraction and biochemical markers of endothelial activation or dysfunction (VCAM-1, ICAM-1 and E-Selectin). They found that the inverse relation between EPA and DHA and VCAM-1 might indicate that these fatty acids have an anti-inflammatory effect on the vascular endothelium, but they saw no effect on sICAM-1 or E-Selectin (Yli-Jama et al., 2002).

The significant negative relationships between omega-3 fatty acids and inflammatory markers were found in NAFLD patients included in the INSYTE cohort more so than in NAFLD patients included in the WELCOME cohort. This may reflect some difference in the phenotype of the individuals, or be related to differences in the sample size.

Thus, the data obtained overall give some support of the third hypothesis, that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and inflammation markers.

5.5 Summary and conclusion

This chapter describes the relationship between the status of omega-3 fatty acids in red blood cells and selected CVD risk factors (body fatness, hyperglycaemia and insulin resistance, and inflammatory markers). Studies provide substantial evidence of the role of adiposity, insulin resistance, and increasing inflammation in CVD risk and CVD outcomes. In this study, in patients with NAFLD several significant associations of omega-3 status with body fatness, insulin resistance, and some inflammatory markers were seen, but in multivariate analysis controlling for age and sex, only some of these associations remained significant and others did not, although strong trends remained in some cases. Higher body mass index and waist circumference were associated with increasing TNF-α, IL-6, IL-8, RANTES and P-Selectin. Higher insulin resistance was associated with increasing TNF-α, IL-8 and P- Selectin. These findings confirm those seen in other groups of patients that obesity (adiposity) and insulin resistance are linked to higher inflammation.

Higher red blood cell DHA and omega-3 index were both associated with lower body mass index and waist circumference. Higher red blood cell DPA was associated with lower insulin resistance in some participants. Higher red blood cell DHA and DPA (and in some cases EPA and omega-3 index) were associated with lower inflammatory markers like IL-8, MCP-1, VCAM-1, and P-Selectin; also, there were trends toward negative relationship with TNF-α and RANTES. There was an opposing effect of DHA and DPA on CRP. While DHA had a negative significant association with CRP, DPA had a positive significant association with CRP.

To conclude, in patients with NAFLD a higher omega-3 status is associated with lower body mass index, lower waist circumference, and lower levels of some inflammatory markers. This may contribute to reduced CVD risk. However in patients with NAFLD there seems to be no strong relationship between omega-3 fatty acids and insulin resistance.

Chapter 6: **Final discussion**

This project focused on the relation between red blood cell omega-3 fatty acids and cardiovascular risk factors in people with non-alcoholic fatty liver disease (NAFLD). The primary cohort studied was patients involved in the INSYTE study, but data available from patients involved in the WELCOME study were used to confirm findings from the INSYTE patients. The status of omega-3 fatty acids was determined by the level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and docosapentaenoic acid (DPA) in red blood cells. The sum of EPA plus DHA in red blood cells, termed the "omega-3 index" is an increasingly recognised marker of long term omega-3 fatty acid intake and has been shown to be related to tissue (e.g. heart) omega-3 fatty acids (Harris et al., 2004) and to risk of cardiovascular disease (Harris and von Schacky, 2004). Cardiovascular disease (CVD) risk in the NAFLD patients was determined using history report, anthropometric measurements, blood lipids, carotid intima media thickness, insulin resistance, and inflammatory markers. The novelty of this research was to identify, for the first time in patients with NAFLD, whether status of omega-3 fatty acids is related to the cardiovascular risk profile. Patients with NAFLD are known to be at increased risk of CVD (Bhatia et al., 2012a, Byrne and Targher, 2015) but they show variation in this risk and in the underlying risk factors. The novel aim of this research was to determine the extent to which the variation in risk factor profile and in risk could be accounted for by variation in omega-3 fatty acid statuss. This project remains timely, since NAFLD affects 20 to 30% of the adult population, its prevalence is increasing, and the discovery of factors to lower risk of development of CVD in patients with NAFLD will be important.

The overarching hypothesis of this work is that "a higher omega-3 status is associated with a better cardiovascular risk factor profile in patients with NAFLD". The research hypotheses being examined in this study are that:

1- NAFLD patients have elevation (compared with what is regarded as normal or healthy) of the following cardiovascular risk factors: body fatness, blood pressure, blood lipids, insulin resistance, inflammatory markers, carotid intima-media thickness and cardiovascular risk score, but that this will vary among the patients.

2 - NAFLD patients have a low omega-3 status. Age and sex will influence omega-3 fatty acid status in patients with NAFLD (increased with age and higher in women).

4 - Within patients with NAFLD a higher the omega-3 fatty acid status will be associated with lower cardiovascular risk: body fatness, blood pressure, blood lipids, insulin resistance, inflammatory markers, carotid intima-media thickness and cardiovascular risk score.

A total of 168 patients with NAFLD were studied. These were 63 patients involved in a randomised controlled trial of a synbiotic intervention (INSYTE study) and the measurements presented were made at baseline in this trial. Moreover, baseline data of 105 NAFLD patients involved in the WELCOME study, a randomised controlled trial of omega-3 fatty acids, were used to confirm findings from the INSYTE patients.

6.1 Summary of research findings

6.1.1 Omega-3 fatty acid status in NAFLD patients

To my knowledge, this is the first study to report the three bioactive omega-3 fatty acids (EPA, DPA and DHA) in red blood cells of patients with NAFLD. EPA, DPA and DHA were 0.9%, 3.0% and 4.7% of total fatty acids and were very similar for patients in both INSYTE and WELCOME studies. Omega-3 index was very similar between the INSYTE and WELCOME cohorts. The index is not especially low compared with other groups of individuals but is low in relation to estimated CV

risk. The results presented in this thesis reject the hypotheses that the NAFLD participants recruited into INSYTE and WELCOME studies have low omega-3 status. Median omega-3 index was 5.5 in both cohorts; this is a level indicating medium risk (omega-3 index 4.1 to 7.9) of CVD; and only 3.2% of patients had an omega-3 index associated with a low CV protection (index $\leq$ 4). However, only 6% of patients had an omega-3 index associated with a high level of CV protection (index $\geq$ 8). This finding indicates that patients with NAFLD do not have a high cardioprotection level of omega-3 fatty acids. In general, this is consistent with the calculated 10-year CV risk.

In both cohort, EPA and DHA and EPA and DPA were strongly related; however there was no relationship between DPA and DHA. EPA and DHA may be strongly related because they have a common dietary source. Although DPA is also found in the diet, it is commonly described that DPA is formed from EPA by elongation (see Figure 1-8). Thus EPA and DPA may be associated because of this metabolic relationship. It is also well described that DPA is not well converted to DHA. Thus, the absence of a clear metabolic relationship between DPA and DHA may explain the lack of an association between these two fatty acids, as observed here.

In the NAFLD patients in this study, age was one of the determinants of EPA, DHA, and omega-3 index but not a determinant of DPA, with higher status at higher age. This is the first time that this has been reported in patients with NAFLD. This confirms the previous finding of a positive correlation between EPA and DHA in red blood cells and age (Walker et al., 2014). Some previous studies have reported higher omega-3 fatty acid status of adipose tissue (Bolton-Smith et al., 1997) and of plasma phospholipids and white blood cells (Rees et al., 2006) in older compared with younger healthy subjects. This may reflect dietary differences between older and younger people such as higher fish intake in older people. However, there may be other explanations. For example, older people may be less

physically active and so may oxidise less fatty acids in general, leaving more omega-3 fatty acids for incorporation into cells and tissues. Body composition differences with ageing may also play a role; for example less lean mass may spare omega-3 fatty acids for incorporation into cell membranes elsewhere. There are likely to be metabolic changes with ageing that might be important in determining partitioning, utilisation or retention of omega-3 fatty acids at different sites in the body. In this regard, a recent study using stable isotopes demonstrated that EPA is metabolised differently between young and old men (Léveillé et al., 2017).

In this study there was no link between sex and omega-3 fatty acid status, i.e., sex was not one of the determinants of omega-3 status in NAFLD patients. This contrasts with some other studies that reported higher omega-3 fatty acid status in females compared to males (Bakewell et al., 2006, Childs et al., 2012, Lohner et al., 2013, Childs et al., 2008, Walker et al., 2014). Some human studies using stable isotopes show that females have a higher ability to synthesise EPA and DHA from alpha-linolenic acid than males (Burdge and Wootton, 2002, Burdge et al., 2002). However, most of these studies, including the latter two using stable isotopes, included only young adults, rather than older people. The absence of sex effects in the NAFLD patients studied here may be due to the older age of these patients; the average age of patients included in both INSYTE and WELCOME studies was 51 years with a wide range (21 to 76 years across the two studies). It will be important to more systematically study EPA and DHA synthesis from α-linolenic acid according to age.

6.1.2 CVD risk factors in NAFLD patients

Overall, the results presented in this thesis from both cohorts confirm the hypothesis that NAFLD patients have elevation (compared with what is regarded as normal or healthy) of the following cardiovascular risk factors: body fatness, blood pressure, blood lipids, insulin resistance, inflammatory markers, and cardiovascular

risk score, but that the extent of elevation varies among the patients. Although no formal statistical comparison was made, findings were very similar for all outcomes between the INSYTE and WELCOME cohorts. Most of the NAFLD patients recruited (with mean liver fat of 27% and 23% in the INSYTE and WELCOME studies, respectively) had metabolic syndrome (body fatness with at least two out of three of dyslipidaemia, hypertension, and hyperglycaemia). Furthermore most had a BMI indicating obesity, a waist circumference indicating excess abdominal fat, and high systolic blood pressure. Many had high blood cholesterol, triglycerides and glucose and a high cholesterol to HDL-cholesterol ratio. According to HOMA-IR more than two-thirds of the patients had moderate or severe insulin resistance. More than one third of the participants in INSTYE and around half of the WELCOME participants had low grade inflammation according to CRP concentration. Therefore, the NAFLD participants recruited into INSYTE and WELCOME have increased CV risk, but the individual factors contributing to CV risk vary among the patients. Mean 10-year CV risk, calculated using the Framingham General Cardiovascular Risk Score, was 10.2 and 11.8% among patients in the INSYTE and WELCOME studies, respectively. However, diastolic blood pressure was not elevated in either cohort and carotid intima media thickness was normal. 60% of the participants in INSYTE and 30% in WELCOME were being treated for diabetes, 50% for hypertension in both INSYTE and WELCOME, and 33% and 50% for hyperlipidaemia in INSYTE and in WELCOME, respectively. This profile among the 168 NAFLD patients studied here supports that NAFLD is associated with other diseases such as hypertension, hyperlipidaemia, type-2 diabetes, and metabolic syndrome. According to British Heart Foundation Statistics having diabetes can double the risk of developing CVD. CIMT is reported to significantly increase with age (Lim et al., 2008, Kotani et al., 2005). This was confirmed in the current study in patients with NAFLD and probably simply represents the progressive buildup of atherosclerotic plaques over time. NAFLD is reported to be associated with increased CIMT (Mishra et al., 2013). However, in the participants in both INSYTE and WELCOME studies the CIMT values were normal (this finding was previously

published for the WELCOME study participants (McCormick et al., 2015). It is possible that the normal CIMT is related to use of lipid lowering medications among these participants.

Overall participants involved in INSYTE and WELCOME are at high metabolic and cardiovascular risk. The identified characteristics of NAFLD patients involved in INSYTE and WELCOME are consistent with what other investigators have reported about the characteristics of NAFLD participants. For example, others have reported the association of NAFLD with diseases like hypertension, diabetes, hyperlipidaemia, metabolic syndrome (Ahmed et al., 2012, Wild and Byrne, 2005) and that NAFLD is associated with increasing CV risk (Ahmed et al., 2012, Anonymous, 2010).

6.1.3 Relationships between omega-3 fatty acids and CVD risk factors in NAFLD patients

To my knowledge, no prior study has related omega-3 fatty acid status to CVD risk in patients with NAFLD without increasing the oral intake of omega-3 fatty acids. The majority of the studies in the research field investigate the effect of increased omega-3 intake on NAFLD with different dose and duration of intervention. However, this study investigated the actual state of omega-3 fatty acids in patients with NAFLD without any intervention.

The results presented in this thesis only partly confirm the hypothesis that a higher omega-3 fatty acid status will be associated with lower cardiovascular risk in patients with NAFLD. A higher omega-3 status was associated with lower body mass index, lower waist circumference, and lower levels of some inflammatory markers. This may contribute to reduced CVD risk in these patients. Differences in omega-3 fatty acid status have modest, if any, effects on blood lipids, blood

pressure, CIMT, insulin resistance and 10-year cardiovascular risk in patients with NAFLD. Any effects of omega-3 fatty acids may be masked by medication use to control risk factors or by the small sample size of both cohorts.

Omega-3 fatty acid status was not associated with blood lipids in this study. Only DPA had a negative relationship with TG and then only in patients in the WELCOME study. This lack of association of omega-3 fatty acids and blood lipids partly agrees with findings of (Mori et al., 2000) who showed that omega-3 supplementation (either EPA or DHA) in overweight men with mild hyperlipidaemia did not affect total cholesterol concentration. Furthermore, LDL and HDL cholestetol concentrations were not affected by EPA, while DHA increased LDL cholesterol concentration (Mori et al., 2000). It may be that omega-3 fatty acid intake is too low in the current study to impact on lipoprotein metabolism. Also many patients were taking medicationsto control blood lipids and the pharmacological effect of these medications may overcome the more mild influence of omega-3 fatty acids.

Overall there were no relations between omega-3 fatty acids and systolic and diastolic blood pressure in the NAFLD participants; this lack of relationship was similar in the INSYTE and WELCOME cohorts. However, consistent with my hypothesis, EPA, DHA and omega-3 index had a negative relationship with systolic blood pressure in hypertensive participants independently of sex and age in the INSYTE study participants. This supports the findings in NAFLD patents of the WELCOME study where 3.6 g EPA plus DHA per day for 15-18 months was able to lower systolic (and diastolic) blood pressure (Scorletti et al., 2015). Thus omega-3 fatty acids may be used to improve blood pressure in hypertensive NAFLD patients. Other studies have shown that the blood pressure lowering effect of omega-3 fatty acids is more commonly seen in hypertensive and older subjects (Morris et al., 1993, Harris et al., 2008, Galassi et al., 2006).

Omega-3- fatty acids status was not associated with cardiovascular 10-year risk score. Findings were very similar for both the INSYTE and WELCOME cohorts. In a published paper from the WELCOME study, it was concluded that high dose of omega-3 fatty acid treatment did not improve measures of microvascular function or vibration perception, but they did not present the effect of omega-3 fatty acids on CVD risk score (McCormick et al., 2015). Also omega-3 fatty acid status was not associated with CIMT in patients with NAFLD; findings were very similar for both the INSYTE and WELCOME cohorts. In a study of healthy Mexican women there were no relation between omega-3 fatty acid status and CIMT (Monge et al., 2016). However, in previously published paper from the WELCOME study, it was reported that 3.6 g EPA plus DHA per day for 15-18 months lead to reduction in NAFLD severity and this was associated with reduced CIMT progression (Bhatia et al., 2016). It may be that the intake of omega-3 fatty acids in the habitual diet is not sufficient to influence the factors related to CIMT thickness, but that a higher level of supplementation does affect the factors related to CIMT progression.

Overall the data obtained from INSYTE and WELCOME do not support the hypothesis that there is negative relationship between omega-3 status and insulin resistance in patients with NAFLD. The absence of improvement in blood glucose or insulin resistance with EPA or DHA has been reported by many studies. For example, in the WELCOME randomised placebo controlled trial high dose purified omega-3 fatty acids (3.6 g / EPA plus DHA per day day) for 15-18 months did not improve IR (McCormick et al., 2015). Also, some studies reported increased glucose or insulin after intervention with EPA and DHA; (Mori et al., 2000) studied the effect of 4 g/day EPA or DHA for 6 weeks in overweight men aged 48.8 years and with mild hyperlipidaemia. Both EPA and DHA increased fasting insulin significantly. Also, in a clinical trial (Godeny et al., 2010) investigating the effect of 3 g/day of EPA and DHA in 40 patients with the metabolic syndrome (20 controls and 20 patients) insulin resistance was increased.

The data obtained from both INSYTE and WELCOME cohorts support the hypothesis that patients with NAFLD will have an inverse relationship between omega-3 fatty acid status and body mass index and waist circumference. Higher red blood cell DHA and omega-3 index were both associated with lower body mass index and waist circumference. The observation of the inverse association between omega-3 fatty acids and adiposity is consistent with the findings of (Thorsdottir et al., 2007) and (Kunesova et al., 2006) who reported reduction in body weight, and BMI with omega-3 fatty acid intervention in combination with exercise or energy restriction. There are two possible mechanisms for this. First, DHA is known to increase fatty acid oxidation in liver, adipose tissue, and small intestine. Second, DHA is known to inhibit hepatic lipogenesis. Both these processes shift the balance of fatty acid metabolism toward oxidation rather than storage. Further, DHA is also known to induce mitochondrial biogenesis (Kalupahana et al., 2011) which would promote oxidative processes. It is also important to consider an alternative explanation, a dilution effect. More obese individuals have greater capacity to remove DHA from the blood and store it. Thus, more obese people might have lower blood omega-3 fatty acids. In other words the relationship that is observed in the current study may be due to the adiposity mass in obese people, rather than any real physiological impact of omega-3 fatty acids on adiposity. However, some previously published findings reported that omega-3 fatty acid intervention decreased adiposity. For example, Thorsdottir at al. found that body weight and waist circumference decreased after 8 weeks of 1.3 g/day of EPA and DHA with an energy restricted diet (Thorsdottir et al., 2007). Also, (Kunesova et al., 2006) reported decreased body weight and BMI with omega-3 fatty acid intervention. These findings together with those from present study, suggest that DHA may have a metabolic effect that results in less adipose tissue in patients with NAFLD.

The data obtained overall give some support of the hypothesis that patients with NAFLD will have a negative relationship between omega-3 fatty acid status and

inflammation markers. Higher red blood cell DHA and DPA (and in some cases EPA and omega-3 index) were associated with lower inflammatory markers like IL-8, MCP-1, VCAM-1, and P-Selectin; also, there were trends toward negative relationship with TNF-α and RANTES. This finding agrees with many cohort studies that report inverse associations between dietary intake of EPA + DHA or blood concentrations of EPA and DHA and the concentrations of a range of inflammatory markers, including cytokines, adhesion molecules, and acute phase proteins (Saravanan et al., 2010, Yli-Jama et al., 2002, Calder, 2015, Masterton et al., 2010).

Correlations were found between inflammatory markers and BMI, waist circumference, and insulin resistance in the patients in INSYTE study. As a novel finding in NAFLD, BMI had a significant positive relationship with TNF-α, IL-6, RANTES and P-Selectin. Also waist circumference had positive significant relationships with TNF-α, IL-6, IL-8, RANTES and P-Selectin and insulin resistance had positive significant relationships with TNF-α, IL-8 and P-Selectin. There has been no previous study that looked at this relationships in patients with NAFLD. The findings suggest that both body fatness and insulin resistance are related to inflammation in NAFLD. Previous studies had reported that obese people have higher blood concentration of several pro-inflammatory mediators like TNF-α, IL-6, IL-8, CRP and MCP-1 compared to normal weight people (Mueller et al., 2002) (Calder, 2015a). Kim et al. conducted a study in 100 subjects (50 overweight and obese (BMI ≥ 25 kg/m^2) and 50 normal weight (BMI < 25 kg/m^2) and found a positive relationship between BMI and waist circumference and circulating concentrations of CRP and other inflammatory markers (Kim et al., 2006). Previous studies had reported that adiposity is strongly associated with low-grade systemic inflammation inducated by CRP, TNF-α and IL-6 concentrations(Thorand et al., 2006).

The reason for lack of agreement in some findings between the INSYTE and the WELCOME cohorts (e.g. for blood lipids, systolic blood pressure, and some inflammatory markers) or between this study and other studies is not immediately clear but may relate to sample size. For example, the WELCOME cohort was larger. Also, it may relate to different use of medications among the participants in the two cohorts; for example, in the WELCOME cohort around 50% of patients were using lipid lowering medications, while in the INSYTE cohort only 30% of patients were using lipid lowering medications. Medication use may remove any relation between omega-3 and the risk factors being studied, such as blood lipids, blood pressure, CIMT, insulin resistance and 10-year cardiovascular risk score.

Moreover, the differences in the results may be explained by the presence of genetic (i.e. genotype) predisposition to omega-3 fatty acid-risk marker interaction (Madden et al., 2011). For example, in a previously published paper from the WELCOME study, there were genetic variations in both patatin-like phospholipase domain-containing protein-3 (PNPLA3) (I148M) and the transmembrane 6 superfamily member 2 protein (TM6SF2) (E167K) that influenced red cell DHA enrichment during the trial (Scorletti et al., 2015) and this has not been accounted for in the present study.

6.2 Study strengths

This study has several strengths.

The determination of omega-3 fatty acid status was made by analysing the fatty acid composition in red blood cells. This will reflect dietary omega-3 fatty acid intake averaged over the red blood cell lifespan (up to 120 days), thus representing a long period of exposure. In contrast, plasma concentrations of fatty acids, as frequently used, reflect the intake over only the last few days to weeks. Also, red

blood cell omega-3 fatty acids have been shown to be highly correlated with omega-3 fatty acid concentrations in tissues such as the heart (Harris et al., 2004), suggesting that this is a physiologically meaningful measurement. Furthermore, omega-3 fatty acids in red cells (omega-3 index) have been associated with CV risk (Harris et al., 2004), suggesting that this is also a clinically meaningful measurement. Also, omega-3 fatty acids in the red blood cell are less affected by any temporary changes in the diet or health than other blood lipid fractions (Harris and Thomas, 2010). In addition, this study examined the associations of red blood cell DPA as well as EPA and DHA; few studies have examined red blood cell DPA, but DPA may have important biological actions (Kaur et al., 2011).

This project depended on the baseline data of the INSYTE and WELCOME cohorts, which reflects the actual state of omega-3 fatty acids in the body as a result of habitual diet and metabolic processes. The majority of studies in the research field investigate the effect of increasing omega-3 fatty acid intake on fatty acid status and on the different aspects of health; in this setting there are many factors that may have an impact in the individual response to the same dose of omega-3 fatty acids (like age, sex, genotype, compliance). In addition, by using the baseline data, a placebo effect is avoided.

Another strength of this study is that many cardiovascular risk factors were assessed: body weight, body mass index , waist circumference, systolic and diastolic blood pressure, fasting blood lipid concentrations (total cholesterol, HDL-cholesterol and triglycerides and calculated LDL-cholesterol), fasting blood concentrations of insulin and glucose and calculation of insulin resistance, presence of the metabolic syndrome, fasting blood concentrations of inflammation markers (CRP, TNF-α, IL-6, IL-8, IL-10, MCP-1, RANTES , VCAM-1, ICAM-1, E-Selectin, and P-Selectin), carotid intima-media thickness and cardiovascular risk

score. Thus, a full picture of the relation between omega-3 fatty acids in red cells and CV risk could be obtained.

Another strength is that the analysis controlled for effects of age and sex both of which affect many of the risk factors measured and affect overall CV risk. This adjustment makes the findings from the study more robust.

A final strength is that two cohorts were compared to see how reproducible any findings were. The INSYTE cohort was the primary cohort under study but availability of data from the WELCOME cohort enabled a second, confirmatory, examination of the relatiosnhips under study. Importantly the characteristics of the two cohorts, including omega-3 fatty acid status and the various cardiovascular risk factors, were very similar

6.3 Challenges and limitations

As with any research involving human participants, there were some challenges and limitations to this project. There was some difficulty in recruitment into the INSYTE study and this reduced the number of participants recruited in to my cohort (to 63) and restricted this project to analysis of baseline data. To mitigate the effect of this, the baseline data from the WELCOME study for 105 NAFLD participants was also used. However, a methodological limitation of this project is the relatively small sample size for this type of analysis. As a result at least some of the analyses are likely to be underpowered, in particular for the more variable outcomes such as inflammatory markers, running a risk of type 2 error (false negative findings).

More than two-thirds of the patients in both INSYTE and WELCOME cohorts were using medications to control CV risk factors. Although this represents the profile of typical patients with NAFLD and high risk of cardiovascular disease, it may have

masked effects of omega-3 fatty acids. In addition there were differences in the profile of medication use between the participants in the two cohorts and this may lead to lack of agreement in some findings between the INSYTE and the WELCOME cohorts.

6.4 Interpretations and recommendations

Taking all the results together, the NAFLD patients studied here, who would be entirely representative of such patients in the UK and in many other countries, have a normal omega-3 fatty acid status compared to other comparable adult populations. The median omega-3 index was 5.5 in both cohorts which indicate an omega-3 status conferring medium cardio-protection. Nevertheless, the NAFLD patients studied here have an increase in CVD risk and this increase in risk varies among them. The associations investigated indicate that among NAFLD patients, the higher the omega-3 fatty acid status the lower is the CV risks according to some risk markers. However, some of the common CV risk factors, such as blood lipids, were not related to omega-3 fatty acid status. The actual omega-3 index of the NAFLD patients is too low in the current study to impact on some of the CV risk factors and so to reduce the CV risk. According to Harris and von Schacky (2004) omega-3 index has to be ≥ 8 to provide high cardioprotection. Therefore, the current study supports that a high omega-3 index is necessary to provide high cardio protection in patients with NAFLD (see Figure 6-1). Further investigation is needed of the effect of increasing omega-3 fatty acid intake and status on cardiovascular risk factors and risk in patients with NAFLD. In the WELCOME study 3.6 g EPA+DHA per day for up to 18 months significantly decreased blood TG concentrations and blood pressure (Scorletti et al., 2015). In the study on the prevention of coronary atherosclerosis by intervention with marine omega-3 fatty acids (SCIMO) (Von Schacky et al., 1999) omega-3 fatty acids (3 g EPA + DHA/day for 3 months followed by 1.5 g/day for 18 months) increased the omega-3 index from baseline values of 3.4% to 8.3% ($P < 0.05$) after 2 years on trial.

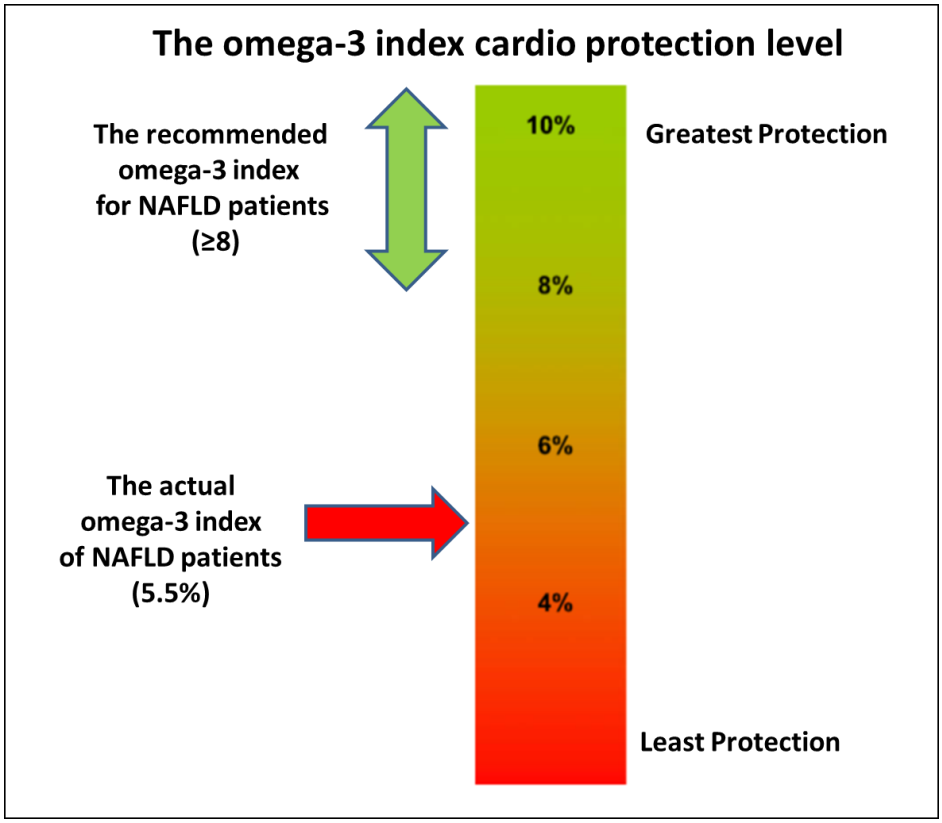


Figure 6-1 The recommended omega-3 index for the NAFLD patients. (the green arrow in the left) as optimal cardio protection level according to the omega-3 index risk profile proposed by (Harris and von Schacky, 2004): low protection (omega-3 index ≤ 4), medium protection (omega-3 index = 4.1 to 7.9), high protection (omega-3 index ≥ 8).

The American Heart Association statement on omega-3 fatty acid use recommends an intake of at least two fish meals per week in patients without documented coronary artery disease, and supplemental therapy with 1 g per day of omega-3 fatty acids for those who have had a myocardial infarction; in patients with high triglyceride concentrations omega-3 fatty acid supplementation at a dose of 2 to 4 g per day is recommended (Kris-Etherton et al., 2002). Increasing omega-3 fatty acid consumption through foods is preferable to using supplements, but it is difficult to reach an intake of 2 to 4 g per day of EPA+DHA through foods. Fish and other seafood (especially cold-water fatty fish, such as salmon, mackerel, tuna, herring, and sardines) are good sources for omega-3 fatty acids especially EPA and

DHA. However, there is limitation of increasing the intake because some types of fish may contain high levels of mercury, dioxins, polychlorinated biphenyls and other environmental contaminants. Fortified foods (such as certain brands of eggs, yogurt, juices, and milk) may help to increase the intake of EPA and DHA, although availability of such foods is limited and the EPA and DHA content is quite low. Nuts and seeds (such as flaxseed, chia seeds, and walnuts) are sources of alpha-linolenic acid but the research conducted to date indicates that the human body can convert limited amounts into EPA and then to DHA. Therefore, it is difficult to get enough omega-3 by diet alone especially for those with disease (such as NAFLD patients). Omega-3 dietary supplements such as fish oil, krill oil, cod liver oil, and algal oil (a vegetarian source that comes from algae) provide a wide range of doses and forms, and so supplementation could be an affective option to increase the omega-3 index for NAFLD patients.

6.5 Possible future work

Firstly, because of the limited sample size of this study, a further study that is better powered should examine the omega-3 fatty acid-CV risk factor relationship studied herein. The findings identified in this thesis indicate that an exploration in a bigger dataset would be worthwhile. Sex and age have been reported to have an effect on EPA and DHA status (Bakewell et al., 2006, Childs et al., 2008, Walker et al., 2014, Rees et al., 2006). An effect of age but not sex was seen in the current study but because of the overall sample size numbers in different subgroups were small. It would be novel to investigate sub-groups of NAFLD patients such as male < 50 years, male ≥ 50 years, female < 50 years, and female ≥ 50 years to find more detail about the effect of sex and age variation on the omega-3 fatty acid-CV risk marker interaction in order to improve control of CV risk in patients with NAFLD.In this context, it would also be novel to reassess the relationships between omega-3 fatty acid status and CVD risk factors after an omega-3 fatty acid interventions (for example at the end of WELCOME study) to assess the effect of increasing the concentration of EPA and DHA on this relation.

Finally, this study invites further intervention with omega-3 fatty acids in patients with NAFLD in order to improve CV risk in these patients.

Chapter 7: **List of References**

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Appendices

Appendix A

Health Research Authority

NRES Committee South Central - Southampton B
Bristol REC Centre
Level 3 Block B
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Lewins Mead
Bristol
BS1 2NT

Telephone: 01173 421383
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06 February 2013

Professor Christopher D Byrne
Professor Endocrinology and Metabolism
University of Southampton
Institute of Developmental Sciences
MP 887, Southampton General Hospital
Tremona Rd, Southampton. Hants
SO16 6YD

Dear Professor Byrne

Study title:	**Investigation of the effects of a synbiotic on liver fat, disease biomarkers and intestinal microbiota in non-alcoholic fatty liver disease.**
REC reference:	**12/SC/0614**
Protocol number:	**RHM MED1071**
IRAS project ID:	**105232**

Thank you for your letter of 6th February 2013. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 10th December 2012

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Reply letter to Committee		01 February 2013
Other: Participant Reply Slip	2	12 December 2012
Participant Consent Form: Participant Consent Form Part 1	3	12 December 2012

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
Covering Letter		15 October 2012
Reply letter to Committee		01 February 2013

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University Hospital Southampton NHS
NHS Foundation Trust

PARTICIPANT CONSENT FORM (PART 1)

Title of study: Investigation of synbiotic treatment in NAFLD

Name of Principal Investigator: Christopher D. Byrne
Study number: RHM MED 1071
REC approval number: 12/SC/0614
Participant ID:

Name of researchers
Professor Christopher D. Byrne (Principal Investigator)
Professor Salim Khakoo (Co-Investigator - Hepatology)
Dr Kathyrn Nash (Co-Investigator - Hepatology)
Dr Nick Sheron (Co-Investigator - Hepatology)
Dr Mark Wright (Co-Investigator - Hepatology)
Dr Eleonora Scorletti (clinical research fellow)
Dr Kate Fayers (Co-investigator – Diabetologist)
Dr Richard Fuller (clinical research fellow)

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

PART 1
PLEASE INITIAL THE BOXES IF YOU AGREE WITH EACH SECTION:

1. I have read the information sheet Version.... dated ..... for the above non-alcoholic fatty liver disease (NAFLD) research study and have been given a copy to keep. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to donate samples of (blood/ urine/stool/tissue) for research in this study and for these samples to be kept in storage for future biomedical research into liver disease, which will be approved by a Research Ethics Committee. I understand how the samples will be collected, that giving samples for this research is voluntary and that I am free to withdraw my approval for use of these samples at any time. I understand that in donating the samples some of them will leave Southampton and be sent to other collaborating centres in the United Kingdom and Europe. These samples will not have any identifiable information about me. ☐

4. I consent to my anonymised data and samples being retained for analyses by the research team in the unlikely event that I loose capacity to give consent during the study. ☐

5. I agree to take the treatment, supplement or placebo, as directed for approximately 12 months. I agree to undergo investigations related to the study and understand how the investigations will take place as detailed in the information sheet ☐

6. I agree to undergo a Magnetic Resonance scan. An NHS consent sheet will be completed at the time of scan. ☐

7. I agree to have blood collected for DNA analysis. I understand that this sample will be stored anonymously; this sample will not be traceable back to me. This sample will be used to investigate the genes involved that may possibly be related to liver disease. To the best of the researcher's knowledge there are no genes that are linked to inherited risk of non-alcoholic fatty liver disease (NAFLD), reproductive decisions or inherited status. The DNA will be simply used to examine clusters of genes that may possibly be linked to the development and consequences of the liver condition. My DNA sample will be kept in storage for future analyses related to my liver disease. ☐

8. I agree to have the adipose tissue biopsy at the beginning and at the end of the study. I understand that these samples will be stored anonymously, and these samples will not be traceable back to me.
These samples will be used to investigate whether there is a relationship between the biochemical feature of adipocytes and pathogenesis and severity of NAFLD. ☐

9. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records for this study and future studies related to my liver disease. I understand that the information will be kept confidential. ☐

10. I understand that my GP will be informed of my participation in the research study. I understand that I will be informed if my test results are abnormal and I require additional investigations or treatment. ☐

11. I understand that I will not benefit financially if this research leads to the development of a new treatment or test. ☐

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University Hospital Southampton NHS
NHS Foundation Trust

Medicine

12. I know how to contact the research team if I need to. ☐

13. I agree to participate in this study ☐

Name of participant	Date	Signature
Name of researcher taking consent	Date	Signature

When completed, 1 copy (original) for Investigator Site File, 1 copy for participant, 1 copy for medical record/hospital notes

Principal Investigator: Christopher D. Byrne, Professor of Endocrinology and Metabolism, Institute of Developmental Sciences/HDH Academic Unit, Faculty of Medicine, University of Southampton, Southampton General Hospital, Mailpoint 887, Tremona Road, Southampton. SO16 6YD. Tel: 023 8120 5006

Main Campus: University of Southampton, Highfield Campus, Southampton. SO17 1BJ. United Kingdom.
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Blood samples collected and delivered to the UHS Pathology laboratory

BLOOD SAMPLE VOLUME AND COLLECTION TUBE	PROCESSED SAMPLE FOR	BRIEF PROCESSING INSTRUCTIONS
2 x 5 ml SST	Fasting biochemistry (Liver, bone and renal profiles, AST, γGT, cholesterol, HDL-cholesterol, triglyceride, HA, P3NP, CRP)	Deliver to Pathology with Trial Form
3 ml EDTA	Full blood count	Deliver to Pathology with Trial Form
3 ml citrate	international normalized ratio (INR)	Deliver to Pathology with Trial Form
3 ml EDTA	HbA1C	Deliver to Pathology with Trial Form
5 ml Fluoride oxalate Tube	Fasting glucose	Deliver to Pathology with Trial Form

Samples processed in the Biomedical Research Laboratory at baseline and end of INSYTE study

ORIGINAL SAMPLE	PROCESSED SAMPLE FOR	BRIEF PROCESSING INSTRUCTIONS	Volume Required
Plasma from blood (3 ml) collected into EDTA	NEFAs Insulin Save sample of EDTA tube	Centrifuge the blood tube at 2000 g for 10 minutes at ambient temperature. Separate the plasma	500 ul 500 ul 2 x 300 ul
Serum from blood (5 ml) collected into serum separator tube (SST) and protected from light	Homocysteine Vitamin K	Allow to clot (> 30 min)] and then centrifuge the blood tube at 2000 g, 10 min at 4˚C. Separate the serum and freeze immediately.	1 ml 1 ml
Plasma from blood (4 ml) collected into Plain tube	Immunoglobulins Amino scids Vitamin A, E, and D	Centrifuge the blood tube at 2000 g, 10 min at ambient temperature. Separate the plasma	500 ul 500 ul 500 ul

Appendix A

ORIGINAL SAMPLE	PROCESSED SAMPLE FOR	BRIEF PROCESSING INSTRUCTIONS	Volume Required
(red top)			
Serum from blood (2 x 8.5 ml) collected into SST	Inflammatory markers Vitamin B12, Folate, Ferritin	Stand at room temperature up to 30 min prior to centrifugation Centrifuge the blood tube at 2000 g, 10 min at ambient temperature. Separate the serum	10 x 500 ul 500 ul
Blood (6 ml) collected into sodium heparin trace element tube	Manganese	Freeze whole blood tube	6 ml
Plasma from blood (6 ml) collected into sodium heparin trace element tube	Cu, Zn, Se, I and Cr	Centrifuge the blood tube at 2000 g, 10 min at ambiente temperature. Separate the plasma	2 x 1 ml
Serum from blood (5ml) collected into SST	Lipopolysaccharide	Stand at room temperature up to 30 min prior to centrifugation Centrifuge the blood tube at 2000 g, 10 min at 4˚C. Separate the serum in hood with sterile endotoxin free material.	100 ul
Serum from blood (5ml) collected into SST	Short chain fatty acids	Stand at room temperature up to 30 min prior to centrifugation. Centrifuge the blood tube at 2000 g, 10 min at 4˚C Separate the serum	100 ul
Serum from blood (2x 8ml) collected into SST	For storage	Stand at room temperature up to 30 min prior to centrifugation Centrifuge the blood tube at 2000 g, 10 min at ambient temperature Separate the serum	10 x 500 ul
Plasma from blood (2 x 8 ml) collected into sodium citrate	Microparticles	Invert blood tubes 3 times and. Centrifuge the blood tubes at 2000 g, 10 min at room temperature. Remove almost all plasma leaving a small amount above the cells. Recentrifuge this platelet poor plasma at 2000 g, 10 min . Separate the plasma	3 x 1 ml

ORIGINAL SAMPLE	PROCESSED SAMPLE FOR	BRIEF PROCESSING INSTRUCTIONS	Volume Required
Plasma from blood (6 ml) collected into EDTA	DNA	Centrifuge the blood with Histopaque at 2000g 10 mins at ambient temperature. Remove buffy layer into 2ml aliquot tube and save 300ul RBC. Store at -80˚C	300ul buffy layer 300 ul RBC
Plasma from blood (8ml) collected into EDTA	Metabolomics	Mix x 5; centrifuge the blood tube at 2500 g, 20 min at 4˚C. Separate the plasma within 15 min. Store at -80˚C (freeze immediately)	1 ml 6 x 250 ul
Plasma from blood (6 ml) collected into sodium heparin	Fatty acids	Centrifuge the blood tube at 800 g, 15 min at ambient temperature. Separate the plasma. Take 0.5 ml of RBC pellet and make this up to 15 ml with PBS; invert. Centrifuge at 450 g, 10 min at room temperature and low brake. Remove PBS and discard. Re-suspend the cell pellet in 15 ml PBS, invert to mix and centrifuge at 450 g, 10 min at room temperature and low brake. Again, remove PBS, wash and then add 0.5 ml PBS. Re-suspend the pellet in PBS and freeze at -80^{o}C.	500 ul plasma 1 x red cell pellet
Plasma from blood (6 ml) collected into sodium heparin	For storage	Centrifuge the blood tube at 2000 g, 10 mins at ambient temperature	4 x 500 ul
Urine	For storage	Aliquot	3 x 1 ml
Urine	Metabolomics	5 x 1 ml Aliquot. Store at -80˚C; freeze immediately	5 x 1 ml
Early Morning Urine	For storage	Aliquot	3 x 1 ml
Urine	Gut permeability by lactulose and mannitol	Aliquot	2 x 10 ml

Appendix A

ORIGINAL SAMPLE	PROCESSED SAMPLE FOR	BRIEF PROCESSING INSTRUCTIONS	Volume Required
Stool	For short chain fatty acids and microbiota determination	Freeze immediately at -80 ˚C	3 aliquots of 0.5 – 1.0 g x pots

Table 0-1 visit 1, 5

		73.5ml FASTING BLOOD						URINE
BLOOD TUBE								
	2 x SST Tube ~ 5ml BIOCHEMISTRY	1 xCitrate Tube ~2ml CLOTTING	2 X EDTA Tube ~2ml FBC, Hb1AC	2 x 6ml EDTA Tubes	Fluoride oxalate Tube ~2ml	1 X Plain Red Top Tube	1 x 8.5ml SST Tube PROTECT FROM LIGHT!!	2 x 8.5ml SST Tube	2 x 6ml Trace Elements Na Heparin	20ml universal Alb/creat
SAMPLE(S)	2 x whole blood for Pathology Renal, liver, bone profiles + AST + γGT + cholesterol , HDL-cholesterol, triglyceride, CRP, HA, P3NP With trial form	1 x whole blood INR With trial form	1 x whole blood Hb1AC FBC With trial form	Process aliquots for 1)NEFA 2)INSULIN 3)Save EDTA	1 x whole blood Plasma glucose With trial form	Process aliquots for 1) Serum Ig's 2) Amino Acids 3) Vitamin A,E,D	Process 2 x 1ml aliquot for both Vitamin K and Homocysteine Protect from light! Wrap blood tube in foil and keep on ice	Process aliquots for 1)Inflammatory Markers 2)B12, folate ,ferritin	Process for Manganese, copper, zinc, selenium, iodine and chromium	Process aliquots for Archive Remainder to Pathology UHS with trial form
CENTRE	Direct to Pathology UHS	Direct to Pathology UHS	Direct to Pathology UHS	Store at- 80°C in SCBR – for analysis later	Direct to Pathology UHS	Store at- 80°C in SCBR – for analysis later	Store at- 80°C in SCBR – for shipping later	Store at- 80°C in SCBR – for analysis later	Store at- 80°C in SCBR – for analysis later	Store at- 80°C in SCBR – for analysis later

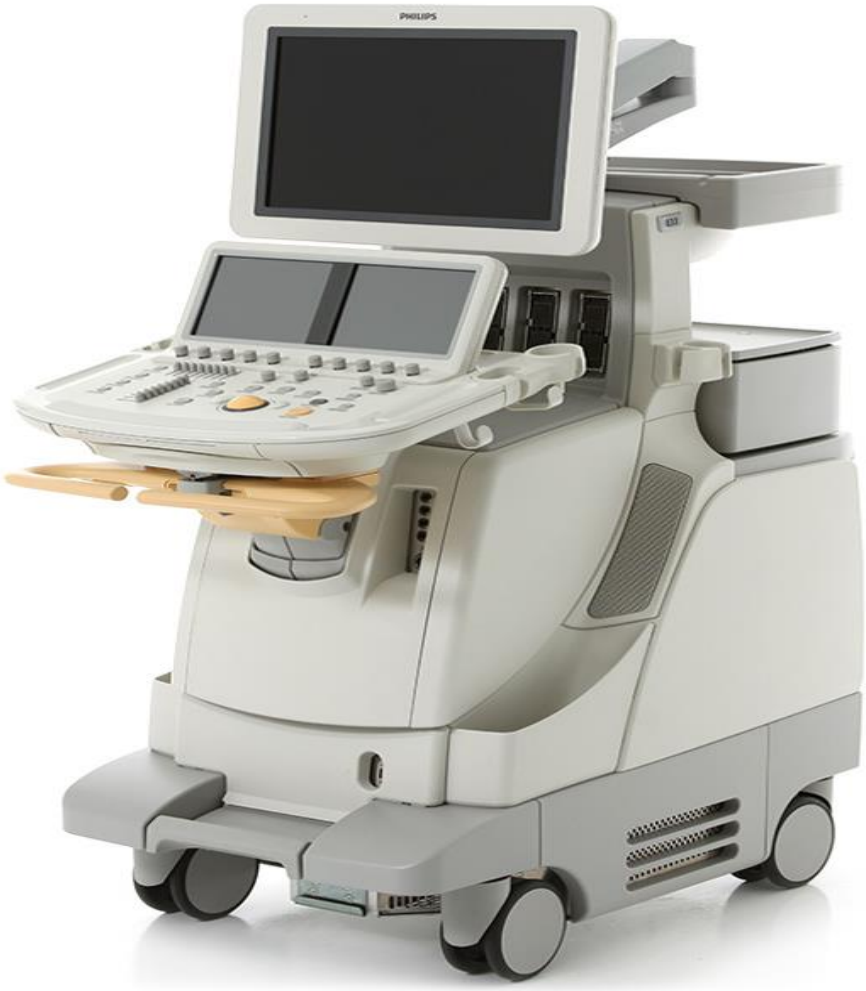
Table 0-2 visit 2, 6 INSYTE study

	70ml FASTING BLOOD						
						URINE	EMU AT HOME
BLOOD TUBE	3 x 8.5ml SST Tube	2 x Citrate Tube No Vacutainer	1 X 6ml EDTA Tube	2 x Lithium Heparin Tube	2 X 6ml EDTA Tube	Universal	Universal
SAMPLE(S)	Markers 1)LPS 2)SCFA 3)Save serum ***Keep one tube on ice for SCFA***	Microparticles	DNA	1) PUFA 2) Save Plasma	Metabonomics	Metabonomics	Archive
CENTRE	Store at- 80°C in SCBR – for analysis later	Store at- 80°C in SCBR – for analysis later	Store at 80°C in SCBR – for analysis later	Store at- 80°C in SCBR – for analysis later	Store at- 80°C in SCBR – for analysis later	Store at- 80°C in SCBR – for analysis later	Store at- 80°C in SCBR – for analysis later

Table 0-3 visit 4 INSYTE study

	5mls FASTING BLOOD			Urine	Stool			
BLOOD TUBE				2 x 1ml	3 x pots			
	1 x SST ~ 5ml BIOCHEMISTRY Tube	1 X 6ml EDTA Tube	1 x SST ~ 5ml					
SAMPLE(S)	? x whole blood for Pathology Renal, liver, bone profiles + AST + γGT + cholesterol , HDL-cholesterol, triglyceride, CRP,	INSULIN	Save serum	*8-epi-PGF2*				
	With Trial Form							
CENTRE	Direct to Pathology UHS							

Appendix B



Ultrasound Machine for carotid intima-media thickness imag