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The effect of n-3 PUFA on metabolic and inflammatory markers in normal weight and obese subjects and the modulation of inflammatory signals by different fatty acids in THP-1 derived macrophages

by

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ABSTRACT

FACULTY OF MEDICINE

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THE EFFECT OF N-3 PUFA ON METABOLIC AND INFLAMMATORY MARKERS IN NORMAL WEIGHT AND OBESE SUBJECTS AND THE MODULATION OF INFLAMMATORY SIGNALS BY DIFFERENT FATTY ACIDS IN THP-1 DERIVED MACROPHAGES

by Carolina Parás Chávez

Obesity has been related to chronic low level inflammation. Furthermore, some studies associated obesity and metabolic disorders with a higher postprandial inflammatory response. This study aims to investigate the acute and chronic effect of omega 3 fatty acids (O3FA) on human metabolism and inflammation in healthy normal weight and obese subjects. In parallel, a macrophage model has been used to study the signalling pathways affected by different fatty acids in vitro.

The human study was a double-bind, randomised controlled trial. Healthy normal weight and obese adults were recruited. They made 3 clinic visits. A 6 hour postprandial test was performed following consumption of a high fat meal (HFM) on each visit. On one of the first two visits the meal included O3FA and the other included placebo (acute effect). Between the second and third visit subjects consumed O3FA or placebo for 12 weeks (chronic effect). Plasma lipids were evaluated using a blood chemistry analyzer. Toll like receptors (TLR) 2 and 4, tumor necrosis factor α and interleukin 1β on isolated cluster of differentiation ($CD14^+$) blood monocytes was determined by flow cytometry. *Ex vivo* production of cytokines in whole blood was analyzed using FlowCytomix kit. Plasma inflammatory markers were measured employing Luminex multiplex kits.

THP-1 cells were differentiated into macrophages using 100 ng/mL phorbol-13-acetate (PMA) and rested for 5 days. The activation of NF-κB and production of inflammatory mediators in response to LPS was evaluated following fatty acid pretreatment.

Fasting and postprandial metabolic and inflammatory markers were higher in obese compared with normal weight subjects. Chronic fish oil supplementation reduced fasting plasma concentrations of C-reactive protein (CRP), P-selectin, intercellular adhesion molecule-1, adiponectin, and adipsin in normal weight subjects, while a reduction in the *ex vivo* production of interleukin-1β, interleukin-6 (IL-6) and interleukin-8 was seen in obese subjects. A reduction in the postprandial concentrations of CRP, IL-6, interleukin-10 (IL-10), resistin and TLR-2 expression was observed in obese subjects after fish oil supplementation, while decreases in the concentrations of P-selectin and triglycerides were observed in the normal weight subjects ($\rho<0.05$). PMA treatment induced the expression of macrophages features on THP-1 cells. Eicosapentaenoic acid (EPA) reduced p65 protein binding, and both docosahexaenoic acid (DHA) and EPA pretreated cells produced lower concentrations of proinflammatory lipid mediators.

The data described provide evidence related to the differences in metabolism and inflammatory markers in normal weight and obese subjects in the fasting and postprandial state and the effect of acute and chronic fish oil supplementation on these markers.

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Figure 8.9 Adhesion molecules responses to a HFM challenge in normal weight subjects after 12 weeks placebo supplementation

Figure 8.10 Adhesion molecules responses to a HFM challenge in obese subjects after 12 weeks fish oil supplementation

Figure 8.11 Adhesion molecules responses to a HFM challenge in obese subjects after 12 weeks placebo supplementation

Figure 8.12a Adipokine responses to a HFM challenge in normal weight subjects after 12 weeks fish oil supplementation

Figure 8.12b Adipokine responses to a HFM challenge in normal weight subjects after 12 weeks fish oil supplementation

Figure 8.13a Adipokines responses to a HFM challenge in normal weight subjects after 12 weeks placebo supplementation

Figure 8.13b Adipokines responses to a HFM challenge in normal weight subjects after 12 weeks placebo supplementation

Figure 8.14a Adipokines responses to a HFM challenge in obese subjects after 12 weeks fish oil supplementation

Figure 8.14b Adipokines responses to a HFM challenge in obese subjects after 12 weeks fish oil supplementation

Figure 8.15a Adipokines responses to a HFM challenge in obese subjects after 12 weeks placebo supplementation

Figure 8.15b Adipokines responses to a HFM challenge in obese subjects after 12 weeks placebo supplementation in normal weight and obese subjects

DECLARATION OF AUTHORSHIP

Academic Thesis: Declaration Of Authorship

I, Carolina Paras Chavez 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.

Title of Thesis: The effect of n-3 PUFA on metabolic and inflammatory markers in normal weight and obese subjects and the modulation of inflammatory signals by different fatty acids in THP-1 derived macrophages

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

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3. Where I have consulted the published work of others, this is always clearly attributed;

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ABBREVIATIONS

AA	arachidonic acid
ADAM-8	metallopeptidase domain-8
AEC	3-Amino-9-EthylCarbazole
AKT	protein kinase B
AP-1	activator protein 1
ASP	acylation stimulating protein
ATGL	adipose tissue lipase
AUC	area under the curve
BMI	body mass index
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
CAMs	cellular adhesion molecules
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CD	cluster of differentiation
CEBPA	CCAAT/enhancer binding protein
CHD	coronary heart disease
CLA	conjugated linoleic acid
CLS	crown-like structure
COX	cyclooxygenase
CRP	c-reactive protein
CVD	cardiovascular disease
cyPG	cyclopentanose prostaglandin

DC	dendritic cell
DGAT	diglyceride acyltransferase
DGLA	dihomo-gamma-linoleic acid
DHA	docosahexaenoic acid
DN	dominant negative
DNA	desoxyribonucleic acid
DRVs	dietary Reference Values
EET	epoxygenase
EIA	enzyme immunoassay
EPA	eicosapentaenoic acid
FA	fatty acid
FABP4	fatty acid binding protein
FAMEs	Fatty acids methyl esters
FAS	fatty acid synthase
FCγRI	fc gamma receptor 1
FFA	free fatty acid
FITC	fluorescein isothiocyanate
FO	fish oil
GC	gas chromatography
GLUT2	glucose transporter type-2
GM-CSF	granulocyte macrophage-colony stimulating factor
GPAM	glycerol-3-phosphate acyltransferase
GPCR	G protein couple receptors
GPIHBP1	glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1

HFD	high fat diet
HFM	high fat meal
HIF-α	hypoxia inducible factor alpha
HOMA	homeostasis model assessment
HSL	hormone sensitive lipase
iAUC	incremental area under the curve
ICAM	intercellular adhesion molecule
IFN-γ	interferon gamma
IKK	Inhibitor κB kinase
IL-1	interleukin 1
IL-10	interleukin 10
IL-4	interleukin 4
IL-6	interleukin 6
iNOS	nitric oxide synthase
IRS	insulin receptor
IκB	inhibitor κB
JNK	c-Jun N-terminal kinase
KO	knock out
LA	linoleic acid
LDL	low density lipoprotein
LOX	lipoxygenase
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LT	leukotriene
LX	lipoxin

LYVE-1	lymphatic vessel endothelial hyaluronan receptor
MAC-1	macrophages antigen-1
MCP-1	monocyte chemo attractant protein 1
MD-2	lymphocyte antigen 96
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP-9	metalloproteinase-9
MN	mononuclear cells
MSR	macrophage scavenger receptor
MUFA	monounsaturated
MyD88	myeloid differentiation primary response gene 88
NADH	nicotinamide adenine dinucleotide
NEFA	non esterified fatty acid
NF-κB	nuclear factor kappa B
NO	nitric oxide
NSB	non-specific binding
PAI-1	plasminogen activator inhibitor-1
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PG	prostaglandin
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLA	phospholipase A
PMA	phorbol 12-myristate 13-acetate

PMC	polymorphonuclear cells
PPAR-γ	proliferator-activated receptor gamma
RMA	repetitive measures ANOVA
ROS	reactive oxygen species
SAA	serum amyloid A
TBS	tris-buffered saline
TIMP-1	TIMP metalloproteinase inhibitor 1
TIR	toll/interleukin 1R
TLR	toll-like receptors
TXB	thromboxane
UPRS	endoplasmic reticulum stress
VCAM-1	vascular adhesion molecule
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein

CHAPTER 1

Introduction

CHAPTER 1

Introduction

1.1 Fatty acids

1.1.1 Fatty acid structure and naming

Lipids are a group of heterogeneous compounds characterized by their relative insolubility in water. They are major structural components of all living cells and are involved in pivotal biological processes including energy storage, cell signaling and regulation of physiological activities. Lipids fall into four major classes: acylglycerides, phospholipids, sterols and fatty acids (FA). FA are components of acylglycerides, cholesteryl esters and phospholipids (Gurr *et al.* 2002).

FAs are hydrocarbon chains formed by 2 or more carbons ending with a carboxyl group and generally with an even number of carbon atoms. They are classified as short, medium and long according to their hydrocarbon chain length. As their carbon chain lengthens, their solubility in water declines and their melting point increases. Other structural features defining the properties of FA are the presence of double bonds. FA are considered saturated (SFA) when their hydrocarbon chain contains only single carbon-carbon linkages, or unsaturated (UFA), when their hydrocarbon chain contains at least one carbon-carbon double bond (Gurr *et al.* 2002).

The double bonds can occur in either the *cis* or *trans* conformations (**Figure 1.1**). When the FA substituent groups are positioned in the same double bond side, they are referred as *cis* isomers, whereas they are considered *trans* when substituent groups are in opposite orientation. FAs that are otherwise identical but with double bonds in different conformations have different biological properties.

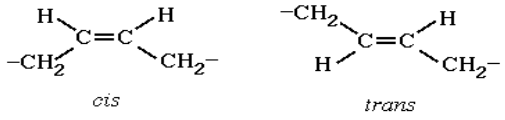


Figure 1.1 FA *cis* and *trans* FA configuration (Gurr *et al.* 2002).

FA nomenclature is based on the hydrocarbon chain length, the number of double bonds (degree of unsaturation) and the position of double bonds. Over the years, Different nomenclatures have been proposed for naming FA. The systematic nomenclature of

IUPAC (International Union of Pure and Applied Chemistry) is the standard and least ambiguous. Using this nomenclature, FAs are named according to their hydrocarbon chain length. The unsaturation position is denoted with a number before the FA name. This number explains the number of carbon atoms between the carboxyl group and the first pair of carbons where the double bond occurs. The double bond isomerism is designated as cis/trans or E/Z and is written before the unsaturation position (Gunstone,1996). An example of this nomenclature is (9Z, 12Z)-9,12-Octadecadienoic acid (common name: linoleic acid). This FA is an 18 carbons acid containing two double bonds in *cis* configuration at positions 9 and 12 (Gunstone, 1996; Gurr *et al.* 2002).

FA can be also named using trivial (i.e. common) names, that refer to the FAs origins (e.g. palmitic acid, major component of palm tree oil), or a shorthand name, where FA are referred to according to their carbon number followed by a colon, the number of double bonds and their position based on the methyl carbon being carbon number 1 (e.g. palmitic acid, 16:0; eicosapentaenoic acid, 20:5 n-3). Shorthanded nomenclature is common but assumes *cis* isomerism and cannot be employed for *trans*, or more complicated FA including those with branched chains or hydroxyl side groups (Gunstone, 1996; Gurr *et al.* 2002). **Table 1.1** shows examples of the nomenclatures for some of the most common FA.

1.1.2 FA metabolism and biosynthesis

FA are naturally occurring molecules that generally exist as components of more complex lipids or coupled to proteins. They can be produced de novo or provided in the diet, usually in triglyceride (TG) form.

After ingestion, TG is hydrolyzed to a monoglyceride and free fatty acids (FFA) by gastric and pancreatic lipases. These products are absorbed by enterocytes, and are re-esterified into TG and assembled into lipoproteins called chylomicrons. Chylomicrons are released first into lymph and then into the bloodstream. Once in the blood stream, they are utilized by different tissues (Frayn, 2010).

Common name	IUPAC	Short name	Stucture
Lauric acid	Dodecanoic acid	12:0	$CH_3(CH_2)_{10}COOH$
Palmitic acid	Hexadecanoic acid	16:0	$CH_3(CH_2)_{14}COOH$
Oleic acid	9-octadecenoic acid	18:1n-9	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
Linoleic acid	(9Z,12Z)-9,12-Octadecadienoic acid	18:2n-6	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$
Linolenic acid	(9Z,12Z,15Z)-9,12-Octadecadienoic acid	18:3n-3	$CH_3CH_2CH=CHCH_2CH=CHCH_2=CH=CH(CH_2)_7COOH$
Arachidonic acid	(5Z,8Z,11Z,14Z)-5,8,11,14-eicosatetraenoic acid	20:4n-6	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
Eicosapentaenoic acid	(5Z,8Z,11Z,14Z,17Z)-eicosa-5,8,11,14,17-pentenoic acid	20:3n-3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
Docosahexaenoic acid	(4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenoic acid	22:6n-3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_2COOH$

Table 1.1 FA nomenclature and structure

When FA are consumed in increased amounts compared with usual consumption they can be incorporated into different tissues and cells (e.g. immune cells) in a dose and time dependent manner (Calder, 2013). Fish oil (FO) supplementation results in incorporation of UFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), into cell membranes. Maximum EPA incorporation into immune cells has been reported after 4 weeks of supplementation, and DHA at week 8 (Yaqoob *et al.* 2000). The increment of n-3 polyunsaturated FA (PUFA) levels in plasma and immune cells is at the expense of arachidonic acid (AA) and dihomo-γ-linoleic acid (DGLA) (Yaqoob, 2000; Rees *et al.* 2006; Damsgaard *et al*. 2008).

1.1.2.1 FA Biosynthesis *de novo*

FA can be synthesized *de novo* and then transformed into more complex molecules. Carbohydrates from the diet represent the main source of carbons for de novo FA synthesis in animals. In the early stages, pyruvate derived from glycolysis is transformed into acetyl-CoA by pyruvate dehydrogenase and in turn acetyl-CoA is transformed into malonyl-CoA under the action of acetyl-CoA carboxylase (Gurr *et al.* 2002).

Malonyl-CoA provides all the carbons for FA formation which is catalyzed by fatty acid synthase (FAS). The end product of FAS activity is palmitic acid (16:0). The formation of longer chain SFA is catalyzed by enzymes called elongases; using these enzymes palmitic acid can be converted to stearic acid (18:0). FA elongation is an essential step for the transformation of essential FA from the diet into more complex PUFAs with potent biological functions Gurr *et al*. 2002). **(Figure 1.2).**

UFA synthesis occurs by the insertion of a double bond into a SFA chain under an oxidation reaction catalyzed by desaturase enzymes and involving nicotinamide adenine dinucleotide (NADH) as a cofactor, and cytochrome B5 (Gurr *et al.* 2002). One such enzyme is stearoyl-CoA desaturase which converts stearic acid (18:0) to oleic acid (18:1n-9). Stearoyl-CoA desaturase is also known as delta-9 desaturase (Gurr *et al.* 2002).

Desaturation on positions 3 and 6 involve $\Delta 12$ and $\Delta 15$ desaturase action, respectively. These enzymes are exclusive to plants. These desaturations produce linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3), respectively (**Figure 1.2**). Since only plants can

produce these two FA, but they are important for animal health, they are considered to be essential FA (Gurr *et al.* 2002). Both, linoleic and alpha linolenic acid, are primary precursors for the biosynthesis of n-6 and n-3 PUFA.

N-6 PUFA and n-3 PUFA are biosynthesized in animal tissues by a series of elongation and desaturation steps as described in **Figure 1.2**. In terms of n-6 PUFA, linoleic acid is transformed in Y-linolenic acid with the introduction of a double bond in the position 6. In further steps this fatty acid is elongated and converted in arachidonic acid by Δ5 desaturase action. The conversion of α-linolenic acid into n-3 PUFA (e.g. EPA and DHA) requires the action of Δ5 and 6Δ desaturases and acyl-conenzyme A esters (**Figure 1.2**). α-linoleic acid is sequentially elongated and desaturated, new double are introduced between existing double bounds and carboxyl groups. DHA formation requires further elongation cycles and retroconverstion (beta-oxidation).

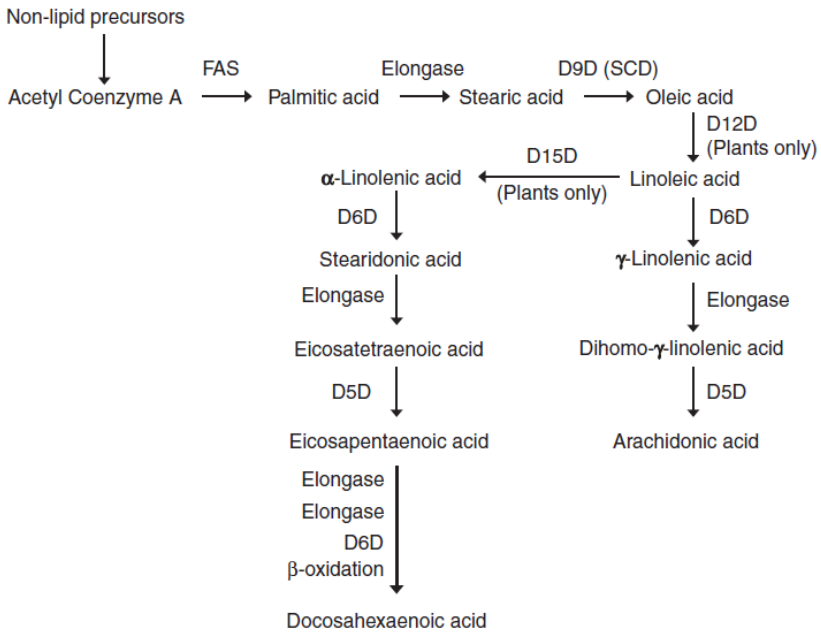


Figure 1.2 FA biosynthesis (Gurr *et al.* 2002)

Long chain FAs (arachidonic acid (AA), DHA and EPA) from cellular phospholipids are precursors of eicosanoids, a diverse family of autacoids fundamental in inflammation physiology (Serhan, 2010). Depending on the external stimuli and the cell FA availability, essential 20C FA are released from membrane phospholipids by phospholipases (PLA_2)

and metabolized through cyclooxygenases (COX), lipoxygenases (LOX), epoxygenase and isoprostane pathways to give rise to different metabolites (Serhan, 2010).

COX family is a group of haemoproteins with cyclooxygenase and peroxidase properties. COX1 and COX2 are found in human cells. COX 1 is ubiquitous, constitutively expressed and involved in house-keeping processes, while COX 2 is induced under specific conditions, including inflammatory signals. Both enzymes utilize FAs, mainly AA, as substrates. Eicosanoids derived from COX activity include prostaglandins, prostacyclins and thromboxanes. These mediators lead inflammatory responses, from initiation to resolution, and are involved in some physiological activities including smooth muscle contraction, platelet stickiness, contraction or dilation of blood vessels (Gurr *et al.* 2002; Serhan, 2010).

Long chain PUFA can be alternatively lipoxygenated by LOX. LOX family subtypes found in mammals include 5-, 8-, 12- and 15-. LOX derived leukotrienes have many biological roles, including promoting chemotaxis of neutrophils and eosinophils, arteriolar constriction and dilatation (Serhan, 2010).

Epoxygenases play a role in tissues lacking COX and LOX expression, such as kidneys. Epoxygenase derivatives (EETs) are involved regulating renal function. Isoprostane pathways is activated by oxidative stress and has been associated with the activation of inflammatory pathway (Vance *et al.* 2008; Serhan *et al.* 2010).

Resolvins, protectins and maresins are a new family of LOX metabolites derived from metabolism of the n-3 PUFA EPA and DHA. These compounds have been associated with anti-inflammatory and pro-resolution properties (Serhan, 2010; Serhan, 2011).

1.1.3 FA sources and intakes

FA can be found esterified to glycerol forming triacylglycerol molecules, representing the main energy storage depot, or forming phospholipids mainly as part of membranes. Membrane FAs, as phospholipid components, contribute to the structural and functional properties of cell membranes.

Most FAs from the diet are in triacylglycerol form, as these are the main fat storage molecules in animals and plants. Since animals can produce SFA and monounsaturated FAs (MUFA), animal tissues commonly contain significant amounts of these FA and much

less PUFA. In general fat from ruminants tends to have a greater percentage of SFA, although the fat composition from single-stomached and ruminant animals can be manipulated through the diet (Gurr *et al.* 2002). Oily fish (e.g. salmon and mackerel) are a good source of n-3 PUFA. These fatty acids are not synthesized by fish but obtained through their diet (algae and other fish). **Table 1.2** shows the FA composition from different products.

Plants can produce the two essential FA, linoleic and alpha-linolenic acid. Thus plant tissues (e.g. leaves, seeds, nuts and seeds) contain high proportions of these FAs. Indeed, seeds and seed oils represent the major sources of essential n-6 and n-3 PUFAs in the human diet. Corn oil, sunflower oils and safflower are very rich in LA which contributes 50-80% of the FA present, while some nuts, linseeds and linseed oil are abundant in α-linolenic acid. Soybean oil is rich in linoleic acid (50% of FA) and provides α-linolenic acid (7% of FA) (Calder, 2013).

Type	SFA	Cis-MUFA	Cis n-3 PUFA	Cis n-6 PUFA	CLA
Cod, raw	38	34	21	7	-
Tuna, raw flesh	39	36	17	8	-
Mackerel, raw	25	44	28	3	-
Salmon, smoked	27	38	27	8	-
Egg	34	47	2	17	-
Milk	73	25	1	-	0.8
Beef	48	47	4	1.0	-
Pork	40	39	2	19	-
Poultry	33	43	1	23	-
Lamb	53	41	2	4	-
**Data expressed as percentage of total FA in 100g; SFA= saturated fatty acids, MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids; CLA=conjugated linoleic acid*					

Table 1.2 FA composition of different products.

(Department of Health UK, 2013; Wood *et al.* 2008)

Typically plants are not good sources of longer chain PUFAs. AA (20:4n-6) is found in meat, eggs and offal. Long chain highly unsaturated omega-3 FAs like EPA (20:5n-3) and DHA (22:6n-33) are produced by some algae and other lower organisms and are found

in algal oils. Fish and other seafood that consume EPA and DHA rich organisms can accumulate the EPA and DHA in their tissues. As a result seafood, especially some types of fish (e.g. salmon, herring, sardine, mackerel, tuna), are good sources of EPA and DHA. Oils derived from marine fish are used as supplements, commonly known as “fish oils” and are also good sources of EPA and DHA (Calder, 2013).

According to the UK Dietary Reference Values (DRVs), fat and FAs from the diet should not exceed 35% of the daily food energy intake. The UK recommendations for adults for SFA, MUFA and PUFA are < 10%, 13% and 6 to 10% of dietary energy, respectively. The Committe on Medical Aspects of Food Policy (COMA) and Scientific Advisory Committee on Nutrition (SACN) recommends a weekly consumption of two portions of fish, of which one should be oily (providing approximately 0.45g of LC n-3 PUFA per day) (SACN, 2004).

1.2 Inflammation

1.2.1 Overview of the immune system

The immune system is a complex physiological network that protects the host against pathogens and insults from the environment. Components of the immune system can be classified as innate or adaptive, depending on pathogen specificity. The innate immune system is composed of elements recognizing evolutionary conserved patterns on a pathogen’s exterior. In contrast, adaptive immunity is antigen specific and provides protective immunological memory (Roitt *et al.* 2013).

Inflammation, which is an innate response, is a controlled process initiated during tissue injury and/or pathogen invasion. Its main objective is to deliver signalling molecules and cells to the site of infection or injury and return it to homeostasis. This response is mediated by plasma cascade systems, bone-marrow derived cells and inflammatory mediators. Host health relies on an adequate regulation of inflammatory signals. Unsolved inflammation results in detrimental effects to the host resulting in tissue damage and leading to autoimmune and metabolic disorders.

1.2.2 Inflammation: Physiology, cellular and chemical components

Inflammation is constantly challenged by insults from the environment. During infection, the first protection against invaders is the physical, chemical and biological

barriers that prevent pathogens entering the organism. If the pathogens successfully outflank these barriers they encounter cellular and chemical components in plasma, initiating an inflammatory response (Roitt *et al.* 2013).

The complement system consists of a series of plasma proteases activated by antigens on the membrane of microorganisms. Complement activation triggers a biochemical cascade to up regulate vascular permeability, opsonize and disrupt the microbial membrane, and finally attract immune cells to the site of infection. Opsonized microorganisms are encountered by macrophages (tissue sentinels and protagonists in the initiation of an inflammatory response) which can destroy them (Murphy *et al.* 2008).

Tissue-resident macrophages are the first line of defence and detect pathogens through evolutionary conserved surface patterns on a pathogen's exterior (PAMPs). Pattern recognition receptors (PRRs), such as TLRs (toll-like receptors), trigger the activation of signalling pathways that lead to changes in cell physiology and phenotype. In the presence of bacterial constituents, cluster of differentiation (CD) 14 scavenger molecules interact with TLR-4 promoting the activation of the nuclear factor κB (NF-κB) signalling cascade (Delves *et al.* 2006).

The NF-κB binding activity modulates the expression of inducible enzymes that include COX, LOX, cytokines, adhesion molecules and nitric oxide synthase. These events lead to the production of cytokines, reactive oxygen species (ROS), AA derived eicosanoids and complement system molecules.

Cytokines act as endogenous pyrogens inducing the synthesis of lipid mediators and initiating the acute phase response. During this phase, opsonins (collectins, pentraxins and ficolins) are secreted by the liver in response to interleukin-1β (IL-1β) and interleukin-6 (IL-6). Acute-phase proteins detect PAMPs and opsonize microorganisms facilitating phagocytosis. C-reactive protein (CRP) is a commonly measured acute phase protein.

The complement system is another system activated by opsonins. It is involved in the opsonization of pathogens (through C3 fragments), recruitment of inflammatory cells (fragments C4 and C5) and pathogen killing (membrane attack).

Another key event regulated by cytokines is endothelium activation (Murphy *et al*. 2008). The endothelium surface is activated while a chemokine gradient induced by macrophages guides leukocytes to the site of infection. Endothelium activation results in an up-regulation of E-selectin and intercellular adhesion molecule (ICAM) -1 expression. Selectins induce endothelium-leukocyte interactions. These links are strengthened by ICAM-1, producing a strong heterodimerization between the leucocyte's integrins and the endothelium. These events facilitate leucocyte extravasation and movement to the focus of infection. Neutrophils are a cellular cascade arriving to the site of infection, followed by monocytes at later stages (Murphy *et al.* 2008).

Lipid mediators derived from inflammatory cells are fundamental in both the progression and resolution of inflammation. Cytokines determine the fluctuation in lipid mediator levels during inflammation onset (Serhan, 2010). Once pathogens have been destroyed, macrophages and newly recruited monocytes initiate the tissue repair process guided by pro-resolution signals (Serhan, 2007). Similar to inflammation induced by infectious agents, housekeeping inflammation is determined by similar signals.

In summary, inflammation is an essential process activated in response to tissue damage and/or infection. Tissue resident macrophages encounter intruders and trigger a chemical inflammatory cascade that initiates the migration of leukocytes to the site of infection. Neutrophil recruitment is accompanied by fluctuation of lipid mediators at the site of infections that determines the progression and resolution of the inflammatory response. In the later stages, monocytes are guided to the site of inflammation where they differentiate to macrophages and help to repair damaged tissue. Pro-resolving lipid mediators produced by immune cells over the inflammatory process set the end of this process. The appropriate orchestration of the elements of inflammation determines host homeostasis.

1.2.3 Cellular elements of the inflammatory response

Monocytes are mononuclear cells derived from hematopoietic stem cells in the bone marrow. During maturation, progenitor cells commit to a monocytic or granulocytic lineage, predicting the preceding transformation into monocytes or neutrophils. Mature

monocytes are released from bone marrow by C-C chemokine receptor type 2 (CCR2) action and distributed in the circulation (**Figure 1.3**) (Lewis and McGee, 1992). Monocytes represent 1-6% of blood leukocytes and remain in the circulation in humans for up to 70 h before they return to the bone marrow or spleen or migrate to different tissues (Lewis *et al.* 1992). Monocytes are amoeboid shaped cells with a kidney-shaped nucleus. They play multiple roles including replenishment of macrophage and dentritic cell (DC) populations, phagocytosis and antigen presentation (Lewis and McGee, 1992).

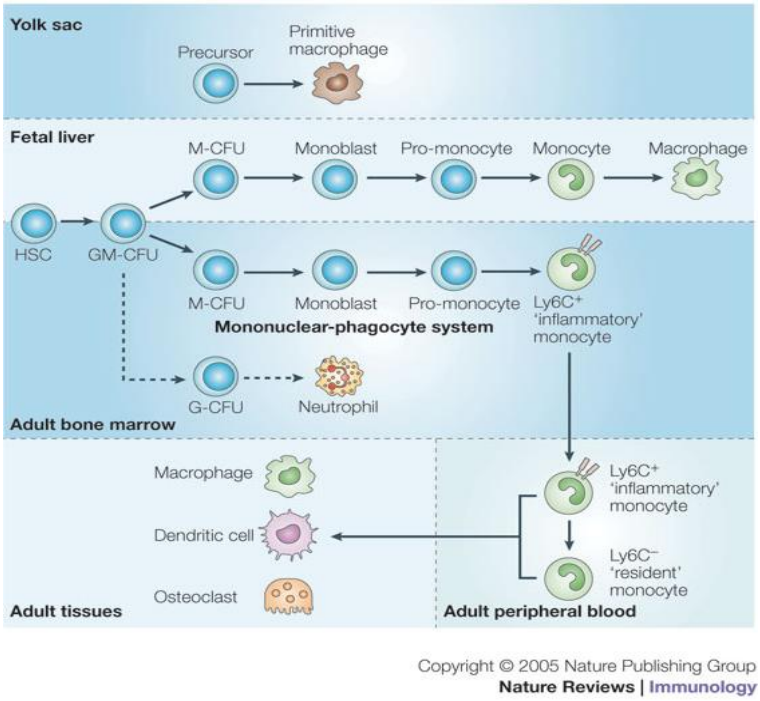


Figure 1.3 Origins of monocytes, macrophages and related cells (Gordon, 2005)

In humans, the peripheral blood monocyte population is identified by expression of the cell surface marker CD14. Distinct monocyte subsets are characterized by different degrees of CD14 expression and ratios between CD14 and CD16 (Passlick *et al.* 1989). In healthy humans, $CD14^+$ $CD16^-$ or "classical monocytes" represent up to 95% of the monocyte population and $CD14^+CD16^+$ or "non-classical" are around 5% (Passlick et al. 1989; Gordon, 2005).

Chemokine and antigen expression differ between monocyte subsets. $CD14^+CD16^+$ monocytes exhibit high major histocompatibility complex (MHC) II and CCR5 while $CD14^+CD16^-$ monocytes are characterized by high expression of CCR2, L-selectin and Fc gamma receptor 1 (FCγRI). Differences in chemokine receptors may determine endothelial transmigration capacity and differentiation into distinct cell populations

once in the tissues (Gordon, 2004). The factors involved in the development of monocyte subsets are not fully understood, but exposure to microbial fractions from gut and lipids from the diet might play a role.

Under physiological conditions monocytes migrate to tissues at low rates (Murphy *et al.* 2008). Up to 95% of tissue macrophages derive from monocytes, although some fate-mapping studies revealed other origins of some tissue macrophages, such as microglia and Langerhans cells (Uchaska et al. 2000). Even though macrophages have been considered to have lost the ability to divide, active macrophage proliferation has been reported after acute inflammation (Davies *et al.* 2011). Some tissue macrophage populations such as those in the lamina propria are highly dependent on peripheral blood monocyte supply to replenish them (Davies *et al.* 2011).

Inflammatory signals intensify monocyte recruitment into tissues. Rolling, adhesion and transmigration of monocytes through the endothelium are facilitated by chemokine gradients and adhesion molecule expression on the endothelium surface, as described in the previous section. Once in the tissue, monocytes differentiate into macrophages or DCs, depending on the environmental conditions.

Ex vivo culture of human monocytes with interferon gamma (IFN-γ), IL-6 or IL-10 skew cells towards a macrophage phenotype, while IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) promote DC features (Allavena *et al.* 1999; Chomarat *et al.* 2000; Delneste *et al.* 2003). These macrophage-like cells show a down-regulation of CD1a (DC marker) and up-regulation of CD68 and CD14 (Allavena, 1998). Once committed to a specific DC or macrophage phenotype, differentiation has been reported to be irreversible in *ex vivo* conditions (Allavena *et al.* 1999; Delneste *et al.* 2003).

Macrophages are key components in immune responses and tissue repair. In the absence of pathogens or immune signals macrophages remain quiescent and perform homeostatic tasks (Lewis and McGee, 1992). Microbial particles and other insults activate macrophages through membrane sensor platforms inducing the production of different enzymes, oxide radicals, lipid metabolites and various peptide mediators

(Murphy *et al.* 2008). The complexity of the organ and micro-environmental stimuli fluctuations influence macrophage phenotype and functionality (Gordon, 2005).

Macrophages can be subdivided into two categories 1) M1, classically activated macrophages, responsible for pathogen killing and production of pro-inflammatory cytokines and 2) M2 or alternatively activated macrophages in control of tissue repair and resolution. Different M2 subclasses have been described by various authors (Gordon, 2013; Olefsky et al. 2010), but it is difficult to simplify macrophage phenotype and commit its behaviour to one or other classification (**Figure 1.4**).

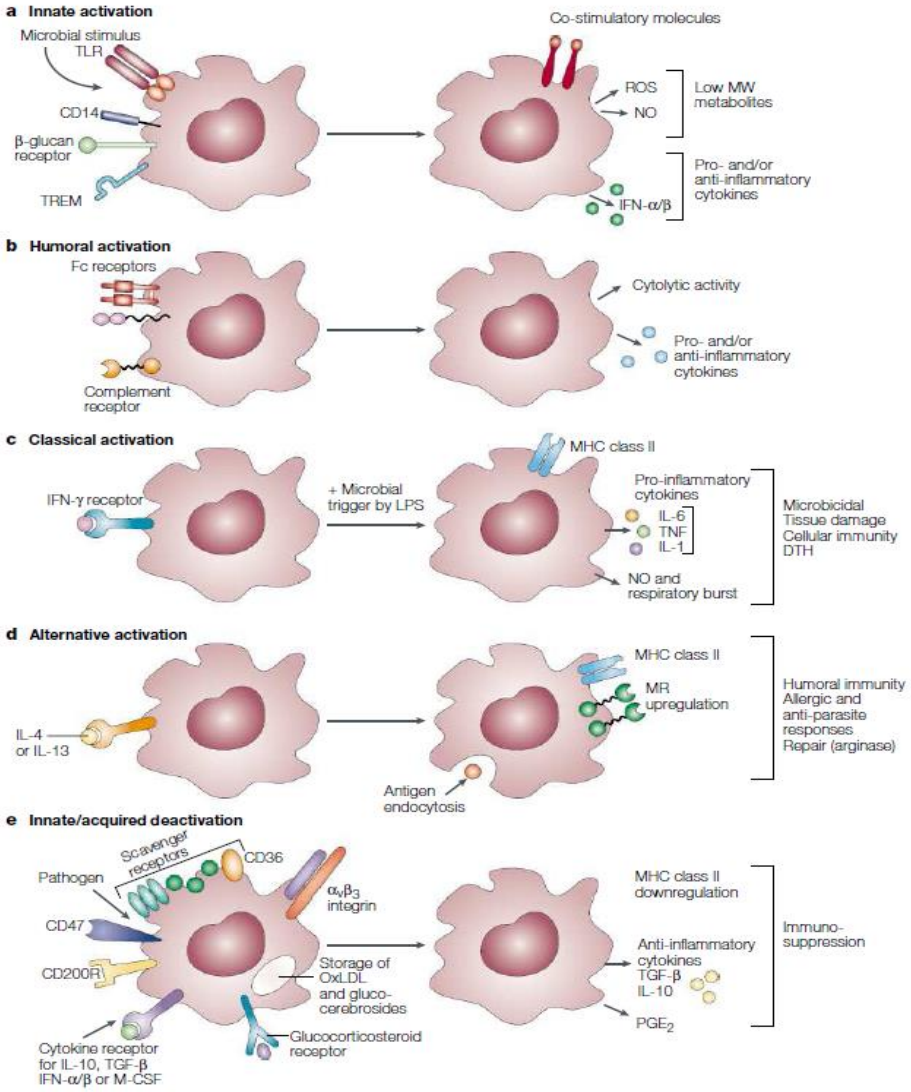


Figure 1.4 Macrophage activation (Gordon, 2003)

Fluctuations in cytokine gradients induce changes in macrophage signature by modifying gene transcription (Lawrence *et al.* 2011). NF-κB and peroxisome proliferator-activated receptor gamma (PPAR-γ) are two transcription factors involved in macrophage differentiation. The relative binding of these two factors to deoxyribonucleic acid (DNA) has been shown to be involved in the development of either M1 or M2 phenotype (Ricote *et al.* 1998; Lawrence *et al.* 2011). Macrophage activation state also determines the repertoire of cytokines and lipid metabolites they produce (Davis *et al.* 2013). **Figure 1.5** illustrates some elements involved in macrophage polarization.

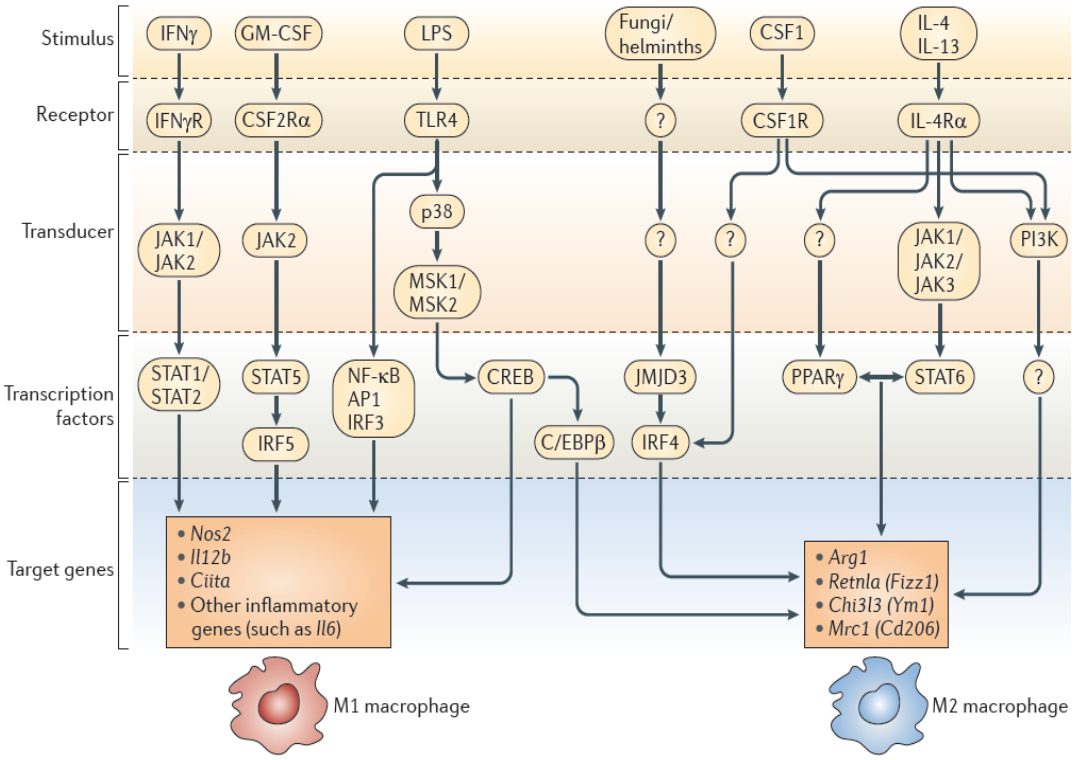


Figure 1.5 Signal transduction pathways involved in macrophage polarization (Lawrence *et al.* 2011)

To conclude, monocytes and macrophages are key elements in the immune system, including the inflammatory component. Macrophage housekeeping tasks ensure proper tissue functioning and activation during infection events drives the course of a controlled inflammatory response. Genetic variations and chronic environmental exposure to inflammatory agents might switch on different profiles determining health or disease.

1.2.4 Inflammatory signals

1.2.4.1 Recognition of inflammatory signals: Toll-like receptors (TLRs)

Inflammation starts with the recognition of non-self-agents through PAMPs on an invader's membrane. PAMPs activate inflammatory signalling pathways through their binding with PRRs on immune cells. TLRs are among the best known members of the PRR family.

TLRs are a family of transmembrane proteins expressed ubiquitously in almost all cell membranes (**Figure 1.6**) (Akira *et al.* 2001). They participate in the initiation of an innate response by recognizing conserved patterns on pathogen membranes. Each receptor has a selective affinity for different pathogen strains. TLR4 and TLR2 are the most studied receptors of this family.

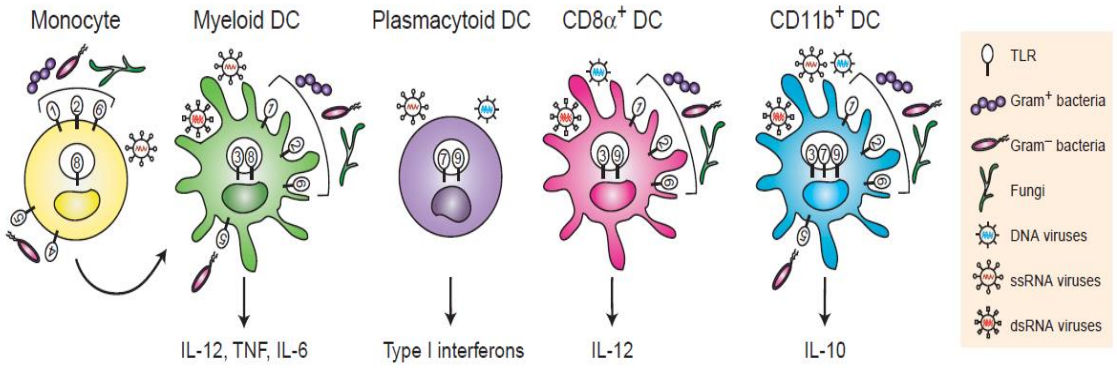


Figure 1.6 TLR expression in different cells from the innate and adaptative immune system (Iwasaki *et al.* 2004)

Some endogenous molecules such as hsp60, fibronectin and plasma lipids initiate TLR activation (Akira *et al.* 2001; Fessler *et al.* 2009). Downstream signals rely on the interaction between cell receptors and lipid A fraction on the bacterial surface. Upon an external stimulus, TLRs form complexes with lymphocyte antigen 96 (MD-2) and CD14. These events are followed by the formation of toll/interleukin 1R (TIR) complexes and activation of Myeloid differentiation primary response gene (88) (MyD88) adaptor proteins in the cytosol. The NF-κB cascade is one of the main downstream components triggered by TLRs (Hwang *et al.* 2001).

TLR4 activation and potency relies on the formation of TLR-MD2-CD14 complex (Akira *et al.* 2001). Another important element upon cell activation is Lipid A on bacteria. Lipid A consists of two glucosamine molecules linked to an acyl chain rich in SFA that accounts for most bacterial toxicity (Murphy *et al.* 2008). Lipid A deacylation or sequestration of its lipid component results in loss of toxicity/inflammatory properties (Mumford *et al*. 1986; Krauss *et al.* 1989). Thereby, the presence of SFAs in Lipid A may play a role in the activation of TLRs.

1.2.4.2 Signal Transduction: NF-κB

NF-κB is a transcription factor family formed by dimers from the rel family (RelA (p65), RelB and cRel), p50 and p52 (Baeuerle, 1994). It works synergistically with other elements such as AP-1 to modulate several homeostatic cell functions including immune responses. Many pro-inflammatory genes involved in the synthesis of cytokines, lipid mediators and adhesion molecules are regulated by NF-κB signalling cascade (Barnes et al. 1997).

The NF-κB pathway is activated by diverse stimuli from the microenvironment. Cytokines, activators of protein kinase C (PKC), pathogens and oxidants represent some of its main upstream ligands (Barnes et al. 1997). Some of the products derived from its own activation auto-regulate the amplification and end of an inflammatory response (Ruland *et al.* 2011).

In resting cells, NF-κB dimers are sequestered by their inhibitor kB (IκB) in the cytoplasm. IκB-α prevents the migration of NF-κB subunits to the nucleus, displaces them from promoter sites and induces their proteolysis (Ruland *et al.* 2011).

The presence of stimuli leads to the activation of IκB kinase (IKKβ). IKKβ phosphorylates IκB-α on Ser32 and 36 and liberates NF-κB dimmers (Viatour et al. 2005). Free dimers migrate to the nucleus, where they interact with κB promoter regions initiating gene transcription (Baeuerle, 1994). **Figure 1.7,** illustrates the NF-κB signalling pathway.

The transcriptional activity of distinct NF-κB dimers induces a different gene expression profile over the course of the inflammatory response in *in vivo* models (Lawrence *et al.* 2001). At early stages, cRel-p50 predominant transcription induces the release of pro-

inflammatory mediators including cytokines. At late stages NF-κB is switched to p50-p50 profile, inducing rather pro-resolution and pro-apoptotic signals (Lawrence *et al.* 2001).

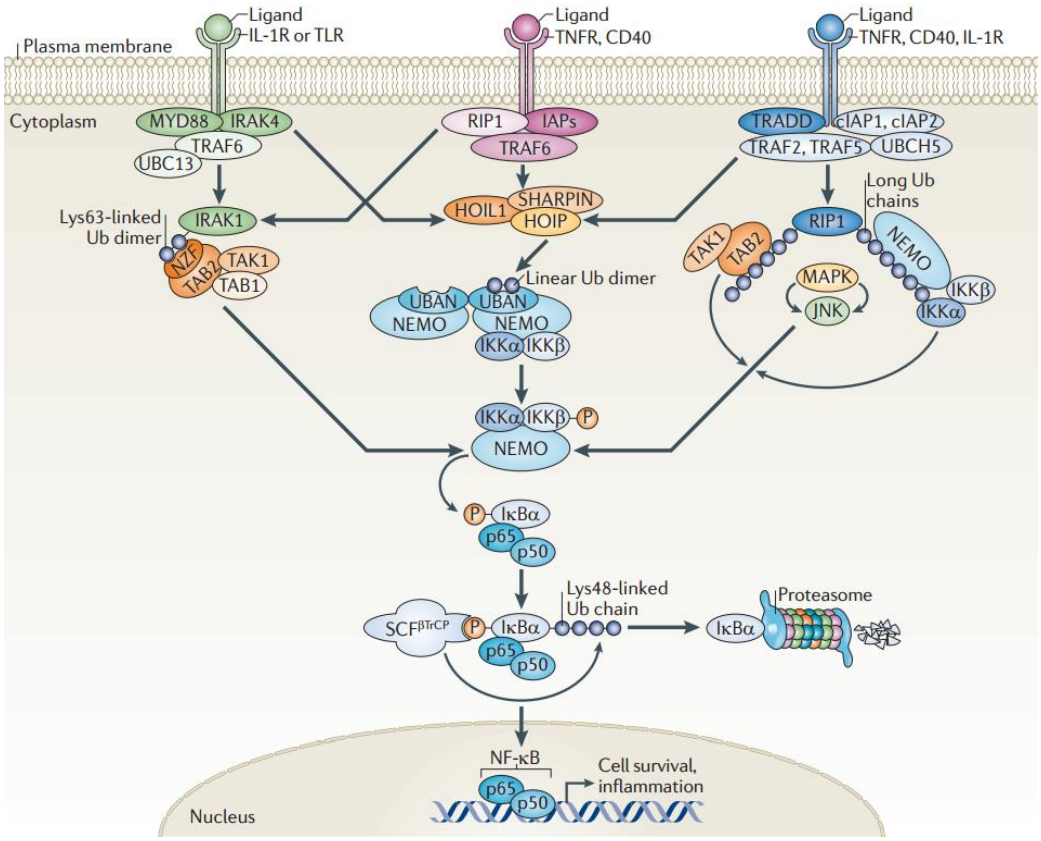


Figure 1.7 NF-κB signalling pathway (Grabbe, 2011)

IκBα may contribute to changes in NF-κB dimer transcription. IκB contains a κB recognition sequence; thus NF-κB signals that prompt its phosphorylation also initiate its *de novo* synthesis (Ruland *et al.* 2011). Newly synthesized IκB-α enters the nucleus to arrest activated NF-κB and transports it back to the cytoplasm (Baeuerle, 1994). IκB-α preferentially arrests cRel. Thus cRel down-regulation followed by other intracellular and extracellular signals may contribute to initiation of the resolution phase of inflammation (Levy *et al.* 2001; Lawrence *et al.* 2001).

Proinflammatory signals are now recognised to be fundamental for resolution. In a carrageenan pleurisy model, NF-κB inhibitors were anti-inflammatory at early stages of inflammation (Lawrence *et al.* 2001). Nevertheless, inhibition of early NF-κB or COX-2 activation led to a sustained recruitment of leukocytes in the site of infection and impaired resolution (Gilroy *et al.* 1999; Lawrence *et al.* 2001; Levy *et al.* 2001).

In conclusion, NF-κB is a key element in the regulation of inflammation. Its activation is meditated by a sequential phosphorylation of cytoplasmic proteins resulting in the migration of NF-κB subunits to the nucleus and subsequent gene transcription. Its prompt regulation results in an appropriate inflammatory response that protects the host against injury and infection.

1.2.5 Amplification of inflammation signals: Cytokines and chemokines

1.2.5.1 Cytokines and chemokines

Cytokines are signaling molecules with autocrine and paracrine effects that modify the behavior and phenotype of cells. They are produced by a wide number of immune cells and also other cell types including adipocytes and play roles ranging from inflammation to regulation of metabolism.

Over the course of an inflammatory response, TNF-α is a predominant molecule induced by NF-κB transcription. The presence of TNF-α is essential for endothelium activation and leukocyte extravasation to the site of infection (Murphy *et al.* 2008). TNF-α dictates the intensity of the immune response by promoting the synthesis of lipid mediators through COX and LOX pathways. Additionally, TNF-α increases body temperature, producing a harmful environment for bacterial multiplication. Along with other cytokines, such as IL-1β and IL-6, TNF-α induces the amplification of the initial inflammatory signal.

Chemokines comprise a group of "communication cytokines" that coordinate the trafficking of cells during homeostatic and pathogenic processes. During a physiological stress, endogenous molecules produced via PPRs induce the synthesis of chemokines that subsequently are delivered to the circulation carrying messages to other cells. Chemokines are recognized by target cells through G protein couple receptors (GPCRs) (Rot *et al.* 2004). **Figure 1.8** illustrates trafficking of different bone marrow cells to the site of inflammation and the role of different chemokines in this process.

Increased numbers of chemokines have been found in patients with autoimmune (e.g. multiple sclerosis) and chronic inflammatory diseases (asthma, rheumatoid arthritis, atherosclerosis) (Gerard *et al.* 2001).

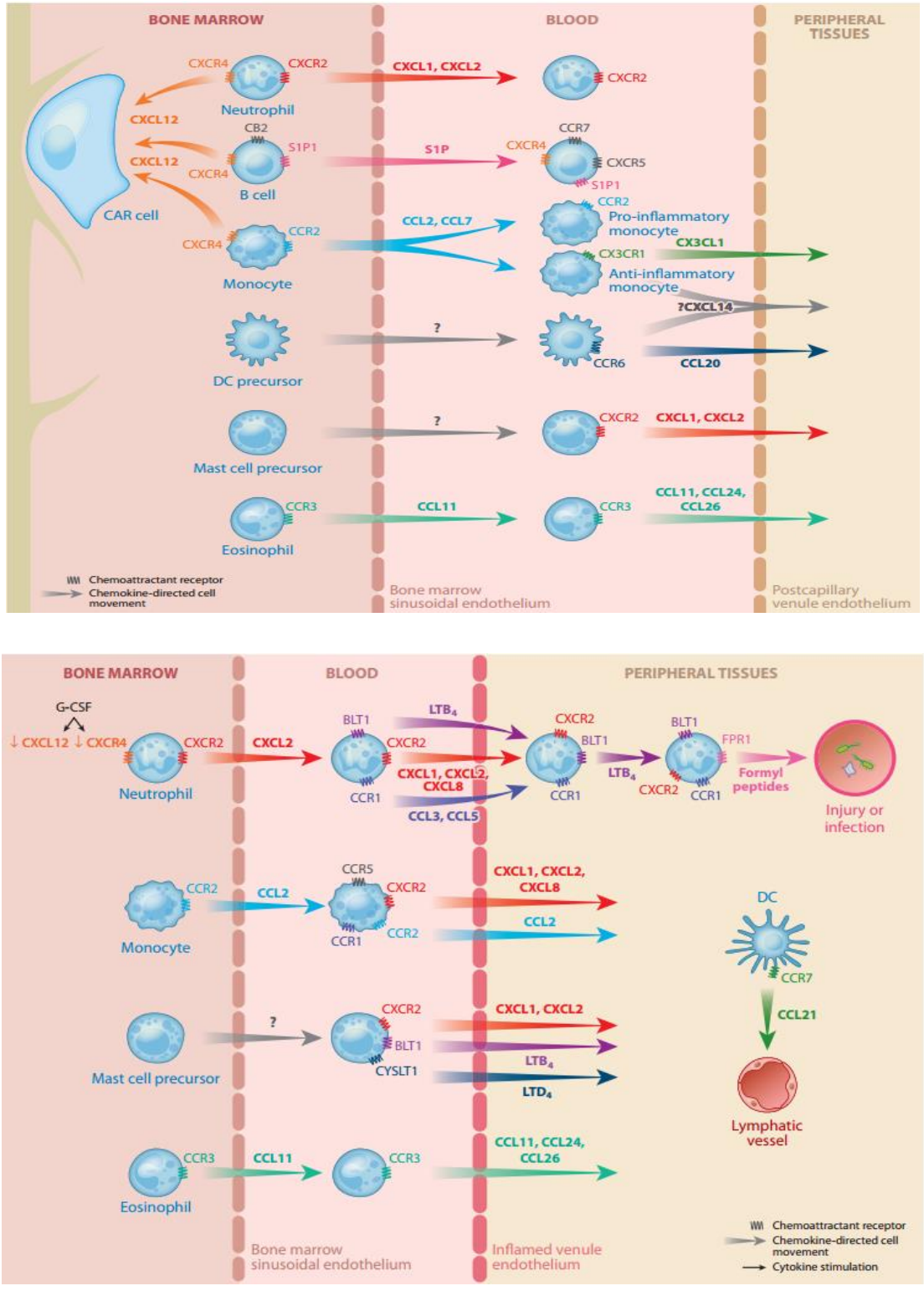


Figure 1.8 Kinetics of immune cells during homeostasis and inflammation triggered by chemokines (Griffith *et al.* 2014)

1.2.5.2 Lipid mediators and its regulation

Lipid mediators are PUFA-derived metabolites produced by enzymatic transformation mediated by COX and LOX. Lipid mediator synthesis occurs *de novo* under an external stimulus. In the presence of cytokines and hydroperoxide molecules AA is released from membrane phospholipids by PLA_2 and the mediator synthesis is activated (Serhan, 2010).

In acute inflammation models, polymorphonuclear cells (PMN) are mobilized to the site of infection peaking after 2-4 hours (Willis *et al.* 1996; Levy *et al.* 2001). The PMN-dominant phase is characterized by an active production of prostaglandins (PGs), leukotrienes (LTs) and nitric oxide derived from COX-2, 5-LOX and nitric oxide synthase (iNOS) (Levy *et al.* 2001; Lawrence *et al.* 2001). Prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) are mainly produced at this stage. The highest concentrations of PGE_2 coincide with the maximum levels of PMN in the site of inflammation.

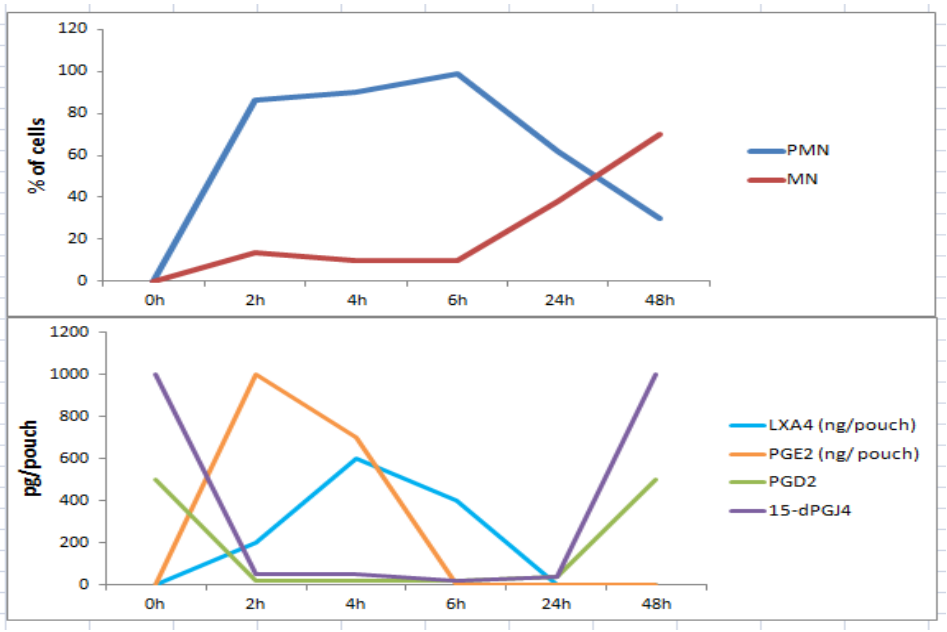
Following these events, PGE_2 levels drop (4 h) while lipoxin A_4 (LXA_4) levels increase rapidly. LXA_4 prevents the recruitment of PMNs at the site of infection and inhibits LTB_4 synthesis (Levy *et al.* 2001). These observations suggest that PGE_2 plays a role in the regulation LXA_4 synthesis (Gilroy *et al.* 1998; Lawrence *et al.* 2001).

After the PMN dominant phase, mononuclear cells (MNCs) are recruited to start the tissue repair process (Willis et al. 1996). MNCs dominant phase is characterized by synthesis of cyclopentanose prostaglandins (cyPGs) of the J series and LXA_4 (Gilroy *et al.* 1998).

NF-κB binding inhibition was shown to affect the course of acute inflammation (Lawrence *et al.* 2001). In mice, NF-κB antagonist treatment inhibited acute inflammation resolution. A similar effect was found when PGE_2 synthesis was blocked by COX-1 and COX-2 inhibition (Gilroy *et al.* 1999). Taken together these findings suggest that the inflammatory mediators produced via NF-κB activation regulate the activation of the resolution phase of inflammation.

Figure 1.9 illustrates the fluctuations of lipid mediator concentrations during the course of an acute inflammatory response and its relationship with the production of cytokines and NF-κB activation. At baseline, PGD_2 and 15-$dPGJ_4$ are detectable in the tissue and there is

no detectable COX-2 activation. After 2 h of the inflammatory response, there is an active mobilization of PMNs to the site of inflammation and a rapid increment of PGE_2 coinciding with COX-2 activation. The progressive migration of PMNs is followed by a delayed production of LXA_4 and drop in PGE_2 levels at 4 h. The following events include the recruitment of MNCs at the expense of PMNs and activation of nitric oxide synthase (iNOS) at 6 h. At 24 h the % of PMNs keeps dropping while MNCs maintain a steady increment. At 48 h, PGD_2 and 15-$dPGJ_4$ and the % of MNCs remain in the tissue, possibly with the objective to repair the remaining damaged tissue. NF-κB DNA binding activity varies at different time points of the inflammatory response.



	0h	2h	4h	6h	24h	48h
COX2 expression	-	++++	++	+		++++
iNOS expression	-	-	-	***	-	-
COX2 activity	-	++++	+++	-	-	-
NF-κB binding				cRel/p50		p50/p50

Figure 1.9 Changes of inflammatory cells, lipid mediators and protein expression during acute inflammation (rat carrageenin-induced pleurisy) (Gilroy *et al*. 1999, Willis *et al.* 1996, Levy *et al*. 2001)

Recently it has been recognized that an inflammatory response is followed by an active "resolution phase" involving newly characterized lipid mediators: resolvins, maresins and protectins (Serhan, 2008).

Resolvins are metabolites derived from DHA (D-series) and EPA (E-series) that were first identified in exudates from murine dorsal air pouches. These compounds are biosynthesized by various immune cells, including leukocytes, via lipoxygenase mediated pathways. These compounds have been linked to regulatory properties and may be an alternative strategy for the prevention and treatment of chronic degenerative diseases (Serhan *et al.* 2008; Levy *et al.* 2010).

To conclude, lipid mediators play an important role in the course and resolution of inflammation. They have inflammatory and regulatory properties contributing to the resolution of an inflammation. Any imbalance in their regulatory signalling pathways may contribute to chronic inflammation.

1.2.6 Inflammation and metabolic diseases

As described in the previous sections, inflammation is a self-limited process activated in response to non-self-agents but also by endogenous signals in an attempt to return to homeostasis. Its course is tightly regulated by its own signalling elements, although unsolved inflammation is a determinant in the progression of some chronic degenerative diseases.

The role of inflammation in the development of metabolic diseases was suggested by early studies with salicylates (Gilgore *et al.* 1960). Since then epidemiological and clinical trials have reported evidence of the role of inflammatory markers in the pathogenesis of diabetes and cardiovascular diseases.

Hotamisligil and colleagues (1995) reported evidence of the role of the pro inflammatory cytokine TNF-α in regulating insulin action in adipose tissue. Recent studies in animal models have given insights into the role of specific components of inflammatory elements in insulin signalling in different tissues (Uysal *et al*. 1997; Lumeng *et al.* 2007c). Transgenic models have provided evidence of the role of TLR4 in the development of diet-induced

insulin resistance. Deletion of TLR4 resulted in an increment in metabolic rates and prevention of insulin resistance in high fat diet (HFD) fed mice (Shi *et al.* 2006; Tsukomo *et al.* 2007; Saberi *et al.* 2009).

Wild-type mice fed on a HFD presented an up regulation of NF-κB pathway adaptor proteins and disruption in insulin signalling pathways in several tissues. This effect was absent in TLR deficient mice (Tsukomo *et al.* 2007). Down regulation of TLR expression also prevented the migration of macrophages and formation of so-called crown-like structures (CLS) in adipose tissue (Saberi *et al*. 2009).

In agreement with previous reports, overfed transgenic animals adipose tissue and liver presented an up regulation of IL-6 and TNF-α mRNA levels (Tsukomo *et al.* 2007; Saberi *et al*. 2009). The deletion of TLR4 may prevent the activation of the NF-κB signalling cascade causing an inhibitory effect on TNF-α and monocyte chemotactic protein-1 (MCP-1) secretion. These changes in tissue activation state may prevent the infiltration of macrophages and other immune cells. In this context, inhibition of NF-κB may improve metabolic parameters partly by modulating lipid metabolism through PPAR signalling.

Studies in TLR4 knock out (KO) mice provided insights into the role TLR4 in insulin signalling. An intravenous infusion of free fatty acids caused a disruption of the insulin receptor in TLR4 KO mice when compared with wild type (WT). Additionally, there was a reduction by 40% in glucose turnover in WT but not TLR KO mice. At the end of a long term HFD intervention, WT adipose tissue presented higher levels of TNF-α, IL-6, SOCS3, MCP-1 and F4/80, an effect not seen in TLR KO mice (Tsukomo *et al.* 2007; Saberi *et al*. 2009).

The antagonistic effect of NF-κB on PPAR expression has been reported. The absence of inflammatory signals for NF-κB activation may keep PPAR-γ active facilitating FA oxidation. PPAR-γ has also been related to the expression of alternative macrophages (M2 phenotype) that mainly produce anti-inflammatory cytokines and proapoptotic molecules (Ricote *et al.* 1998; Odegaard *et al.* 2007). Taking this evidence together, suggests that alteration of the NF-κB pathway contributes to the improvement of FA and glucose metabolism mainly by

preventing TNF-α induced insulin resistance and increasing FA turnover through PPAR-γ signalling pathways

1.3 Obesity

1.3.1 Generalities

Obesity has been defined as an energy-imbalance disorder, characterized by an excessive accumulation of adipose tissue and changes in metabolic parameters. According to World Health Organization (WHO) and National Institute of Health (NIH), a subject is considered overweight if body mass index (BMI) lies between 25.0-29.9 kg/m^2 and obese if its BMI exceeds 30kg/m^2. The prevalence of obesity has tripled the last decade, representing one of greatest public health challenges (WHO).

In addition to the metabolic disturbances associated with obesity, reports in recent years have associated obesity with systemic low-grade inflammation. Inflammation found in obese individuals is the sum of different elements including metabolic adaptations to hypoxia and lymphocyte infiltration in adipose tissue. Unsolved chronic inflammation in obese people may contribute to insulin resistance and endothelium dysfunction that leads to obesity-related co morbidities.

1.3.2 Adipose tissue

1.3.2.1 Adipose tissue physiology

Adipose tissue is a complex organ, whose main parenchymal cells are adipocytes. Adipose tissue is involved in body temperature regulation, immune responses and a metabolic regulation. More than being just an energy compartment, adipose tissue represents a central organ where energy metabolism and inflammation regulation converge (Cinti *et al.* 2012). Adipokines, a family of signalling molecules secreted by adipose tissue, are engaged in endocrine and immune processes. The main adipokines and their functions are summarized in **Table 1.3**.

Name	Source	Inflammatory and metabolic effects
Leptin	Adipose tissue (leptin receptor)	Regulation of feeding behavior ↑ TNF- α, IL-6 and CCL in macrophages Regulates T cell balance (↑ Th1 phenotype, ↓Th2) Positively correlated to adiposity
Adiponectin	Adipose tissue (AdipoR1 receptor)	↑ FA oxidation ↓ glucose synthesis in liver ↓ phagocytosis, IL-6, TNF-α and ↑ IL-10 production in macrophages
Resistin	Adipocytes, macrophages and other cells	Related to insulin resistance in mice but not human Induce/induced by TNF-α and IL-6 Correlated to adhesion molecules levels (ICAM1)
Visfatin (NAMPT)	Adipose tissue and neutrophils	Insulin mimetic adipokine Modulation of B cell differentiation Important role in insulin secretion ↑ p38 and ERK & production of IL1β, TNF-α & IL6
RBP4	Adipose tissue and macrophages	Negative correlation with GLUT4 Inhibits insulin-induced phosphorylation of IRS1 Positive correlation with metabolic syndrome

Table 1.3 Adipokines and their function

(Tilg *et al*. 2006; Lago *et al.* 2009; Ouchi *et al.* 2011)

1.3.2.2 Metabolic aspects of adipose tissue

AT is a highly vascularized tissue susceptible to continuous nutrient influx. The post-prandial state is the period following food intake; it is characterized by an elevation in circulating glucose, insulin and TG concentrations. FAs and glucose from the diet are buffered by adipose tissue, proving an efficient energy reservoir to store and release energy when required. During this period a number of biological responses occur; these aim to promote appropriate nutrient uptake and to maintain hemostasis (Frayn, 2002).

During the fed state, FAs and other nutrients from the diet are hydrolysed and absorbed in the gut. Lipids are transported in the blood stream initially by chylomicrons and triglyceride-rich lipoproteins. Simultaneously, insulin exerts its anti-lipolytic effects by suppressing no

-esterified fatty acids (NEFA) release from adipocytes and triggering FA uptake. In conjunction with insulin, acylation stimulating protein (ASP), another element in this cascade, stimulates FA esterification and uptake in adipocytes (Frayn, 1998). Glycogenesis and FA storage metabolic routes are switched on. FA from chylomicrons and very low density lipoproteins (VLDL) are captured in the adipocytes and myocytes (muscle cells) by lipoprotein lipase (LPL) action and stored as TG (Frayn, 1998; Frayn, 2010) **Figure 1.10**.

Upon energy deprivation, NEFA derived from adipose tissue represent an important source of energy for muscle and other organs (Frayn, 2010). During the fasting state a lipolytic cascade is activated by catecholamines, promoting the increment of cyclic adenosine monophosphate (cAMP) levels followed by phosphorylation of hormone sensitive lipase (HSL) by protein kinase A (PKA) action. 95% of lipid hydrolysis is attributed to HSL and adipose TG lipase (ATGL) (Shweiger *et al.* 2006; Frayn, 2010). Intracellular TG is hydrolyzed and mobilized from adipose tissue to the circulation by HSL action in a rate-limiting manner.

To summarize, FA deposition in and release from adipose tissue are tightly regulated by hormonal signals that trigger FA esterification and mobilization from adipose tissue. The dysregulation of any of these pathways leads to an increment of circulating NEFA and ectopic fat deposition that has been positively correlated to metabolic disorders, such as insulin resistance, and dyslipidemia

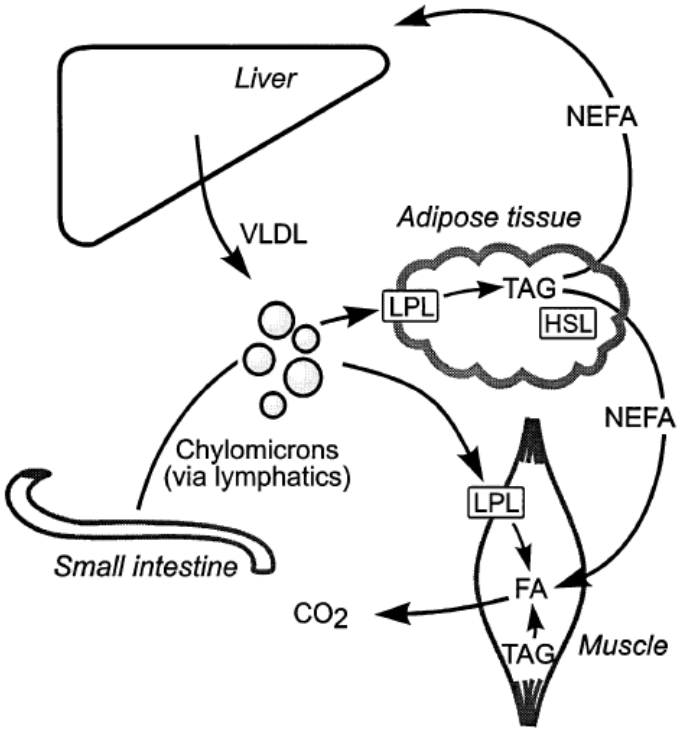


Figure 1.10 Scheme of FA metabolism and movement in white AT and liver (Frayn, 1998)

1.3.2.3 Origins of inflammation in adipose tissue

Inflammation found in adipose tissue from obese subjects has been associated with insulin resistance and development of chronic degenerative diseases. Immune cells infiltrating adipose tissue play a major role in insulin resistance, via inhibition of its signalling cascade.

There are various theories explaining the origins of adipose tissue inflammation and the presence of lymphocytes in this tissue. This process can be simplified by the following steps: 1) in the early stages, adipose tissue overgrowth increases oxygen demands; hypoxic signals trigger angiogenesis 2) tissue remodelling and angiogenesis signals are accompanied by inflammatory signals and changes in adipose tissue population 3) macrophages populate AT in junction with other inflammatory cells in an attempt to repair damage 4) chronic AT

inflammation is fed by inflammatory resident cells metabolites and chronic exposure to components from other organs and the diet (obesogenic environment) (**Figure 1.11**).

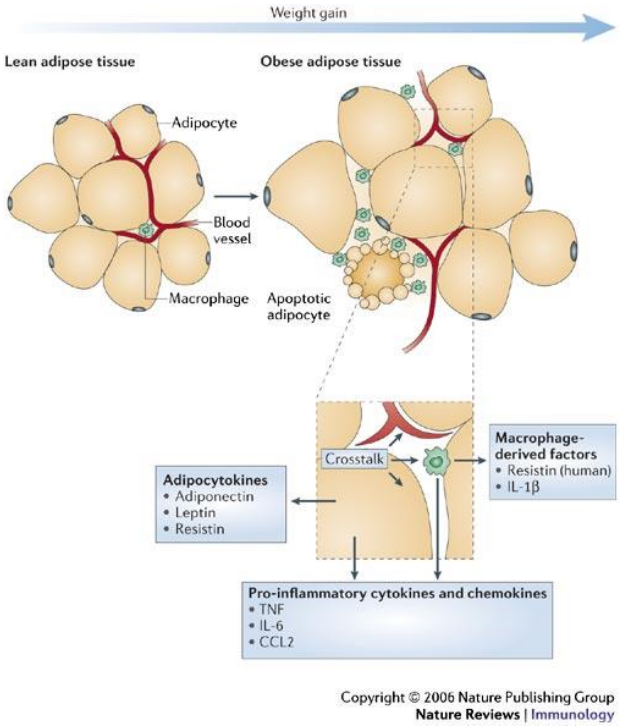


Figure 1.11 Development of AT inflammation

(Tilg, 2006)

1.3.2.3.1 Hypoxic signals

The first response during adipose tissue overgrowth is the development of new storage units (adipocytes) and vessels to ensure tissue oxygenation. Recent studies have explored the role of hypoxia in adipose tissue homeostasis. Both, rodent and human studies, have agreed that excess adiposity results in dysfunctional blood flow and insufficient pO_2 in this tissue (Pasarica *et al*. 2010; Ye *et al.* 2011; O'Rourke *et al*. 2011). When adipose tissue blood flow and pO_2 is measured, normal weight subjects have 2-fold higher oxygenation than obese (Pasarica *et al.* 2010). Hypoxia triggers angiogenesis through NF-κB pathway activation preceded by activation of hypoxia-inducible-factor 1a (HIF-1a) (Rius *et al*. 2008). These events are accompanied by endoplasmic reticulum stress (UPRS) and inflammatory signals that subsequently attract leukocytes and inflammatory cells to cope

with tissue remodelling challenges. Under an inflammatory environment PPAR-y is antagonized, provoking an interruption of insulin signals and leading to alterations of FA storage and oxidation, reflected by excessive release of FFA in the circulation (Odegaard *et al.* 2007).

1.3.2.3.2 Immune cells recruitment

The molecules produced from hypoxic cells trigger changes in adipose tissue population and macrophage recruitment to deal with hypertrophic tissue and moribund adipocytes.

Lymphocytes represent 10% of the total adipose tissue population, and play an important role in tissue homeostasis (Feuerer *et al.* 2009). In animal models, these lymphocytes display an active mobilization to adipose tissue determined by microenvironment changes (Feuerer *et al.* 2009).

T cells seem to be one of the first cell populations to be affected during the development of obesity. In *in vivo* models, the $CD4^+FoxP3^+$ (T-reg) population multiplies from birth to adulthood, and its levels are depleted during obesity development (Feuerer *et al.* 2009). In contrast, $CD8^+$ and $CD3^+$ cells are progressively recruited in adipose tissue during weight gain (Kintscher *et al.* 2008; Nishimura *et al*. 2009). These population changes coincide with a steady increment of inflammatory and chemoattractant genes (metallopeptidase domain-8 (ADAM-8), macrophages inflammatory protein (MIP1A), MCP-1, macrophages antigen-1 (MAC-1), F4/80 and CD68) (Xu *et al.* 2003).

It has been suggested that $CD8^+$ T cells dictate the mobilization of macrophages to the adipose tissue by humoral signals that occur during the development of obesity (Nishimura *et al.* 2009). *In vivo* models have demonstrated that CD8 specific antibody reduces the numbers of $CD8^+$ and M1 macrophages without affecting $CD4^+$ and M2 macrophages populations (Nishimura *et al.* 2009). In obese rodents, $CD8^+$ T cells are localized in hypoxic areas coinciding with macrophages (F4/80) (Rausch *et al.* 2008).

These transgenic mice findings have been confirmed in human studies, where visceral adipose tissue of obese subjects presented a higher numbers of $CD3^+CD8^+$ and $CD3^+CD4^+$

cells than normal weight (O'Rourke *et al*. 2011). Additionally, FoxP3 mRNA has been found in lower levels in obese than normal weight individuals (Feuerer *et al.* 2009).

MCP-1, a chemotactic molecule and a potent $CD8^+$T cells activator, has been postulated as another factor determining a sustained macrophages trapping in AT (Kanda *et al.* 2006). In transgenic mice, deletion of MCP-1 gene partially prevents the macrophage recruitment in AT and insulin resistance induced by HFD (Kanda *et al*. 2006). MCP-1 KO mice had a reduction $F4/80^+/CD11^+$ macrophage population (Lumeng *et al*. 2007a). These results suggest that MCP-1 KO mice are protected from insulin resistance due to alterations of the M1 macrophage population.

Once established, most of the adipose tissue inflammation and related insulin resistance has been attributed to cytokines derived from resident macrophages (Hotamisligil *et al.* 1995; Bourlier *et al.* 2008). Macrophages are dominant among the immune cells residing in adipose tissue. In mice macrophages represents about 10% and 40-50% of the total AT population; a similar pattern has been reported in human (Weisberg *et al.* 2003).

The tissue resident macrophage profile is heterogeneous and defined by micro environmental factors. The M1/M2 population ratio varies between individuals (Weisberg *et al*. 2003; Lumeng *et al.* 2007a, Zeyda *et al.* 2007, Bourlier *et al*. 2008). Animal models have demonstrated mobilization of macrophages $F4/80^+$ to adipose tissue during obesity development (Weisberg *et al.* 2003; Kintscher *et al.* 2008) In lean mice, adipose tissue macrophages present a high expression of M2 macrophage features (chitinase 3-like 3 (Ym1), arginase and IL-10), while obese mice adipose tissue contains a high expression of M1 macrophages (F4/80, CD11c, TNF-α and iNOS) (Lumeng *et al*. 2007a; Lumeng *et al.* 2008; Patsouris *et al.* 2008). These reports have given valuable information about the mobilization of macrophages to adipose tissue. Nevertheless, some of these findings should be analysed carefully when translated to humans.

Recent clinical trials reported mixed M1/M2 macrophage phenotype in normal weight and obese subjects. In humans, total adipose tissue macrophages exhibit low levels of arginine metabolism/iNOS and high levels of $CD14^+$ and lymphatic vessel endothelial hyaluronan

receptor (LYVE-1) markers (Bourlier *et al.* 2008). BMI was correlated to adipose tissue macrophage populations (Bourlier *et al.* 2008, Spencer *et al.* 2010). Obese subjects presented higher levels of $CD206^{+}$ than normal weight and the majority of these macrophages in adipose tissue were associated to fibrotic areas (Bourlier *et al.* 2008; Spencer *et al.* 2010).

Macrophages are accumulated in specific compartments, the majority being recruited in visceral adipose tissue, and in a lesser extent in subcutaneous adipose tissue (Clément *et al*. 2004, Cancello *et al.* 2006; O'Rourke *et al.* 2011). The presence of macrophages in adipose tissue is positively correlated with liver fibro-inflammatory lesions and lipid metabolism imbalance (Cancello *et al.* 2006).

Basal secretion of pro-inflammatory cytokines is higher in obese than normal weight subjects (Hotamisligil *et al*. 1995; O'Rourke *et al.* 2011). *Ex vivo* culture of adipocytes from obese subjects showed higher secretion of TNF-α, IL-6, MCP-1 and IL-10 (O'Rourke *et al.* 2011). TNF-α secretion levels were correlated to insulin sensitivity and TGs plasma levels (Hotamisligil *et al*. 1995). TNF KO mice were protected against obesity induced insulin resistance (Uysal *et al.* 1997).

*In viv*o, the presence of macrophages in adipose tissue triggered the production of TNF-α, IL-6 and IL-1, causing a disruption of glucose transporters and limiting glucose uptake by adipocytes (Lumeng *et al.* 2007b, Lumeng *et al.* 2007a). The majority of TNF-α produced in adipose tissue has been attributed to macrophages. Deletion of the TNF gene in different diabetes and obesity models resulted in an improvement in glucose uptake and insulin sensitivity but not total alleviation of insulin resistance (Uysal *et al.* 1997). One of the mechanisms of action is the role of TNF-α in the serine kinases activation and further c-Jun N-terminal kinase (JNK) phosphorylation that has been implicated in the regulation of insulin signalling pathways (insulin receptor substrate (IRS), etc.); this action is also correlated with disruption of PPAR-γ function (NF-κB involved). Thereby, insulin resistance in obese subjects can be partly explained by the inflammatory molecules produced by macrophages.

The heterogeneity of the macrophage population and the use of different markers in in vivo and human studies make interpretations complicated. Enzymatic activity (arginase/COX) ratio and cytokine levels may be good markers to consider when determining macrophage activation state.

These reports give insights of the elements involved in inflammation development and recruitment of immune cells in adipose tissue. In a non-obesogenic environment, adipose tissue resident T cells and macrophages play housekeeping roles, dealing with remodelling and metabolic homeostasis. Tissue overgrowth leads to signals aiming to solve tissue overgrowth requirements. Excessive efflux of FA from the diet prompts structural and cell population adjustments, having a negative long-lasting effect on adipose tissue equilibrium and cell signature.

1.4 FA and inflammation

1.4.1 FAs and cell membrane microdomains (rafts)

Membranes are lipid bilayers composed mainly of phospholipids and trans-membrane proteins. Cell membrane composition heterogeneity gives rise to a mosaic of lipid micro-domains that affects its functionality, nutrient flux and responsiveness (Shaikh, 2010).

Lipid rafts are sphingolipid /cholesterol-rich membrane clusters serving as signalling platforms. According to biophysical and microscopy studies, lipid domain distribution and composition is altered in nanoseconds after external stimuli (Shaikh, 2010). Under an inflammatory response, adaptor proteins and transmembrane receptors translocate in lipid rafts. Rafts coalesce giving rise to large assemblies serving as a platform for cell communication (Yaqoob, 2009).

SFA and PUFA alter membrane fluidity and raft distribution. *In vitro* studies suggest that palmitic and stearic acids positively interact with cholesterol, facilitating lipid raft construction and amplification of inflammatory signals by anchored protein recruitment, such as c-Src (Holzer *et al.* 2011). Similarly, lauric acid mimics lipopolysaccharide's (LPS) effect, recruiting TLRs and adaptor molecules in lipid rafts (Wong *et al.* 2009). DHA and EPA

prevented the translocation of these molecules to rafts, presumably by altering cholesterol domains and creating non raft domains, disrupting the optimal raft signalling conditions (Wong *et al.* 2009, Shaikh, 2010).

1.4.2 TLR modulation by FA

Lipid A, a molecule acylated with SFAs, is a component of LPS that accounts for some of its inflammatory properties. Manipulation of Lipid A structure by removal of acylated SFA results in loss of its toxicity (Akira *et al.* 2001).

Studies by Hwang and colleagues gave insights of the effect of different FAs in NF-κB modulation through TLRs (Hwang *et al.* 2001; Lee *et al.* 2003a). Genetic manipulation was performed to produce cells with constitutive activation of TLR4 (Lee *et al.* 2003a; Lee *et al.* 2003b). Additionally, dominant negative (DN) cells for different downstream molecules involved in NF-κB signalling were produced to provide more information about the mechanisms of action involved (Lee *et al.* 2003a; Lee *et al.* 2003b).

These *in vitro* studies showed an agonistic effect of lauric acid on NF-κB and COX2 signalling Lee *et al.* 2003b). Some PUFA and MUFA had a lower effect on these pathways compared with lauric acid. Activation of NF-κB and COX2 induced by lauric was prevented in TLR4 DN cells, suggesting that the inflammatory effect of lauric acid was related to TLR4 activation. Under LPS challenge and constitutive TLR4 activity, phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) DN cells prevented NF-κB binding activity, but not myeloid differentiation primary response gene 88 (MyD88) DN. NF-κB binding activity induced by lauric acid was also blocked in PI3K DN cells.

DHA inhibited NF-κB induced by constitutive activation of TLR4 and LPS in a dose dependent manner (Lee *et al.* 2003b). Additionally, it reduced the expression of iNOS and IL-1 and antagonized the expression of IκBα (Lee *et al.* 2003a). DHA effects of TLR and NF-κB signalling were associated with prevention of AKT phosphorylation.

A study reported by Shi *et al* (2006) provided a better understanding of the role of dietary FAs in TLR regulation and metabolic homeostasis *in vivo*. Similarly to Lee *et al* (2003), Shi

and colleagues demonstrated that lauric acid and other SFA induced IL-6 mRNA expression in RAW murine macrophages. A combination of oleate and pamitate mimicked the effect of LPS on TNF mRNA levels in a dose dependent manner. DHA had no effect on inflammatory cytokine expression and prevented the inflammatory effect induced by SFA on TNF mRNA levels (Shi *et al.*2006). In contrast with Lee et al findings, this study concluded that MyD88 was involved in TLR-FA inflammatory signalling. An explanation for this discrepancy in the literature might be the use of different cell lines and FA doses.

In the same study, FFA (palmitate/oleate) intravenous infusion in WT mice induced high NF-κB binding activity in AT when compared with TLR4 KO mice. Obese diabetic rodents presented higher expression levels of TNF-α, IL-6 and MCP-1 compared with the WT.

In human, TLRs activation has been studied in normal weight subjects during a high fat challenge (Ghanim, 2009). TLR2 and 4 were upregulated in mononuclear cells in the postprandial state during the first 3 h after a HFM challenge.

These results suggest that FAs are able to modulate TLR and have an effect on cell transcription. As mononuclear cells and AT are constantly exposed by FFA from the diet, TLR activation and its inflammatory signals may have detrimental effects on insulin homeostasis and inflammation status.

1.4.3 FA in cytokines and gene expression regulation

In a basal state n-9 PUFA and n-3 PUFA present little or no effect on the NF-κB signalling pathway. Lauric, palmitic, stearic and AA are able to induce inflammatory signals without inflammatory stimuli (Camandola *et al.* 1996; Lee *et al.* 2003; Schumann *et al.* 2010). As described previously this effect is explained by FA-TLR interactions that trigger endotoxin-like signals.

Macrophages maintained in culture with different FAs present a different response under LPS challenge. SFAs, especially lauric acid, potentiates LPS effect by inducing NF-κB and COX, which in turn increases inflammatory cytokine gene expression and prostaglandin synthesis (Lee *et al.* 2003).

Under LPS challenge, n-3 PUFA prevent IκBα phosphorylation trapping NFκB subunits in the cytoplasm (Camandola *et al.* 1996; Lee *et al.* 2003; Schumann *et al.* 2010).

AA has been reported to exert pro and anti-inflammatory effects on NF-κB activity depending on the stimulus (Camandola *et al.* 1996; Novak *et al*. 2003; Lee *et al.* 2003; Schumann *et al.* 2010). Schumann *et al* (2010) showed that PUFA incubation time results in a different cytokine profile Long term supplementation of DHA and AA were more effective reducing NF-κB activity. These studies together suggest the role of FA in the modulation of macrophages inflammatory profile. SFA induce a pro-inflammatory cytokine repertoire while n-3 PUFA prevent cell activation.

The effect of feeding mice with different FAs was studied by Yaqoob *et al* (1995). This study explored the effect of olive oil, safflower oil, coconut oil and menhaden (fish) oil on peritoneal macrophage function (Yaqoob *et al.* 1995). After the feeding period, macrophages were isolated and treated with LPS. The FO group presented a significant reduction in PGE_2, TXB_2 and 6-keto PGJ_2 production when compared with the other oils. TNF-α level after LPS treatment were lower in olive oil, safflower and FO than low fat diet group. Coconut oil diet seemed to potentiate LPS effect by inducing an increment in TNF-α production. Only FO diet reduced IL-6 levels at the basal state and under LPS challenge.

These results coincide with findings from some human studies. Caughley *et al.* (1995) explored the effect of sunflower, safflower and FO on cytokine and some eicosanoid synthesis in healthy men. After 4 weeks supplementation with safflower and FO, n-3 PUFA were incorporated into cell membranes and the levels of TNFα, IL-1β, TXB_2 and PGE_2 were reduced significantly; sunflower did not change any of these markers. FO was rapidly incorporated in mononuclear cells and induced a potent reduction of the molecules mentioned above. These studies and others conclude that FAs have different effects on the immune response.

Genomic analysis of PBMC and AT revealed the impact of chronic exposure to different FAs in mature adults (>45y) in diverse pathways.

SFA diet up regulated genes related to immune system, inflammation and lipogenesis and downregulated ADIPOQ and PPAR-γ gene expression (Bouwens *et al.* 2009, van Dijk *et al.* 2012). MUFA and n-3 PUFA down regulated SREBP1, suggesting an anti-lipogenic effect.Chronic FO supplementation induced a reduction of hypoxia markers in PBMC and AT in young and mature adults (Bouwens *et al.* 2009; Itariu *et al.* 2012).

1.5 Knowledge gaps

As described in previous sections, chronic inflammation is the sum of various elements including overfeeding. Previous reports have described the basal and postprandial inflammation after acute exposure to n-3 FAs (van Dijk et *al.* 2012, Itariu *et al.* 2012). However, the changes in inflammatory markers in middle age obese and normal weight subjects after acute and chronic consumption of n-3 PUFA has not been reported.

NF-κB, one of the transcription factors involved in inflammation initiation, has been recently linked to its resolution (Lawrence *et al.* 2001). SFA, but not PUFA, mimicked endotoxin effect on NF-κB and COX activation in murine macrophages (Lee *et al.* 2001). Lo *et al* (1999) found an inhibitory effect of EPA on p65 dimers resulting in a reduction of inflammatory cytokines production, although p50 binding was not affected. These reports suggest that NF-κB cascade is modulated differently by FAs. Although the changes in NF-κB dimers and its cascade elements induced by different dietary FAs are not fully understood.

My project aims to further explore the anti-inflammatory effects of n-3 PUFA with an emphasis on the post-prandial response and on possible differences between normal weight and obese subjects. In parallel, work will be done with a cultured cell line, to enable a deeper evaluation of molecular mechanisms involved.

CHAPTER 2

Modulation of inflammatory signals by different fatty acids in THP-1 derived macrophages

CHAPTER 2

Modulation of inflammatory signals by different fatty acids in THP-1 derived macrophages

2.1 Introduction, Objective Hypotheses and Aims

2.1.1 Introduction

Macrophages are tissue mononuclear cells derived mainly from monocytes circulating in the blood stream. As part of the innate immune system, macrophages play a role as an early line of defence against pathogens and hazards from the environment. In the absence of inflammatory stimuli, macrophages remain quiescent and have homeostatic roles. Macrophage housekeeping tasks ensure proper tissue functioning and their activation during infection drives the course of a controlled inflammatory response.

Tissue resident macrophages are programmed by external stimuli leading to the production of regulatory or inflammatory molecules. The fluctuations in inflammatory molecules also switch macrophage phenotype into M1 which are inflammatory or M2 which are regulatory (**Chapter 1**). The balance between the activation of nuclear factor kappa B (NF-κB) and of peroxisome proliferator-activated receptor γ (PPAR-γ) is involved in the differentiation to the M1 or M2 phenotype respectively.

NF-κB is a transcription factor induced during inflammation and cell survival processes. During an inflammatory response, NF-κB is activated through downstream signals triggered by TLR activation. In turn, NF-κB activation initiates the transcription of genes encoding inflammatory cytokines and lipid metabolism enzymes (COX and LOX) leading to lipid metabolite production. These elements act together to guide the progression and then the resolution (i.e. the end) of the inflammatory response (**Chapter 1**).

Fatty acids (FAs) have the ability to modulate the activation of NF-κB and so to change inflammatory gene transcription (Lee *et al*. 2001). Some in vitro studies have reported the ability of saturated fatty acids (SFA) to directly trigger the activation of NF-κB through TLR-

4. Exposure of murine macrophages to SFAs induced an up regulation of NF-κB binding activity, while this effect was counteracted by polyunsaturated fatty acids (PUFA) especially the n-3 PUFA, DHA (Lee *et al.* 2001; Shi *et al.* 2006). The mechanisms of action involved in these effects have been related to the similarity of SFA with Lipid A present in LPS (lipopolysaccharide), the natural agonist of TLR4, and an effect on membrane lipid rafts organization (Akira *et al.* 2001; Wong *et al.* 2009). N-3 PUFA act directly on raft membrane organization and might inhibit NF-κB activation by switching the production of inflammatory mediators into more regulatory molecules like resolvins (Serhan *et al*. 2008; Wong *et al.* 2009; Yaqoob, 2009). However, the role of different FAs in the modulation of the NF-κB pathway in macrophages and the subsequent production of peptide and lipid mediators has not been fully explored. The work described in this chapter establishes laboratory procedures to investigate the effect of different FAs on NF-κB activation and subsequent cytokine and lipid mediator production in macrophages derived from THP-1 monocytes.

2.1.3 Aim

The aim of the present work is to further explore the role of fatty acids in the modulation of NF-κB signalling cascade and the production of inflammatory molecules (both cytokines and lipid mediators).

2.1.2 Hypotheses

The hypotheses to be tested in the work described in this chapter are that incorporation of fatty acids into membranes of THP-1 derived macrophages:

- will modify the activation of NF-κB;
- will modify the production of inflammatory molecules; and
- that different fatty acids will have different effects

2.1.4 Objectives

The specific objectives of the work described in this chapter are:

1. To establish a macrophage model derived from the THP-1 monocytic cell line.
2. To evaluate the activation of the NF-κB pathway and of lipid metabolism enzymes (COX) during an inflammatory response in THP-1 macrophages
3. To evaluate the production of cytokines and lipid mediators during an inflammatory response in THP-1 macrophages
4. To analyse the incorporation of eicosapentanoic acid (EPA), docosahexanoic acid (DHA), arachidonic acid (AA), linoleic acid (LA), stearic acid, lauric acid and palmitic acid in THP-1 macrophages.
5. To evaluate the effect of these fatty acids on the NF-κB signalling pathway
6. To evaluate the effect of these fatty acids on the production of inflammatory molecules.

2.2 Methods

2.2.1 Reagents and materials

Reagents and materials used for experiments are described in **Appendix I**.

2.2.2 THP-1 Cell Culture and differentiation

THP-1 cells were cultured at 5-7 x 10^5 cells/mL in RPMI 1640 medium supplemented with 10% FBS, L-glutamine (0.14 mM), penicillin (50 U/mL), streptomycin (50 µg/mL), HEPES buffer (15 mM) and were maintained at 37°C in 5% CO_2. To differentiate THP-1 cells into macrophages, they were stimulated with supplemented medium containing 100 ng/mL phorbol 12-myristate 13-acetate (PMA) over 72 h (5 x 10^5/mL). After PMA stimulation, cells were washed three times (twice with phosphate buffered saline (PBS) and once with supplemented medium). Differentiation was followed by 5 days cell maturation (**Figure 2.1**). During this period cells were cultured in supplemented medium (not containing PMA). Cells' medium was changed every second day. Cell viability and adherence were determined by cell counting with trypan blue staining (0.4%). Only cultures with more than 90% viability were used in further experiments. LPS and fatty acid experiments were performed after cell maturation as described in **Figure 2.1**.

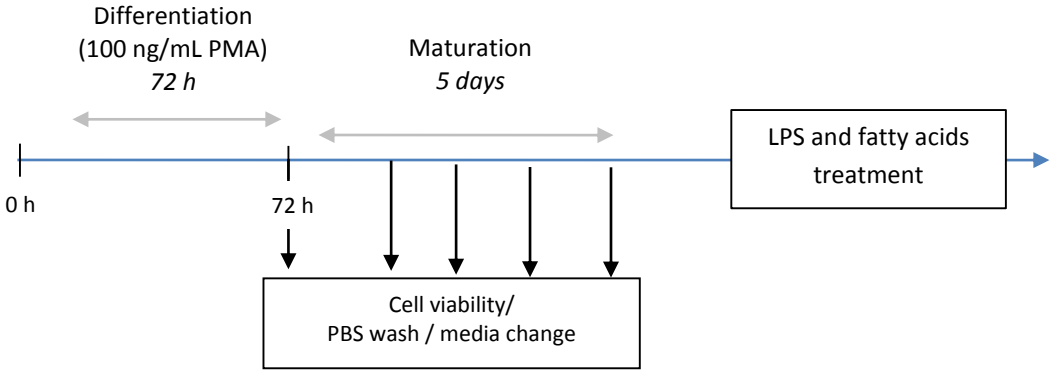


Figure 2.1 Experimental design: THP-1 monocytes differentiation

2.2.3 Assessment of the morphology of THP-1 monocytes and macrophages and of surface markers

2.2.3.1 Microscopy

Cell morphology was monitored over the differentiation period using an AxioVert 200® microscope and images were recorded using AxioVision® software.

2.2.3.2 Cell surface marker analysis by flow cytometry

2.2.3.2.1 Principle

Flow cytometry is a technique based in biophysical technology that allows the analysis of molecules' physical characteristics and properties. The flow cytometer is composed of 1) a fluidic system, that creates a single file of particles leading them to a laser beam 2) optics systems, particles pass through beams of light (lasers) and light signals generated are directed by optical filters to specific detectors and 3) electronic systems: when light signals hit the detector a small current is generated and this voltage is translated to electrical signals and processed in a computer software (**Figure 2.2**) (BD Bioscience, 2000).

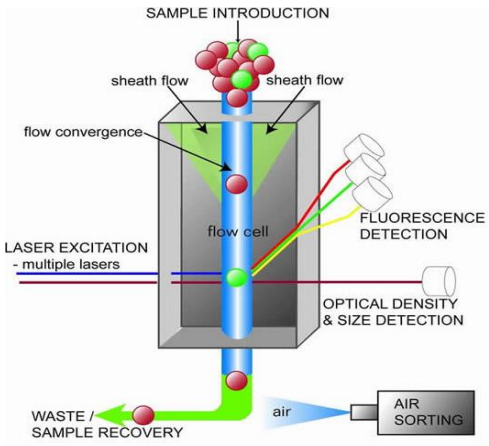


Figure 2.2 Flow cytometer system for sample analysis

2.2.3.2.2 Analysis of cell surface expression of CD11c and CD14

Following PMA incubation and resting in culture, THP-1 monocytes and macrophages were harvested and surface marker expression assessed by flow cytometry. Cells were detached from the flask by incubation in 500 µL of cell dissociation solution for 5 min at 37°C. Cell viability was monitored and density was adjusted to 1 x 10^6 /mL in PBS. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-human monoclonal antibodies against CD14, CD11c, CD206, IgG1,κ1 and IgG_{2b},κ at 10 µg/mL for 30 min at 4°C in the darkness. An unstained negative control was run for each stained sample. After incubation, samples were washed with FACS cell wash, centrifuged at 500 x g, resuspended in 200 µL of FACS buffer and analyzed in a FACS Calibur flow cytometer (Becton Dickinson) employing CellQuest Pro software.

Forward and side scatter were analyzed to determine cell size, granularity and viability. A total of 10,000 viable events were collected per sample and FITC fluorescence from cells isolated by gating was used for further analysis.

2.2.3.3 Macrophage identification by immunohistochemistry (IHC)

2.2.3.3.1 Principle

IHC is a technique that combines immunological and biochemical techniques to identify molecules and elements present in tissues and cells. Using the antibody binding principle, samples are stained with antibodies of interest and the signal is amplified by coupling antibody to an enzyme complex capable to produce a color-producing reaction (**Figure 2.3**).

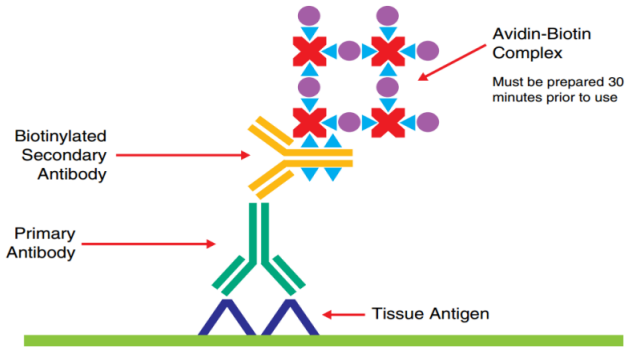


Figure 2.3 Schematic representation of protein signal amplification (ABC complex) (Kurmar and Rudbeck, 2009))

2.2.3.3.2 CD68 staining

THP-1 monocytes were adjusted to 0.7 x 10^6/mL and kept on ice. 70 µL of cell suspension was placed in cytospin inserts and centrifuged at 800 *g* for 5 min at room temperature to attach cells onto a glass cover. Cytospins were allowed to dry for an hour before staining was started. As macrophages are adherent cells, circular glass covers coated with polyglycine (30 min polyglycine treatment, 3 washes with sterile water and UV light treatment for 2 h) were placed in the culture wells used for differentiation. Then, the glass covers with THP-1 monocytes (cytospins) or macrophages (glass covers) were stained using the following procedure. Cells were fixed with methanol (10 min) and blocked for non-specific binding with blocking solution for endogenous peroxidases (30 min), before incubation with avidin (20 min), biotin (20 min) and culture medium (60 min). Slides were incubated with monoclonal mouse anti-human CD68 diluted in tris-buffered saline buffer (TBS) at 1:50 (vol : vol) for 60 min. Signal was amplified by using a biotinylated rabbit anti-mouse antibody in tris-buffered saline (TBS) at 1:1000 (vol : vol) for 60 min followed by avidin biotinylated peroxidase complex solution (ABC solution prepared 1 h before use. Slides were then incubated with 200 µL 3-Amino-9-EthylCarbazole (AEC) substrate for 20 min to visualize staining followed by counterstaining with Meyer's haematoxylin for 60 sec. After this process, the slides were covered with 250 µL of aqueous mounting medium and baked at 80°C for 20 min. The slides were covered with pertex and a coverslip. Images were collected in an Axiovert 200® microscope using AxioVision® software.

2.2.4 Fatty acid treatment of THP-1 macrophages

2.2.4.1 Producing albumin: fatty acid complexes

Fatty acids were bound with fatty acid free albumin according to the method of Mahoney *et al.* (1977). Briefly, fatty acids were diluted in a solution containing chloroform and 0.015 M KOH, dried under nitrogen (N_2) and heated at 70^{o}C for 60 min in a N_2 atmosphere. Fatty acid free albumin was diluted in Ca^{+} and Mg^{+} free PBS and added to the K^{+} salt of the fatty acid (fatty acid: albumin ratio= 1:1 molar). This solution was flushed with N_2, capped and placed in a shaker for 24 h at 37^{o}C. After 24 h incubation, pH was adjusted to 7.2 with KOH and volume adjusted with supplemented medium to achieve 50 µM of fatty acid.

2.2.4.2 Incubation of THP-1 macrophages with fatty acids

Following differentiation, THP-1 derived macrophages were incubated with supplemented medium (control), vehicle (supplemented medium with albumin) or supplemented medium containing albumin: fatty acid complexes for 5 h. Fatty acid concentrations in the medium and their incorporation into cells were evaluated as described in section **2.3** and **Appendix I.**

After incubation with fatty acids or control, cells were treated with medium containing or not LPS at a concentration of 0.1 µg/mL for different durations as previously described in **Figure 2.4**. Supernatant and cells were harvested and stored at -80°C until analysis.

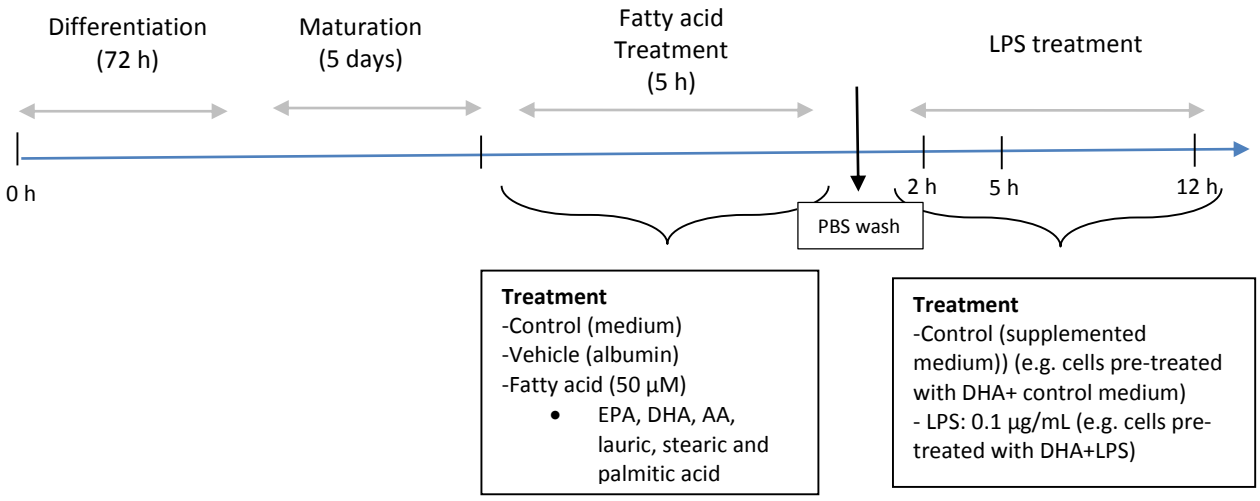


Figure 2.4 LPS and fatty acids treatment

2.2.5 THP-1 fatty acid analysis by gas chromatography

2.2.5.1 Principle of gas chromatography

Gas chromatography (GC) is a method employed to separate fatty acids based on their affinity to a silica capillary column. This depends on the carbon chain length and the number of double bonds and their position. Fatty acid-silica lining interactions increases with chain length and the opposite occurs with the number of double bonds. The gas chromatograph contains a heated injection port, a silica capillary column situated in an oven and a detector (**Figure 2.5**). Fatty acids methyl esters (FAMEs) are injected into the gas chromatograph port and heated at high temperatures to become volatile.

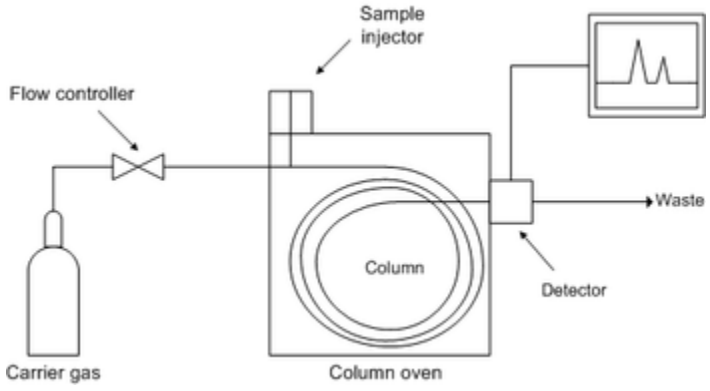


Figure 2.5 Schematic representation of gas chromatograph system

FAMEs are transported by a helium or hydrogen flow through the capillary column, which has a lower temperature than the injection port allowing FAMEs to condense on the column lining. The column is then heated and FAMEs are dissociated from the column according to their affinity to the silica lining. A flame ionization detector held at 250°C is located at the end of the column. FAMEs combustion generates an ion current proportional to the amount of FAMEs in the sample. This process results in a chromatogram containing peaks, each corresponding to a different FAME (**Figure 2.6**).

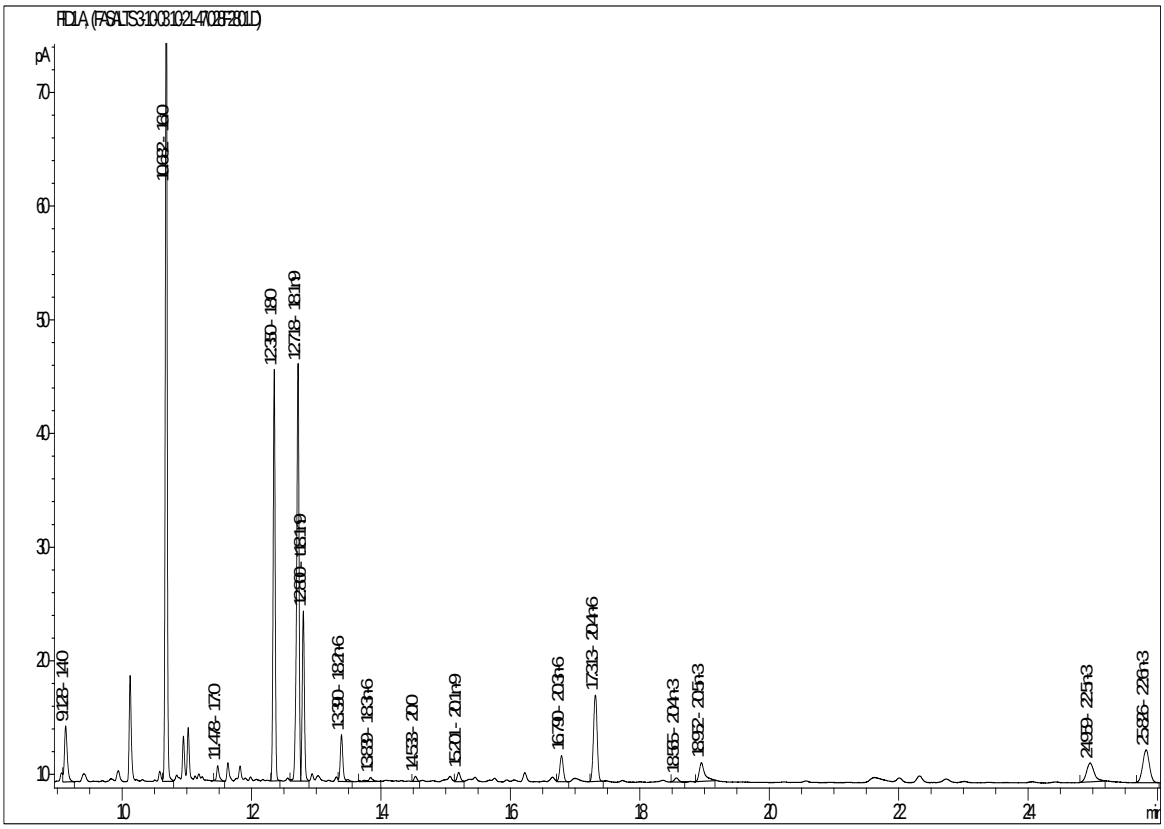


Figure 2.6 Gas chromatography trace of THP-1 macrophage fatty acids

2.2.5.2 Lipid extraction and formation of FAMEs

Total lipid was extracted from cell pellets and culture medium into chloroform : methanol (2 : 1, vol : vol). Lipid was dried under N_2 and then resuspended in toluene. To obtain the FAMEs, the lipid extracts were incubated at 50 ^{o}C for 2 h in the presence of methanol containing 2% (vol : vol) H_2SO_4. Following methylation, the samples were left at room temperature for approximately 5 min to cool and then neutralized with a solution containing $KHCO_3$ and K_2CO_3. The FAMEs were diluted in hexane and dried under N_2. Samples were resuspended in hexane and transferred into small vials for GC analysis.

2.2.5.3 GC analysis

GC analysis was performed in a Hewlett-Packard 6890 gas chromatograph fitted with a SGE BPX-70 capillary column (30 m x 0.2 mm x 0.25 μm) and a flame ionising detector. Split ratio was programmed to 2:1 to allow a higher sample uptake in the column. Injector port was set at 300°C and helium was used as the carrier gas for FAMEs transport. To condense FAMEs, the oven was kept at 115°C for 2 min increasing 10°C / min; a maximum temperature of 200°C was held for 18 min. After this cycle, the column reached 245°C. FAME histograms produced were analysed with Agilent ChemStation software. 37 FAMEs and menhaden oil standards were used to identify fatty acids according to FAME retention time and for software calibration.

2.2.6 Cytokine production analysis by fluorescent bead immunoassay

Th1 and Th2 cytokine concentrations were measured by flow cytometry using FlowCytomix® kits according to the manufacturer's instructions. Briefly, a mixture of beads coated with antibodies to Th1 and Th2 cytokines were incubated with samples and standards. A biotin-conjugated second antibody was added, to bind molecules captured by primary antibodies. Streptavidin-phycoerythrin was added and fluorescence signals emitted were analyzed by running the samples on a FACS Calibur flow cytometer and using CellQuest® software.

Bead populations were separated into two sets, distinguished by different size allowing simultaneous quantification in one fluorescence channel. Setup beads were used to adjust FSC and SSC to visualize the two bead sets. A region was gated per set and a dot plot was produced for each. The voltages of X and Y-axes (PE) were adjusted to position the beads in the left side of the dot plots and to separate bead populations (**Figure 2.7**). At least 300 events per analyte were recorded and data were analyzed using FlowCytomix® Software. Flow cytometer set up, compensation and assay sensitivity are described in **Appendix I**.

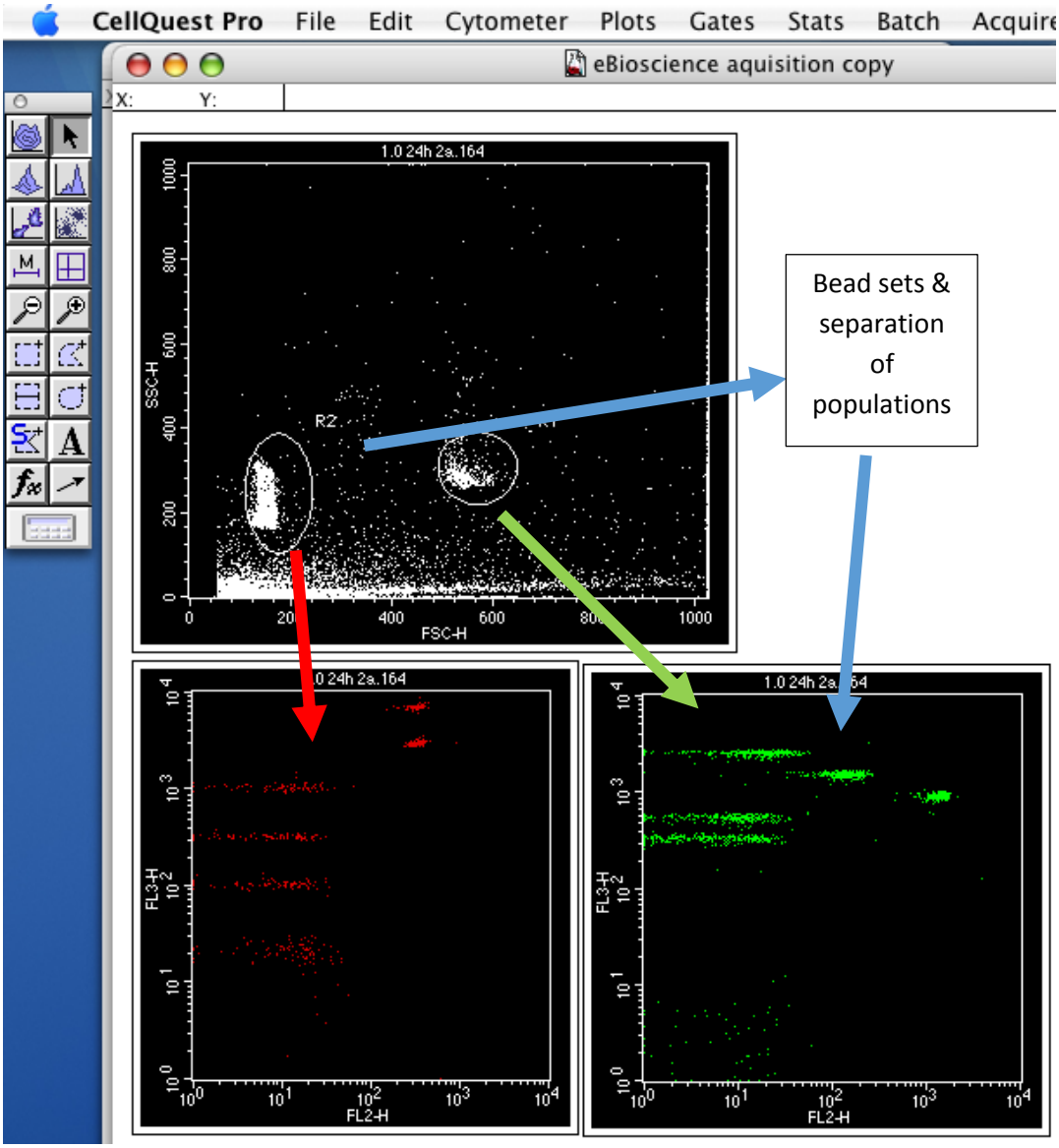


Figure 2.7. Example of bead populations and separation for multiple cytokine analysis

2.2.7 Measurement of lipid mediators by enzyme immunoassay (EIA)

2.2.7.1 Measurement of PGE_2 and TXB_2 concentrations using a competitive-enzyme immunoassay (EIA)

2.2.7.1.1 Principle

Competitive EIA is based on the competition between the analyte of interest and the enzyme conjugated version of the same analyte (tracer) for a small number of binding sites. Tracer concentrations are constant in all wells while analyte concentrations vary according to sample concentration. The amount of tracer is inversely correlated to the amount of analyte in the sample. Thereby, as more analyte is present in the sample less tracer will bind antibody binding sites (**Figure 2.8**).

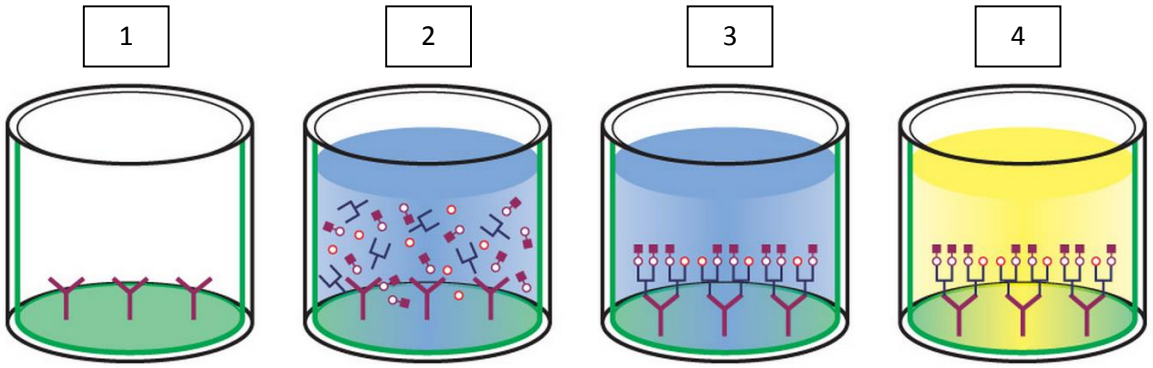


Figure 2.8 EIA assay 1) wells coated with blocking protein and Ab, 2) antiserum, analyte/tracer and analyte or standard are placed in coated wells, 3) wells washed to remove unbound material and 4) color development with Ellman's solution.

2.2.7.1.2 Measurement of PGE_2 and TXB_2 concentrations

Lipid mediator concentrations were measured using a competitive EIA according to the manufacturer's instructions. Briefly, EIA standard was reconstituted in EIA buffer and further diluted with the same buffer to obtain different concentrations (10 ng/mL to 15 pg/mL). Tracer and primary antibody were reconstituted in 6 mL EIA buffer each. Plates were covered with plastic film and incubated for one hour at RT on a shaker.

After incubation, wells were washed five times with wash buffer. 200 µL of Ellman's solution was added to each well, plates were covered with plastic film and incubated 60-90 min at RT with gentle shaking. Plates were read in at a wavelength of 405-420 nm.

Non-specific binding (NSB) absorbance value was substracted from B_0 absorbance (corrected maximum binding). Sample maximum bound (%B/B_0) was calculated by subtracting NSB from sample/standard absorbance and dividing by the corrected maximum binding value. For the standard curve, log standards and concentrations were plotted and a linear regression was performed. Sample concentration was determined using the equation obtained from the standard curve.

2.2.8 NF-κB binding and protein expression analysis in THP-1 derived macrophages

The NF-κB DNA binding activity and the expression of this signalling pathway molecules were evaluated in the nuclear and cytosolic fractions from THP-1 derived macrophages after incubation with LPS and different FA.

2.2.8.1 Cell collection and protein extraction

Nuclear and cytoplasmic fractions were freshly isolated from cells using Active Motif kit (5 x 10^6 cells per sample replicate) as described below.

After treatment with LPS, fatty acids or both, THP-1 macrophages were washed twice with 2.5 mL of ice cold PBS containing phosphatase inhibitors. Cells were gently removed from the dish using a scraper and transferred to a pre-chilled tube. Cells were spin for 5 min at 600 *g* in a centrifuge pre-cooled at 4^{o}C. Supernatant was discarded and pellet was resuspended in 250 µL of 1x hypotonic buffer and incubated for 15 min on ice. After incubation, 12 µL of detergent was added and cells were vortex for 10 sec at the highest setting. At the end of this incubation, cells were spin for 30 sec at 14, 000 g. Supernatants were stored at -80C (this correspond to the cytoplasmic fraction).

Pellets were resuspended in 25 µL of complete lysis buffer and vortex for 10 sec at the highest setting. Samples were sonicated for 15 min on ice and incubated for 15 min on a rocket platform set at 150 rpm. Samples were vortex and spin for 10 min at 8000 *g* in a

micro centrifuge pre- cooled at 4°C. Supernatants were stored at -80°C (this correspond to the nuclear fraction).

2.2.8.2 Protein concentration by Bradford assay

Protein concentrations in cell lysates were determined by Bradford assay. Nuclear and cytoplasmic extracts were diluted 1:1 in dH_2O. Standard curve and diluted cell fractions was prepared and added to a 96 well plate as described in **Table 2.1**. Bradford reagent was diluted in dH_2O (1:4) and 250 µL were added to the samples and standards. Plate was incubated for 10 min and read at 595 nm within 5 min. Total protein concentration was calculated using the standard curve.

Dilution	Final [g-globulin] (mg/mL)	µL H2O miliQ	µL g-globulin stock (1.38mg/mL)	Sample Volume (µL)	Final volume (µL)	Bradford reagent volume (µL)
P0	2.78	0	10	-	10	245
PI	1.39	0	5	-	5	250
PII	0.834	2	3	-	5	250
PIII	0.278	4	1	-	5	250
Blank	0	5	0	-	5	250
Samples	-	-	-	5	5	250

Table 2.1 Standard curve for Bradford assay

2.2.8.3 NF-κB fractions binding assay

2.2.8.3.1 Principle

Trans-AM is an ELISA-based method designed to detect and quantify NF-κB subunits activation. This assay is based in the preferential binding of the activated fractions of NF-κB to its DNA consensus sequence. Each well is coated with an immobilized oligonucleotide containing the NF-κB consensus site (5 ' –GGGACTTTCC-3 '). Active subunits bind its specific oligonucleotide. The primary antibodies specific for each fractions are used to recognize each subunit (p65, p50, p52, c-Rel and RelB) that are accessible only when NF-κB is active. A HRP- conjugated secondary antibody was used to quantify binding levels by spectrophotometry.

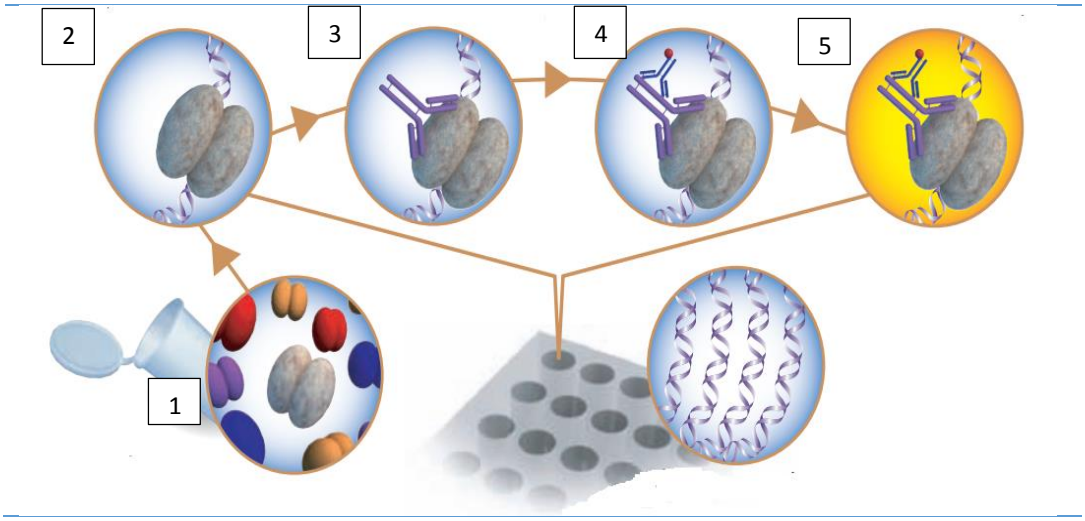


Figure 2.9. *Trans AM kit method.* 1) Sample containing active NF-κB dimers, 2) oligonucleotide bound, 3) primary antibody bind NF-κB active forms, 4) primary antibody is amplified by secondary antibody and 5) colorimetric reaction.

2.2.8.3.2 Methods

Briefly, buffers were prepared 1 h in advance and kept at 4°C. 30 µL of complete lysis buffer was added to each plate well followed by 3.5 µg of nuclear extract diluted in complete lysis buffer, standards, positive controls or blanks. The plate was sealed and incubated for 1 h at room temperature with mild agitation. Wells were washed 3 times with 200 µL of wash buffer. Then, all wells were treated 100 µL of different NF-κB subunits antibodies (1:1000 dilution). Plate was cover and incubated for another hour at room temperature without agitation. Plate was washed after primary antibody incubation as described previously. Wells were treated with 100 µL of HRP-conjugated antibody (1:000 dilution) and incubated for an hour at room temperature. Wells were washed three times and wells were incubated with 100 µL of developing solution for 5 min at room temperature protected from the light. Colorimetric reaction was stopped with 100 µL of Stop Solution. Absorbance was measured within 5 minutes at 450 nm.

A standard curve using recombinant NF-κB p65 was generated to estimate the amount of NF-κB in the samples.

2.2.9 Immunoblotting

The changes of protein expression in the cytoplasmic fractions were evaluated using immunoblotting assay as described in **Appendix I**. Briefly, 20 µg of cytoplasmic proteins were reduced and denatured by boiling for 10 min at 95^{o}C in one volume of 1 x Laemmli buffer containing 2% 2-*mercaptoethanol*. Denatured lysates were fractionated by 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and the membranes were treated with 5% blocking buffer (skimmed milk for total protein and bovine serum albumin for phosphorylated proteins) for 1 hour to avoid unspecific staining.

Membranes were probed for different antibodies at different times. Primary antibody incubation was followed by three washes with solution C, B and PBS for 10 min each. Membranes were treated for 1 h at room temperature with the respective secondary antibody. Protein signal detection was performed by chemiluminescence in a Biorad platform and using the SuperSignal substrate from Pierce. Bands density was measured using Image J software. Detailed protocol and antibody treatment times and dilutions are described in **Appendix I**.

2.2.10 Statistical analysis

Data are expressed as mean ± standard error; data collection and analysis were performed in SPSS and Excel. The significance of the mean differences between samples/treatments was calculated using Student's t-test. When multiple groups were compared, the differences were analysed using One way ANOVA.

2.3 Results

2.3.1 Morphology of THP-1 monocytes and macrophages and surface markers changes during differentiation

2.3.1.1 Cell granularity and morphology

Incubation of THP-1 cells with PMA and then resting in culture was accompanied by an enhancement in cell granularity, as observed by microscopy and confirmed by side scatter analysis (SSC) using flow cytometry (**Figure 2.10**). Flow cytometry analysis revealed that undifferentiated THP-1 cells had lower SSC (i.e. granularity) when compared with THP-1 macrophages. Cell viability was higher than 90% after PMA treatment, while during the resting period 10% of cells detached from the flask.

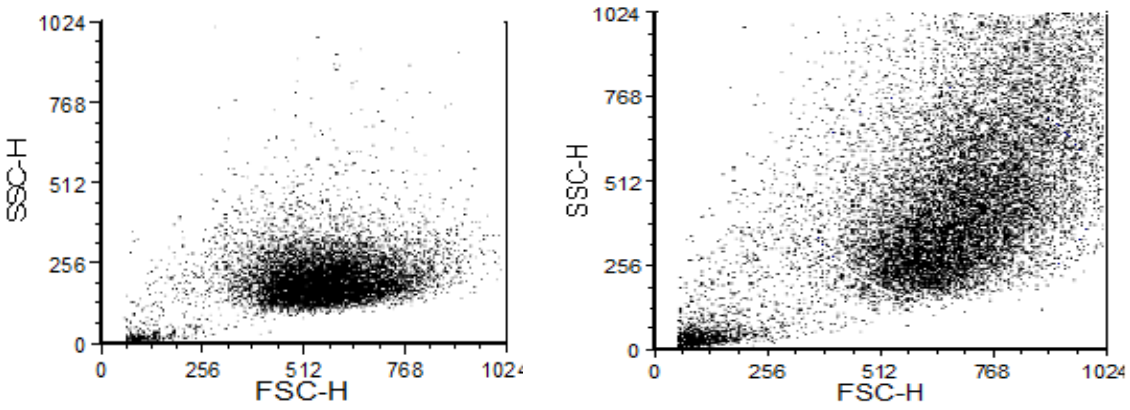


Figure 2.10 Side scatter and forward scatter analysis of THP cells. THP-1 monocytes (*left*) and THP-1 derived macrophages (*right*).

PMA-incubation led to significant changes in cell morphology. After 48 h, THP-1 cells adhered to the flask and started to form clumps, but there were no apparent changes in cell size at this time point. As illustrated in **Figure 2.11**, cells began to flatten out and increase their cytoplasmic space after 72 h of PMA treatment. Over 5 days resting in culture, more than 90% of the cells were attached to the flask and presented an ameboid shape. The nucleus/cytoplasm ratio was higher when compared with the monocytes (**Figure 2.11)**.

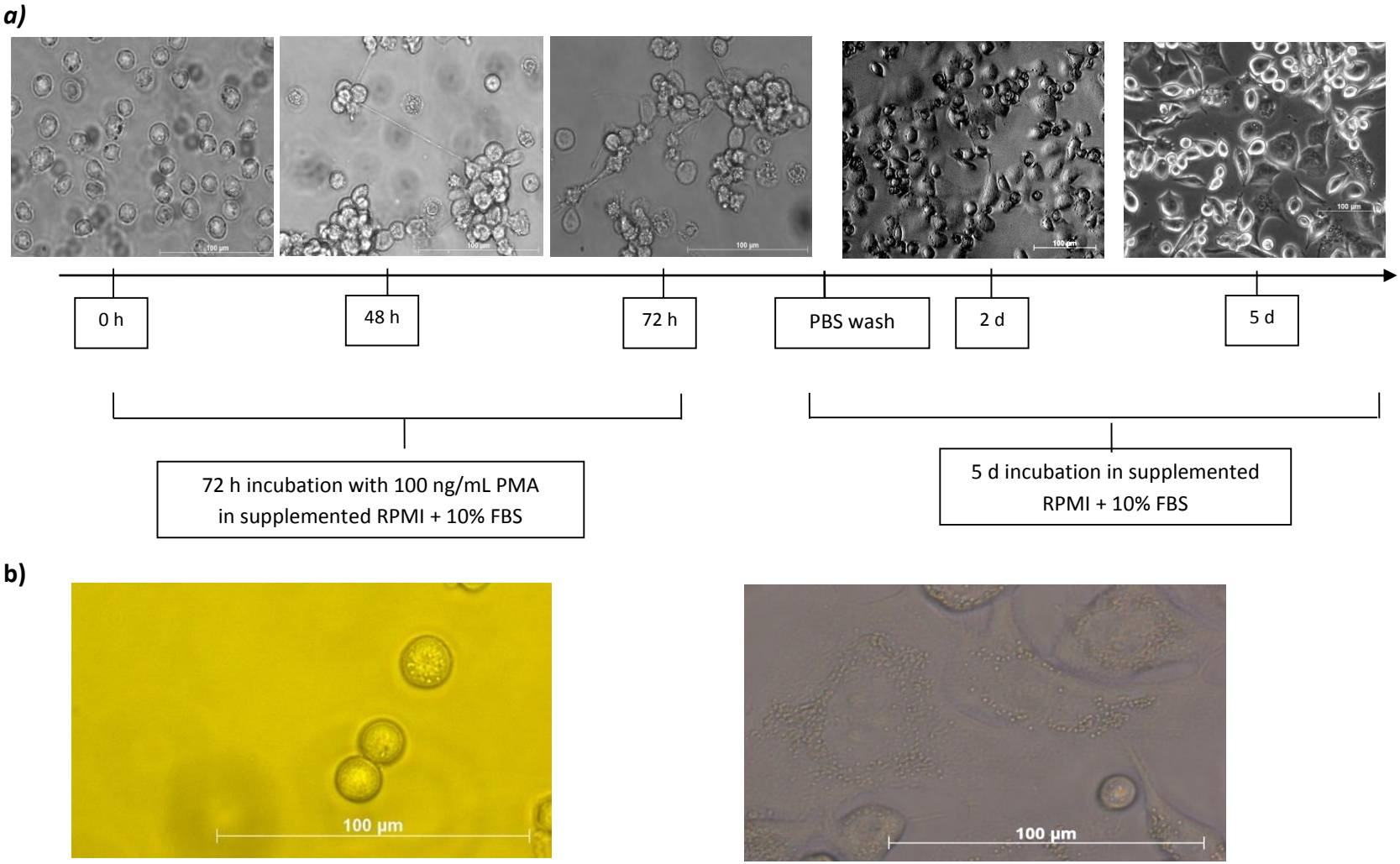


Figure 2.11 Changes in cell morphology after PMA incubation and resting. a) changes in morphology (20x) and b) THP-1 monocytes (*left*) and THP-1 derived macrophages (*right*) (40x) (n=6).

2.3.1.2 Surface marker changes during THP-1 cell differentiation

Monocyte differentiation leads to a series of morphological and surface marker changes which define the macrophage phenotype. CD14, CD11c, CD68 and CD206 expression were analysed to confirm the cell phenotype.

Using immunohistochemical staining, it was seen that more than 60% of THP-1 derived macrophages expressed CD68 (**Figure 2.12**).

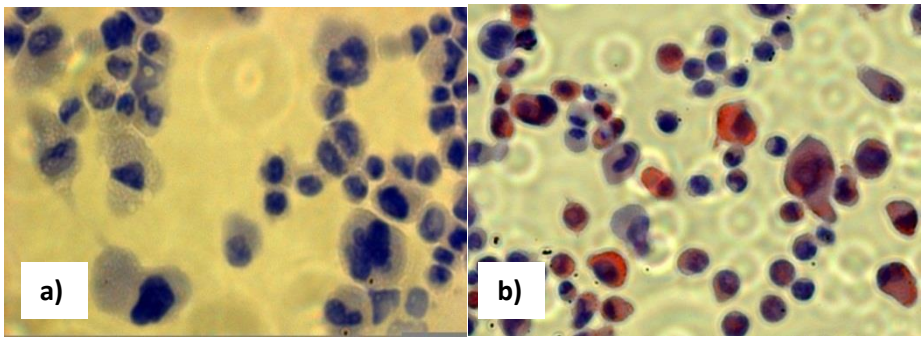


Figure 2.12 CD68 expression in THP-1 macrophages. a) negative control and b) anti-CD68 stained.

Flow cytometry analysis showed no expression of CD206 on either THP-1 monocytes or macrophages (**Figure 2.13**). THP-1 monocytes were positive for CD14 and CD11c. PMA treatment resulted in an upregulation of both these markers. THP-1 macrophages were characterized by high levels of CD14 and CD11c expression when rested in culture (**Figure 2.14**).

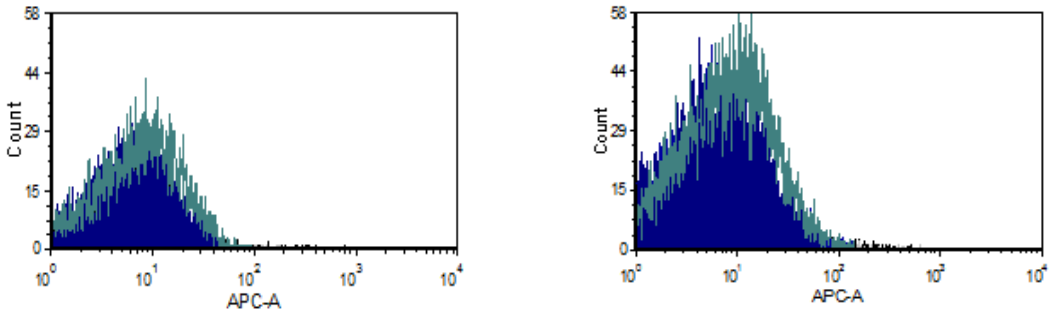


Figure 2.13 CD206 expression (APC-A) on THP-1 cells. THP-1 monocytes (left) and THP-1 derived macrophages (right); IgG in green and CD206 in blue.

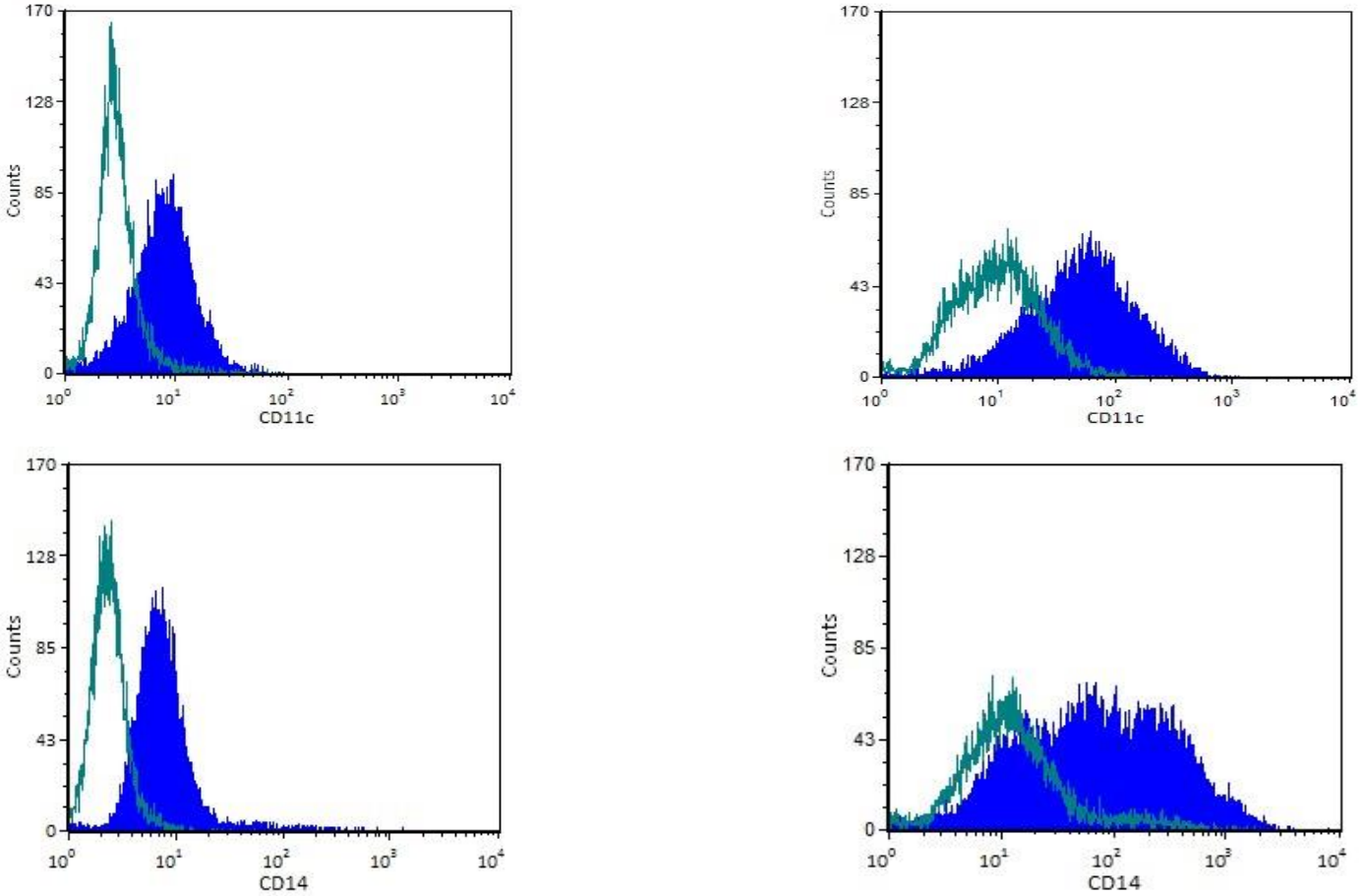


Figure 2.14 CD11c and CD14 expression on THP-1 cells. THP-1 monocytes (left) and THP-1 derived macrophages (right); IgG in green and CD11c or CD14 in blue (n=5).

2.3.2 Fatty acid incorporation into cells

FAs were complexed to albumin to allow incorporation into the membrane of THP-1 derived macrophages. **Figure 2.15** and **Table 2.2a** and **Table 2.2b** show the changes in FA composition after 5 h treatment with SFAs and PUFAs at a concentration of 50 μM.

Upon fatty acid treatment, PUFAs were easily taken up by THP-1 macrophages and induced the mobilization of other fatty acids (**Figure 2.15 and Table 2.2**). For example, cell treatment with n-6 PUFA (AA) induced a significant reduction of the % of DHA and DPA (**Table 2.2a**), while, n-3 PUFA induced changes in the concentrations of other FA such as, 15:0 and 18:1n-9 (oleic acid).

Figure 2.15 shows the enrichment of n-3 PUFA and n-6 PUFA in THP-1 macrophages. Basal levels of AA were high on THP-1 macrophages when compared with DHA and EPA. All fatty acids were efficiently incorporated in cell membranes ($\rho<0.05$).

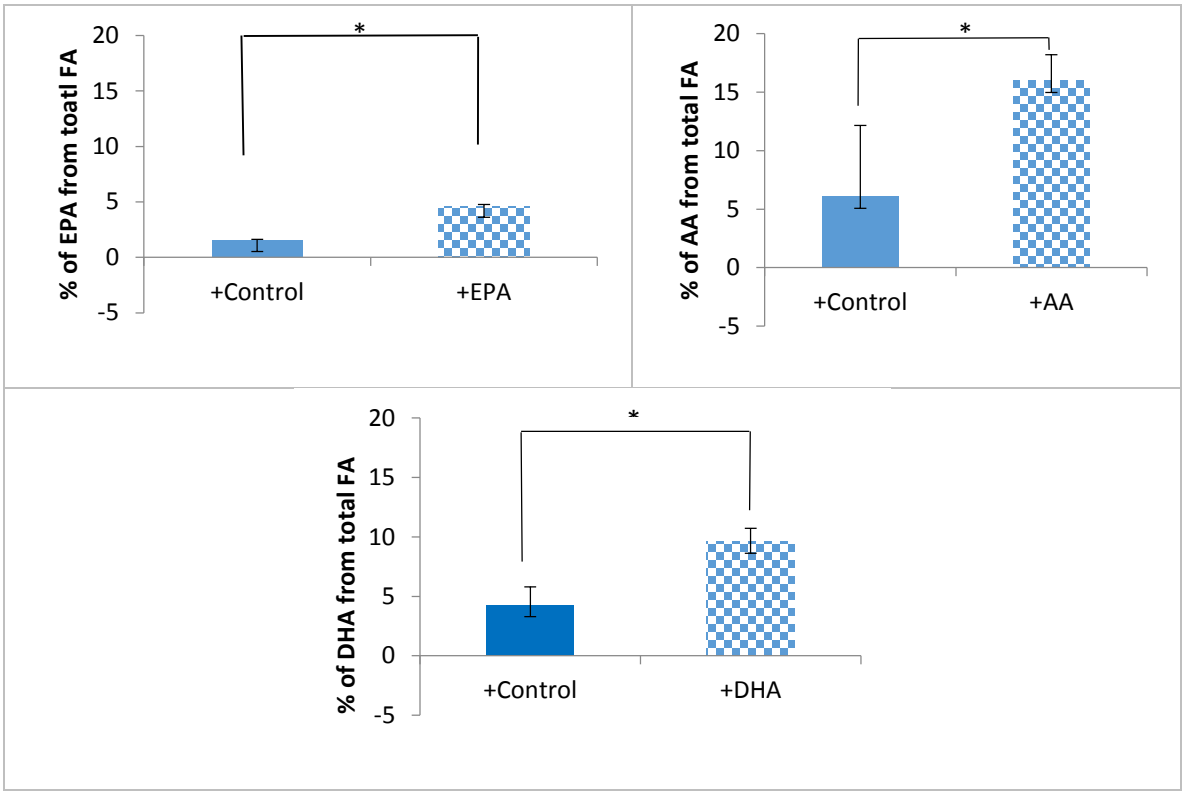


Figure 2.15 Percentage of PUFAs in THP-1 macrophages after treatment. THP-1 macrophages were treated with EPA, DHA, AA and LA for 5h at 50µM; *p-value<0.05, (n=6)

SFA treatment had a modest effect on cells fatty acid composition; fatty acids of interest were not detected to increase significantly after treatment, although, there were changes in the concentrations of other fatty acids upon SFA treatment. FA concentrations in the medium are described in **Appendix I.**

	Control	Δ after EPA	ρ value	Δ after DHA	ρ value	Δ after AA	ρ value
12:00	0.26 ± 0.08	-0.25 ± 0.08	0.14	-0.11 ± 0.23	0.52	0.12 ± 0.08	0.63
14:00	2.72 ± 0.36	-0.01 ± 0.32	0.97	0.88 ± 0.35	0.07	0.48 ± 1.17	0.63
16:00	30.31 ± 0.43	0.64 ± 0.06	0.06	0.36 ± 1.89	0.82	-0.91 ± 2.23	0.63
17:00	0.79 ± 0.08	-0.10 ± 0.07	0.29	0.51 ± 0.93	0.52	2.13 ± 3.13	0.43
18:00	16.08 ± 0.84	0.59 ± 1.03	0.57	-0.49 ± 1.54	0.74	1.08 ± 0.14	0.11
18:1n9	20.02 ± 0.57	-0.96 ± 0.80	0.34	-2.47 ± 1.59	0.14	-4.14 ± 0.26	0.23
t18:1n9	7.12 ± 0.17	-0.48 ± 0.25	0.22	0.01 ± 1.34	0.99	-0.36 ± 0.47	0.41
18:2n-6	2.20 ± 0.07	-0.21 ± 0.09	0.19	0.00 ± 0.00	0.15	-1.99 ± 0.21	**<0.01**
18:3n-6	0.83 ± 0.83	-0.58 ± 0.82	0.50	1.42 ± 0.18	**<0.05**	1.60 ± 0.02	**<0.04**
18:3n-3	0.30 ± 0.00	-0.06 ± 0.00	**<0.05**	0.16 ± 0.36	0.59	-0.08 ± 0.15	0.52
20:1n-9	0.52 ± 0.01	0.03 ± 0.00	**<0.01**	1.69 ± 1.69	0.27	-0.13 ± 0.33	0.64
20:2n-6	0.57 ± 0.02	-0.01 ± 0.01	0.58	0.66 ± 1.59	0.62	-0.20 ± 0.20	0.29
20:3n-6	1.78 ± 0.06	-0.19 ± 0.08	0.19	1.28 ± 1.11	0.22	0.43 ± 0.18	**<0.05**
20:4n-6	5.98 ± 0.35	-0.24 ± 0.77	0.73	0.53 ± 0.45	0.26	10.23 ± 1.95	**<0.01**
20:5n-3	1.75 ± 0.06	3.53 ± 0.00	**<0.01**	0.25 ± 0.05	0.32	-0.84 ± 0.45	0.14
22:5n-3	1.20 ± 0.38	0.58 ± 0.33	0.24	-1.28 ± 0.06	**<0.01**	-1.23 ± 0.23	**<0.05**
22:6n-3	4.17 ± 0.11	-0.48 ± 0.15	0.14	5.60 ± 0.98	**<0.01**	-1.77 ± 0.19	**<0.01**

Control data expressed as % of FA from total fatty acid ± SD; n=6; Δ after refers to the changes in % of FA after different FA supplementation; p value= comparison of Control vs different fatty acids treatment

Table 2.2a Changes in THP-1 macrophages fatty acid composition after 5 h treatment with different PUFAs at 50 µM.

	Control	Δ after Stearic Acid	ρ value	Δ after Palmitic Acid	ρ value	Δ after Lauric Acid	ρ value
12:0	0.26 ± 0.08	-0.25 ± 0.08	0.14	-0.25 ± 0.08	0.14	0.03 ± 0.08	0.72
14:0	2.72 ± 0.36	0.16 ± 0.33	0.63	0.08± 0.34	0.78	0.56 ± 0.31	0.24
16:0	30.31 ± 0.43	1.89 ± 0.24	0.06	1.13 ± 0.25	0.10	1.47 ± 0.02	**<0.01**
17:0	0.79 ± 0.08	-0.16 ± 0.08	0.21	1.65 ± 0.07	**<0.05**	0.62 ± 0.06	**<0.05**
18:0	16.08 ± 0.84	1.27 ± 0.94	0.31	1.29 ± 0.93	0.30	-0.32 ± 1.04	0.74
18:1n9	20.02 ± 0.57	0.48 ± 0.69	0.50	0.79 ± 0.68	0.35	-0.04 ± 0.82	0.96
t18:1n9	7.12 ± 0.17	-0.03 ± 0.21	0.91	-0.47 ± 0.20	0.19	-0.22 ± 0.26	0.43
18:2n-6	2.20 ± 0.07	0.09 ± 0.08	0.37	0.09 ± 0.08	0.36	0.02 ± 0.09	0.78
18:3n-6	0.83 ± 0.83	-0.60 ± 0.83	0.49	-0.48 ± 0.82	0.56	-0.59 ± 0.82	0.50
18:3n-3	0.30 ± 0.00	-0.01 ± 0.00	0.42	0.28 ± 0.00	**<0.01**	-0.06 ± 0.00	0.07
20:1n-9	0.52 ± 0.01	-0.07 ± 0.00	**<0.01**	0.20 ± 0.00	**<0.01**	-0.03 ± 0.00	0.23
20:2n-6	0.57 ± 0.02	-0.41 ± 0.02	**<0.05**	-0.40 ± 0.02	**<0.05**	-0.01 ± 0.01	0.52
20:3n-6	1.78 ± 0.06	-0.14 ± 0.07	0.22	-0.31 ± 0.07	0.10	0.01 ± 0.08	0.88
20:4n-6	5.98 ± 0.35	0.07 ± 0.32	0.81	0.18 ± 1.43	0.89	0.16 ± 0.49	0.72
20:5n-3	1.75 ± 0.06	0.14 ± 0.05	0.17	-0.43 ± 0.60	0.49	0.25 ± 0.04	0.08
22:5n-3	1.20 ± 0.38	-1.09 ± 0.36	0.15	-0.89 ± 0.36	0.18	-0.48 ± 0.34	0.30
22:6n-3	4.17 ± 0.11	-0.36 ± 0.13	0.16	-0.63 ± 0.13	0.09	-0.20 ± 0.16	0.33
Control data expressed as % of total fatty acid ± SD; n=6; Δ after refers to the changes in % of FA after different FA supplementation; p value= comparison of Control vs different fatty acids treatment							

Table 2.2b Changes in THP-1 macrophages fatty acid composition after 5 h treatment with different SFA at 50 μM.

2.3.3 Effect of fatty acid treatment on the activation of NF-κB in response to LPS

In order to investigate the effect of different fatty acids on the modulation of NF-κB signaling pathway the expression of downstream proteins involved in NF-κB activation and the activity of different NFκB dimers were evaluated.

LPS treatment resulted in dynamic changes in protein expression as illustrated in **Table 2.3**. In parallel, NF-κB dimers changed in response to LPS (**Figure 2.16**). NF-κB p65 protein binding increased significantly after cells were treated with LPS at 2 h and 5 h ($\rho<0.05$). The amount of active NF-κB p65 was significantly lower in cells pretreated with EPA prior to LPS stimulation than those treated with LPS only at 2 h.

Similarly, NF-κB p50 protein was significantly increased in response to LPS at 2 h in cells pretreated with DHA, AA, Lauric or Control. After 5 h of LPS treatment, NF-κB p50 was significantly increased in cells pretreated with different fatty acids and control ($\rho<0.05$) (**Figure 2.16a**).

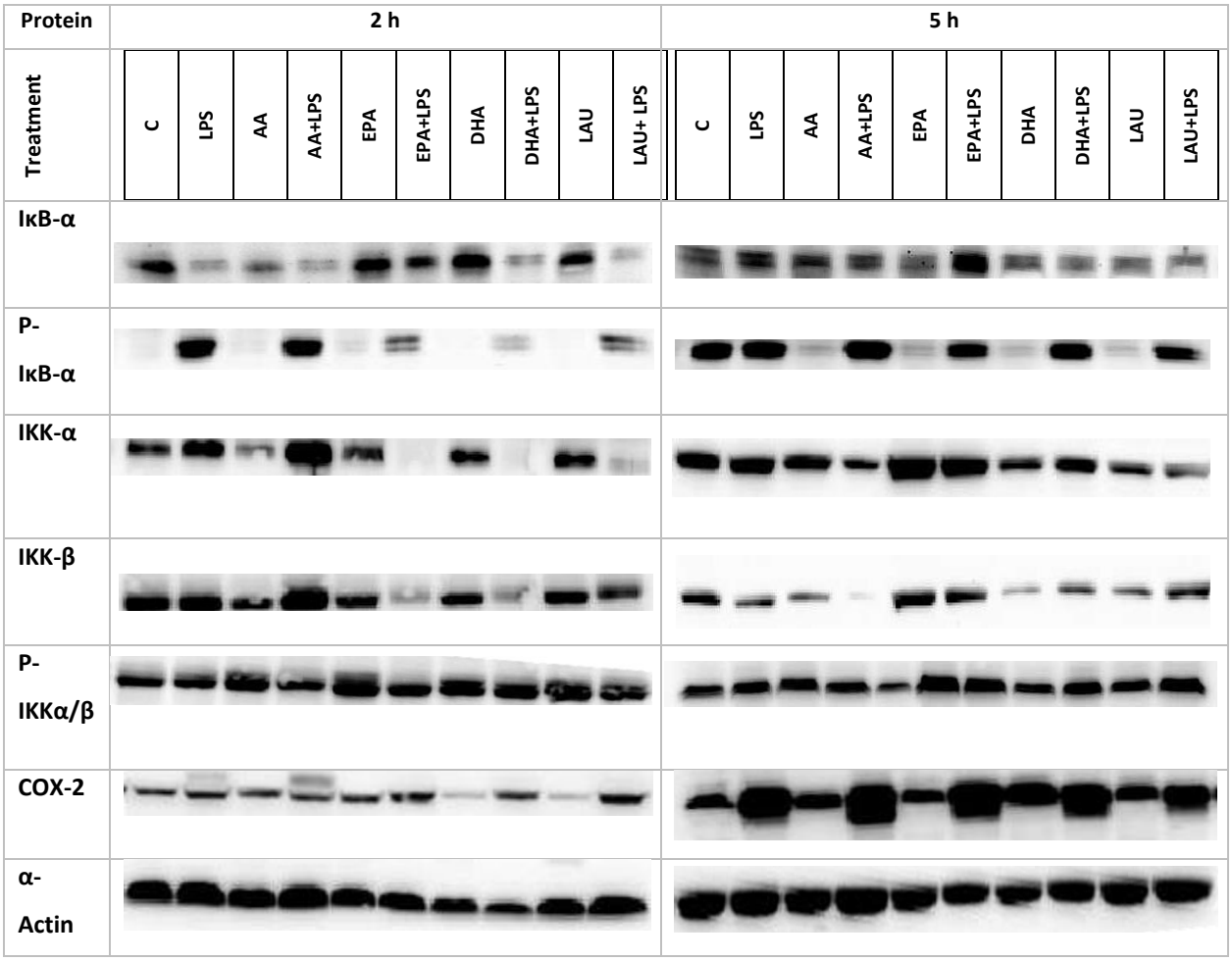
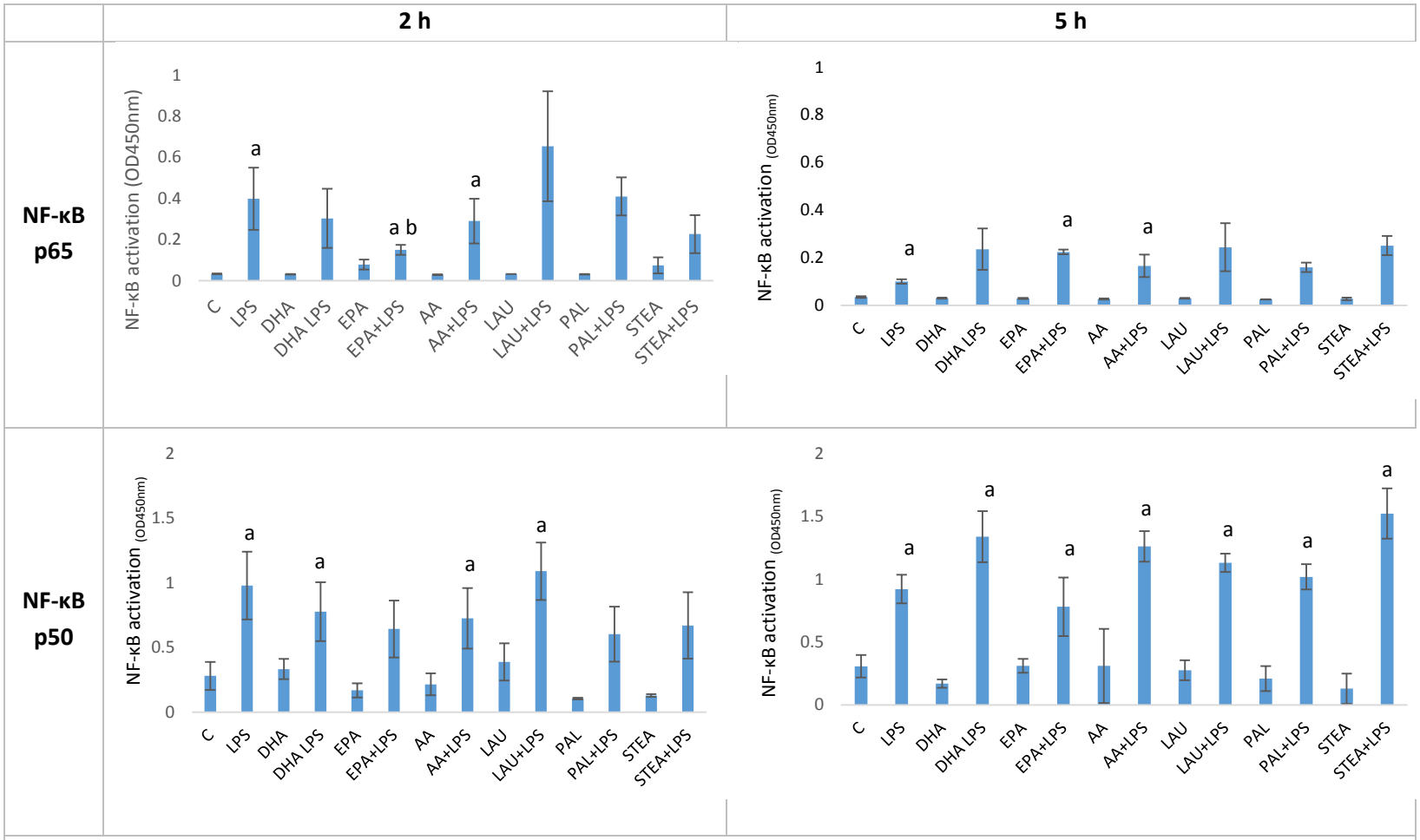
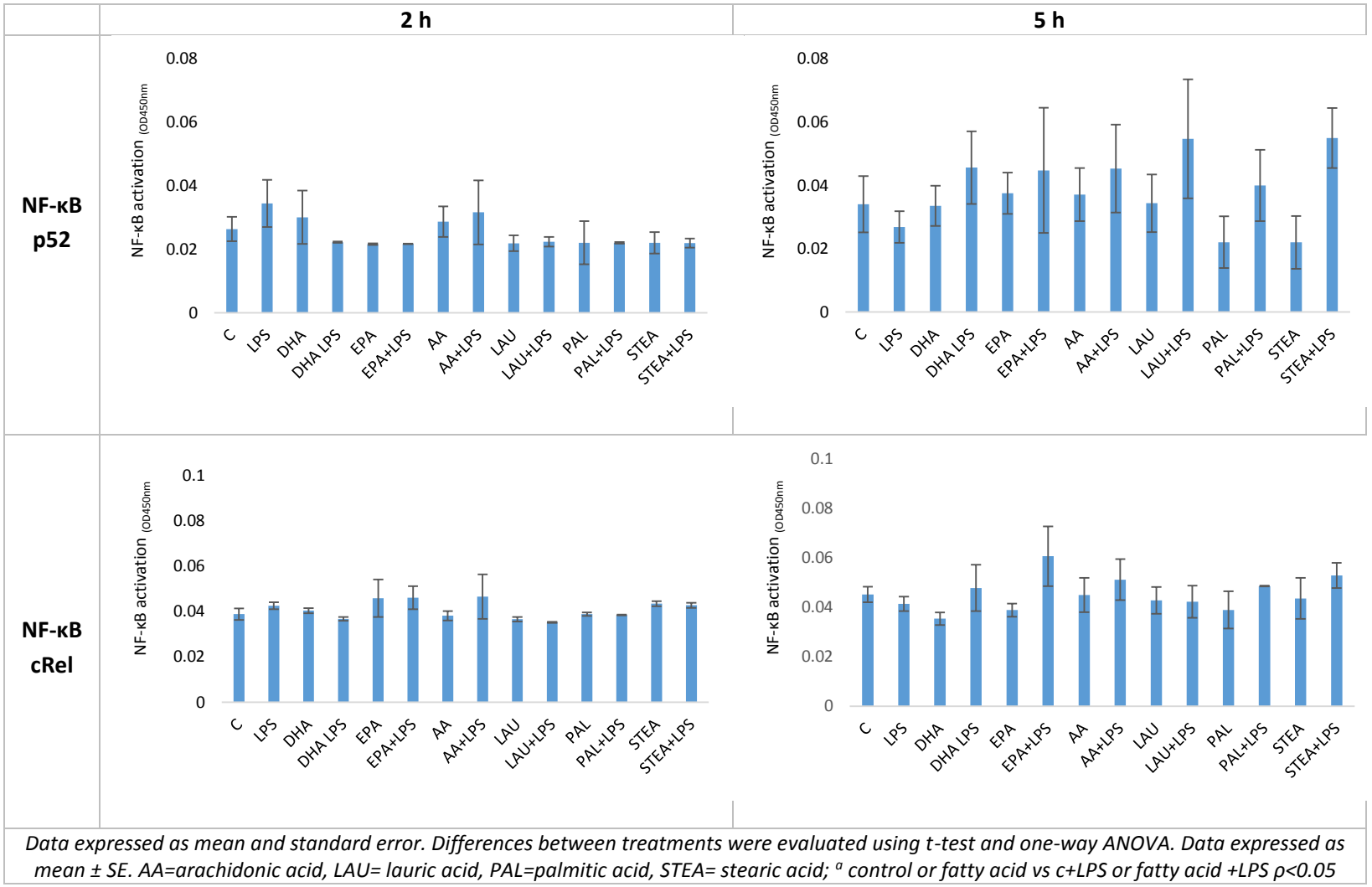


Table.2.3 Representative blots illustrating the changes in protein expression during LPS stimulation of THP-1 macrophages pretreated with different fatty acids (n=3)



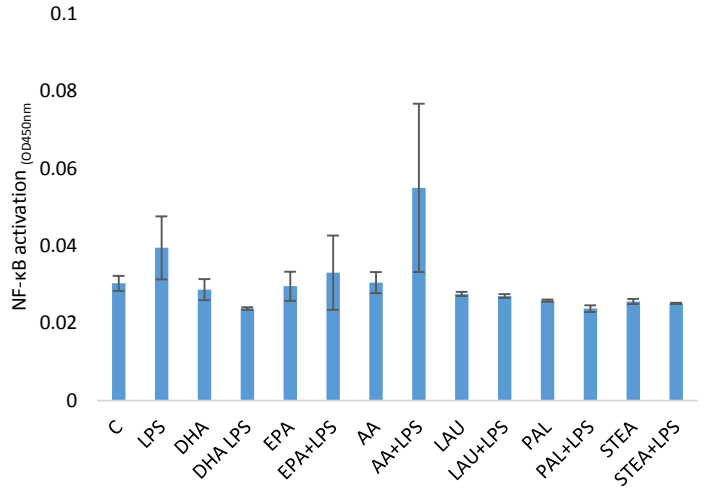
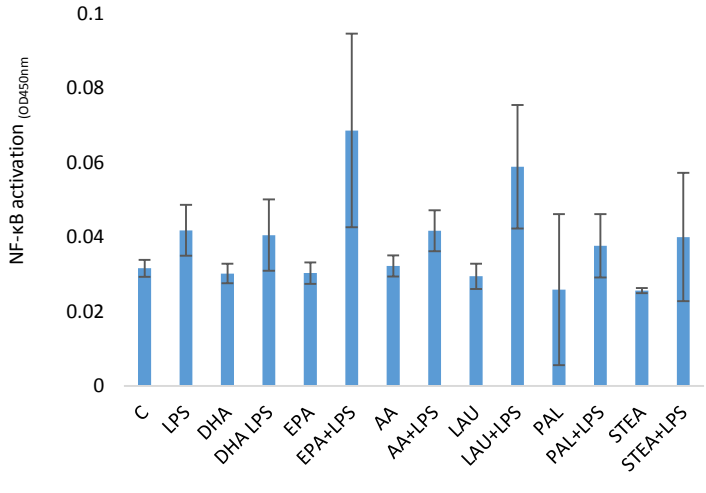
Data expressed as mean and standard error. Differences between treatments were evaluated using t-test and one-way ANOVA. Data expressed as mean ± SE. AA=arachidonic acid, LAU= lauric acid, PAL=palmitic acid, STEA= stearic acid; [a] control or fatty acid vs c+LPS or fatty acid+LPS p<0.05; [b] c+LPS vs EPA+LPS

Figure 2.16a Changes in NF-κB activation during LPS stimulation in THP-1 macrophages pretreated with different fatty acids (n=5)



Data expressed as mean and standard error. Differences between treatments were evaluated using t-test and one-way ANOVA. Data expressed as mean ± SE. AA=arachidonic acid, LAU= lauric acid, PAL=palmitic acid, STEA= stearic acid; [a] control or fatty acid vs c+LPS or fatty acid +LPS $p<0.05$

Figure 2.16b Changes in NF-κB activation during LPS stimulation in THP-1 macrophages pretreated with different fatty acids (n=5)

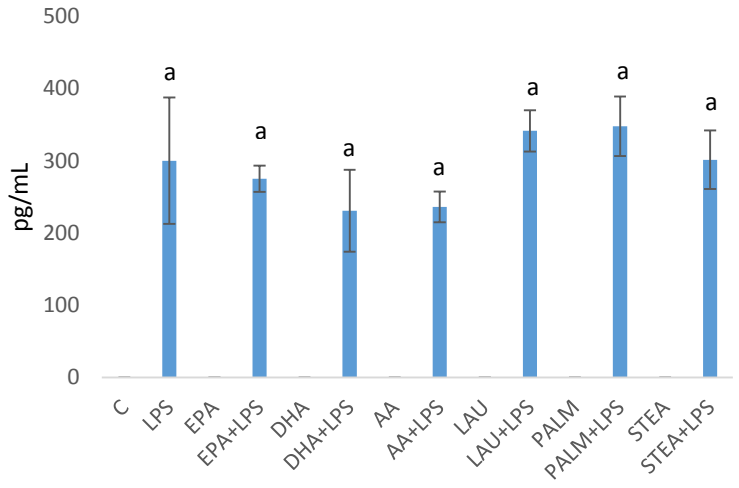
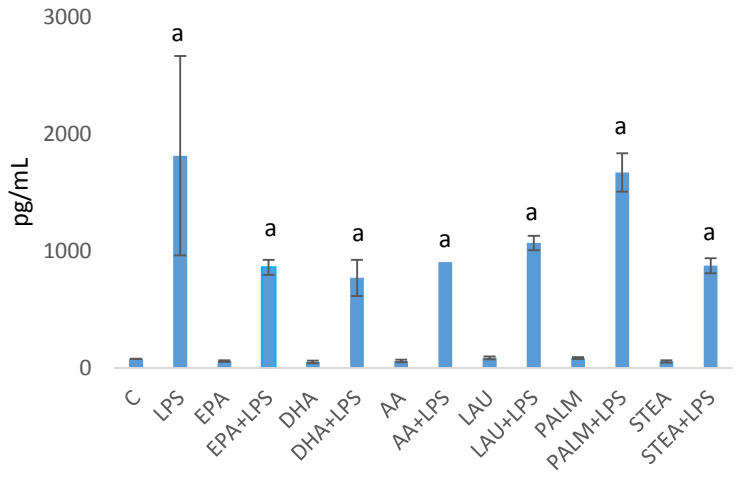
	2 h	5 h
NF-κB cRel		

Data expressed as mean and standard error. Differences between treatments were evaluated using t-test and one-way ANOVA. Data expressed as mean ± SE. AA=arachidonic acid, LAU= lauric acid, PAL=palmitic acid, STEA= stearic acid; [a] control or fatty acid vs LPS $\rho<0.05$

Figure 2.16c Changes in NF-κB activation during LPS stimulation in THP-1 macrophages pretreated with different fatty acids (n=5)

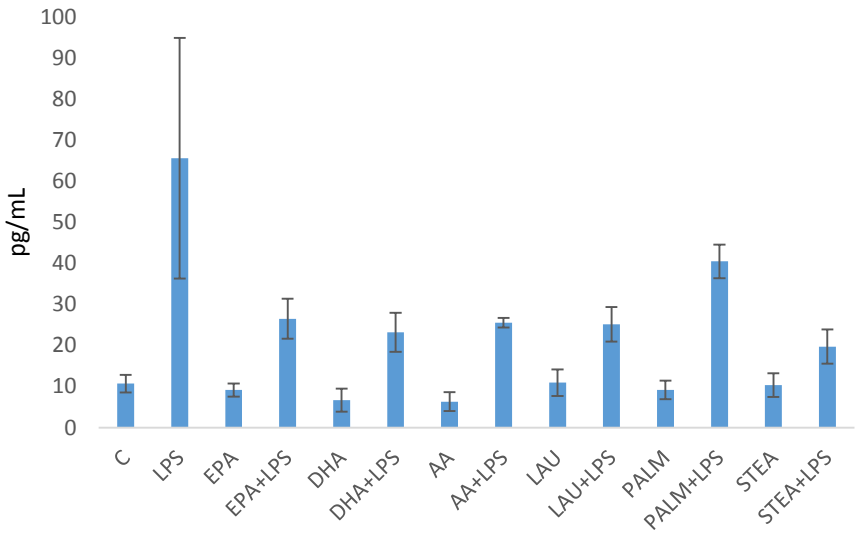
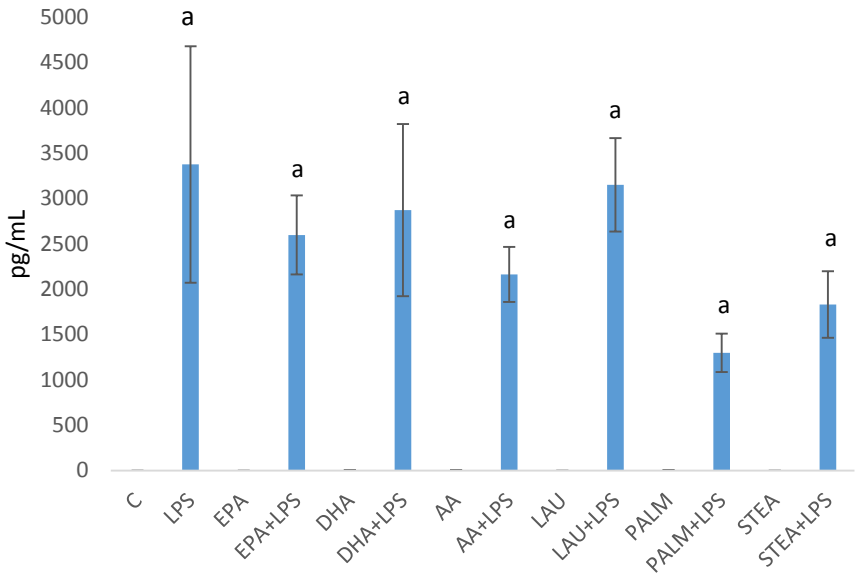
2.3.4 Effect of fatty acid treatment on the production of cytokines in response to LPS

Following pretreatment with different fatty acids, the production of cytokines after 12 h incubation with LPS was evaluated. LPS induced a significant production of IL-6, IL-1β and TNF-α regardless of the fatty acid treatment (ρ<0.05) (**Figure 2.17a** and **Figure 2.17b**).

	12 h
IL-6	
IL-1β	

Data expressed as mean and standard error. Differences between treatments were evaluated using t-test. Data expressed as mean ± SE. AA=arachidonic acid, LAU= lauric acid, PAL=palmitic acid, STEA= stearic acid; [a] control or fatty acid vs LPS ρ<0.05

Figure 2.17a Cytokines production during 12 h LPS stimulation in THP-1 macrophages pretreated with different fatty acids (n=6)

	12 h
IL-10	
TNF-α	

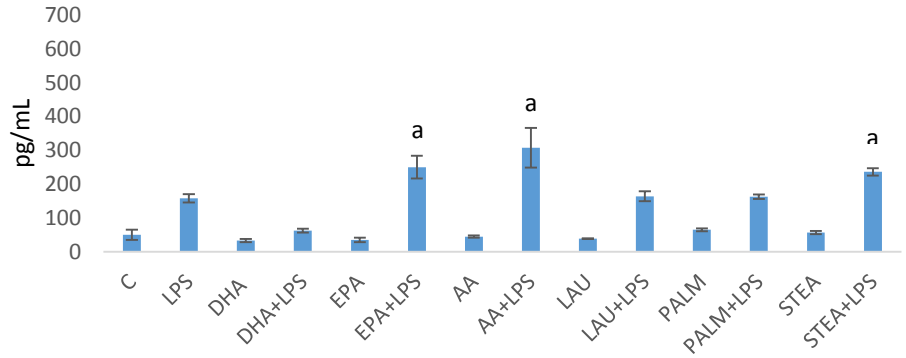
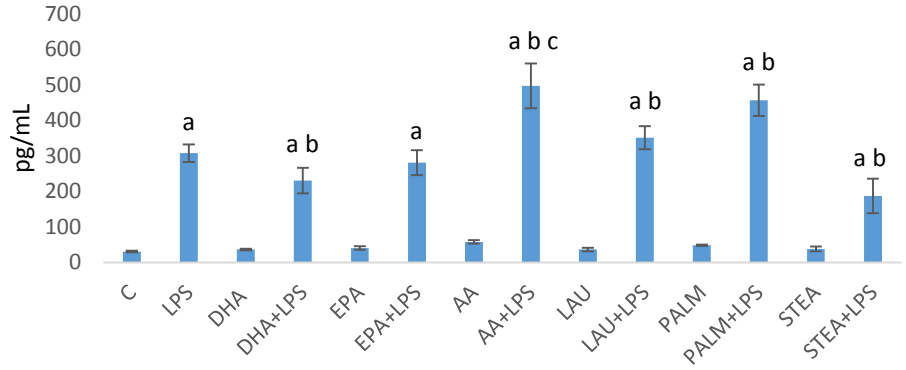
*Data expressed as mean and standard error. Differences between treatments were evaluated using t-test. . Data expressed as mean ± SE. AA=arachidonic acid, LAU= lauric acid, PAL=palmitic acid, STEA= stearic acid; *c or fatty acid vs LPS ρ<0.05*

Figure 2.17b Cytokines production during 12 h LPS stimulation in THP-1 macrophages pretreated with different fatty acids (n=6)

2.3.5 Effect of fatty acid treatment on lipid mediators production in response to LPS

Figure 2.18 and **2.19** show the changes in PGE_2 and TXB_2 in response to LPS stimulation in THP-1 macrophages pretreated with different fatty acids. TXB_2 concentrations increased significantly at 5 h in cells pretreated with EPA, AA and stearic acid compared with their control ($\rho<0.05$). Furthermore, LPS treatment induced a significant increment in TXB_2 concentrations in all samples ($\rho<0.05$).

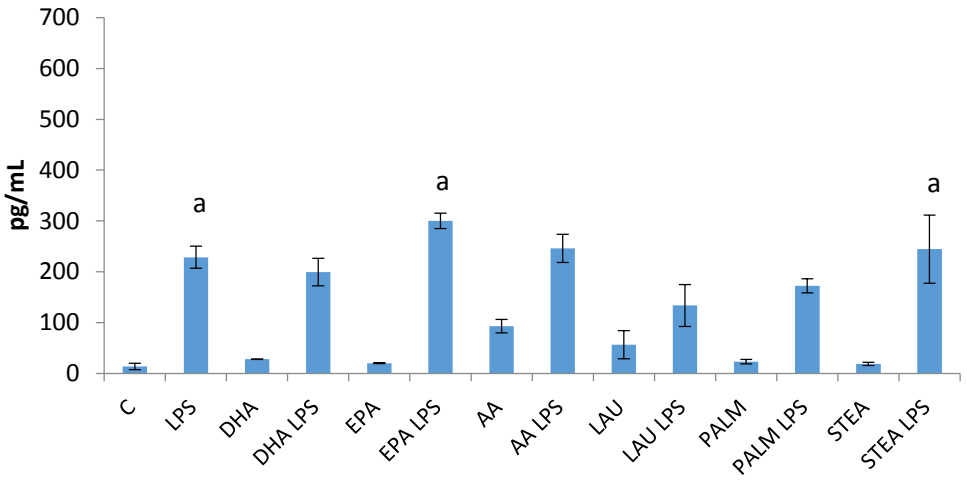
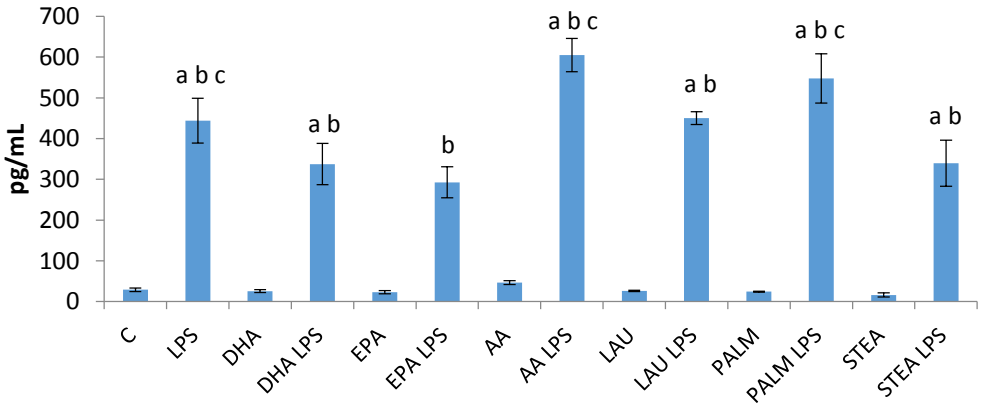
After 12 h, the concentrations of TXB_2 was significantly higher in all the samples treated with LPS compared with control ($\rho<0.05$) (**Table 2.18**). The concentrations of TXB_2 increased significantly during LPS treatment from 5 h to 12 h in cells pretreated with DHA, AA, lauric acid, palmitic acid and stearic acid. Moreover, cells pretreated with DHA produced significantly less TXB_2 compared to those pretreated with AA in response to LPS ($\rho<0.05$).

	TXB_2
5 h	
12 h	

Data expressed as mean and standard error. Differences between treatments were evaluated using t-test. . Data expressed as mean ± SE. AA=arachidonic acid, LAU= lauric acid, PAL=palmitic acid, STEA= stearic acid. [a]Control or fatty acid vs LPS; [b] 5 h vs 12 h or [c] AA+LPSvs DHA+LPS, ρ<0.05.

Figure 2.18a TXB_2 production after 5 h and 12 h LPS stimulation in THP-1 macrophages pretreated with different fatty acids

After 5 h of LPS stimulation, PGE_2 concentrations increased significantly in cells pretreated with LPS only, EPA and stearic acid. PGE_2 concentrations were significantly higher between 5 h and 12 h in cells pretreated with LPS only, DHA, AA, palmitic acid and stearic acid. While, the synthesis of PGE_2 was reduced at 12 h compared with 5 h when cells were pretreated with EPA. Moreover, concentrations of PGE_2 at 12 h were significantly lower in cells pretreated with EPA than AA, lauric acid and palmitic acid ($\rho<0.05$).

	PGE_2
5 h	
12 h	

Data expressed as media and standard error .Differences between treatments were evaluated using t-test. . Data expressed as mean ± SE. AA=arachidonic acid, LAU= lauric acid, PAL=palmitic acid, STEA= stearic acid. [a]Control or fatty acid vs LPS; [b] 5 h vs 12 h or [c] vs DHA+LPS, ρ<0.05.

Figure 2.18b PGE_2 production after 5 h and 12 h LPS stimulation in THP-1 macrophages pretreated with different fatty acids

2.4 Discussion

The course of the inflammatory response relies on the interactions of the different cell subsets involved in the synthesis and disappearance of signalling mediators. Macrophages are tissue sentinels involved in the early innate responses and the signalling molecules they synthesise contribute to the outcome of an inflammatory response (Lewis and McGee, 1992). Some of these molecules contribute to the on-going inflammatory process and some to its resolution.

In this study THP-1 monocytes were used to obtain a macrophage model to study the mechanisms of action of different fatty acids in an inflammatory response. The main objective of these experiments was to obtain a macrophage model that resembles tissue-resident macrophages. Several features were studied in these cells before, during and after differentiation, including morphology, cell surface markers and responsiveness to LPS.

As a result of stimulation with PMA and subsequent rest, cell morphology changed, cells became adhesive, and surface CD11 and CD14 expression were up regulated. These results agree with previous findings where PMA treatment was found to induce changes in cell morphology and surface markers of THP-1 cells (Takashiba *et al.* 1999; Park *et al.* 2007; Dumrese *et al.* 2009; Daigneault *et al.* 2010).

Cell surface marker expression reflects tissue resident macrophage phenotype and their repertoire under a stress response (Gordon, 2003; Gordon, 2005). CD68 (tissue macrophage marker) and CD206 (anti inflammatory/regulatory macrophage marker) have been widely used to discriminate between regulatory and inflammatory macrophage phenotypes. In this study, CD68 and CD206 were explored to obtain a better characterization of the THP-1 macrophage phenotype. In keeping with prior publications reporting CD68 expression on THP-1 macrophages (Ramprasad *et al.* 1996), CD68 was found on THP-1 macrophages; the percentage of positive cells was higher than before differentiation.

Previous studies have reported conflicting results in terms of CD206 expression on these cells. Tjiu *et al.* (2009) reported CD206 expression in THP-1 cells treated with 320 nM PMA for 24 h while no expression was reported by Daigneautl *et al* (2010). Although the same

methods were used for cells surface staining, differences in PMA concentration used during differentiation have been suggested to affect cell physiology and phenotype (Park *et al.* 2007; Maeß *et al.* 2014). In this study, CD206 expression was not observed.

It is concluded that the objective of obtaining a macrophage model that resembles tissue-resident macrophages was achieved in the current work.

As described in previous sections, fatty acids have diverse properties dictated by their unique structure (**Chapter 1**). Fatty acids induce changes in cell membrane organization and fluidity that have been shown to affect receptor organization and may change their affinity and interactions with extracellular molecules (Yaqoob, 2009). FA present in cell membranes also constitute a substrate pool for the synthesis of lipid mediators with regulatory or inflammatory properties (Serhan, 2010).

Given the importance of fatty acids in immune cell function, the effect of different fatty acids on signalling and on synthesis of inflammatory mediators was explored using the THP-1 macrophages. First, the viability and enrichment of SFA and PUFA in this macrophage model was studied.

The method of Mahoney *et al.* (1977) was employed for fatty acid incorporation (Mahoney *et al.* 1977). Compared with methods reported by others where solvents were employed for FA incorporation, the use of FA salt: albumin complexes offer the advantage to resemble more physiological conditions and prevent harmful effects of solvents on cells.

The enrichment of different fatty acids using the same method was reported by Calder *et al.* (1990) for cultured murine peritoneal macrophages. Similar to this study, PUFA were enriched in macrophages when they were provided in the medium, although in this study no significant incorporation of SFA was detected. The differences may be explained by the differences in cell type, cell metabolism and the incubation times. In this report 5 h and 12 h incubation were used while Calder *et al.* (1990) incubated the cells with fatty acids for 48 h.

Following fatty acids enrichment, their effects on the inflammatory cascade were evaluated. LPS treatment induced IκB-α phosphorylation at 2 h and this effect was

attenuated in cells treated with DHA and EPA at 2 h. This effect was reflected in a significant reduction in p65 activation at 2 h in cells treated with EPA. Comparable results are reported in the literature (Mullen *et al.* 2000).

Fatty acids pre-treatment prior to LPS incubation had no significant effect on cytokine synthesis. Previous studies have reported a reduction of mRNA and cytokine synthesis in THP-1 derived macrophages pre-treated with DHA and EPA (Mullen *et al.* 2000; Zhao *et al.* 2005). Those studies have employed higher concentrations of fatty acids (25 mM) (Mullen *et al.* 2000) or fatty acids have been used during both pre-treatment and the LPS challenge (Zhao, 2005). Similarly, palmitic and stearic acid (100μM) have been found to induce the secretion of TNF, IL-8 and IL-1β (Haversen *et al.* 2009). The contrast between these studies and the results described in this chapter may be explained by the differences in the doses used to treat the cells and the duration of the exposure to fatty acids.

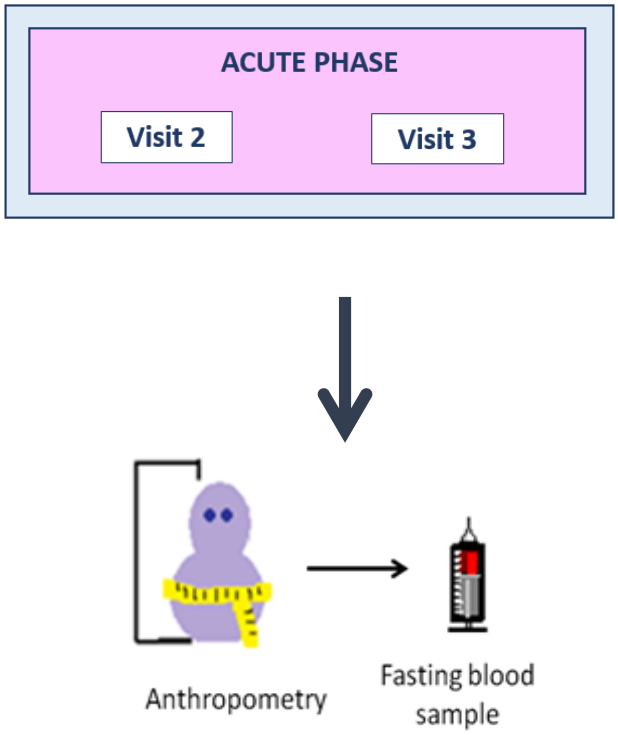
In parallel with the production of cytokines, the synthesis of lipid mediators was modulated by n-3 PUFA. N-3 PUFA pre-treatment resulted in a reduction of TXB_2 and PGE_2 synthesis in response to LPS at 12 h. These observations are in agreement with previous studies suggesting that n-3 PUFA inhibit the metabolism of arachidonic acid to eicosanoids (Smitz *et al.* 2009).

2.5 Conclusions

To conclude, the macrophage model presented in this chapter has similar features to tissue-macrophages, including morphology, surface marker expression and responsiveness. The cells were found to incorporate PUFA well but incorporation of SFA was not seen. In addition, the incorporation of EPA on THP-1 macrophages downregulated p65 binding and both DHA and EPA reduced the synthesis of TXB_2 and PGE_2 compared with other fatty acids (AA and palmitic acid) and LPS itself. Thus, n-3 PUFA has the ability to modulate the activation of NF-κB and reduce the production TXB_2 and PGE_2.

CHAPTER 3

Study design and subjects' baseline anthropometric characteristics and metabolic profile



CHAPTER 3

Study design and subjects' baseline anthropometric characteristics and metabolic profile

3.1 Introduction, objective, hypothesis and aims

3.1.1 Introduction

Obesity is a condition generally caused by a positive energy balance inducing changes in body composition and subsequently in metabolic regulation. Body mass index (BMI) has been widely used to classify subjects with obesity, although this measurement does not allow for discrimination between lean and fat mass and does not take account of body fat distribution. Epidemiological studies have shown that other markers such as waist circumference and total body fat can be useful tools to predict risk of metabolic disease in both normal weight and obese subjects (Pouliot *et al*. 1992).

Abdominal obesity can be defined as having an abdominal circumference greater than 94 cm for men and 80 cm for women (International Diabetes Federation worldwide consensus, 2006). Studies of subjects with different body fat distribution observed that abdominal fat was associated with impairment in glucose response and a substantial increase in metabolic complications (Jensen *et al.* 1988; Pouliot *et al*. 1992; Fox *et al.* 2007; Zang *et al*. 2008).

In accordance with these reports, studies of metabolism of adipocytes from different body depots revealed differences in lipolysis rate, secretory profile of adipokines and insulin sensitivity (Hirsch and Goldrick, 1970; Frayn, 2002; Fain *et al.* 2004). Thus, the proximity of abdominal fat to metabolically active tissues such as the liver and the influx of its molecules to the periphery may contribute to the onset of insulin resistance.

Metabolic homeostasis relies on an adequate equilibrium between energy intake, expenditure and efficient storage. In physiological conditions, carbohydrates (mainly poly, di and monosaccharides) and lipids (mainly triglycerides) from food are absorbed by enterocytes and transported in the circulation (Frayn, 2010). In terms of dietary carbohydrates, after being hydrolysed by various enzymatic reactions (catalysed for example by amylases and disaccharidases) they are absorbed in the form of

monosaccharides by enterocytes and transferred from the enterocytes into the circulation by monosaccharide transporters (e.g. glucose transporter type-2 (GLUT2)) (Frayn, 2010).

Glucose entering the bloodstream reaches the hepatic portal vein. The liver takes up a significant proportion of this glucose to protect other organs from spikes in the plasma glucose concentration and to replenish its glycogen stores. The presence of glucose in the bloodstream stimulates insulin secretion by beta cells of the pancreas. Insulin stimulates glucose uptake into cells (especially skeletal muscle cells and adipocytes) by triggering the translocation of the glucose transporter type-4 (GLUT4) to the cell surface via IRS/PI-3 kinase pathway (Saltiel *et al.* 2001).

In parallel, fatty acids are hydrolysed from dietary triglycerides by pancreatic lipases and then taken up into the enterocytes. Here triglycerides are reformed and these are assembled into large complexes with phospholipids and proteins. These complexes are called chylomicrons and they are one type of lipoprotein. The chylomicrons are released from the enterocyte into the lymphatic circulation. They bypass the liver and enter the circulation. Fatty acids from triglycerides in chylomicrons are accessed by the enzyme LPL. LPL is present in the endothelium of most tissues and it facilitates fatty acid uptake from lipoprotein triglycerides; thus LPL activity is an important determinant of triglyceride clearance. Insulin upregulates LPL activity in various tissues, including adipose tissue, thus promoting fatty acid uptake and storage as triglycerides in the fed state. Fatty acids are stored mainly in adipose tissue in triglyceride form and they are released into the circulation during energy deprivation in the form of NEFA by the action of HSL. The chylomicron remnants that remain after removal of triglycerides by LPL are cleared by the liver and their products are reassembled in the liver into denser lipoprotein structures (Frayn, 2010). This system is tightly regulated by feedback loops keeping substrates within normal concentration ranges.

The differences in metabolic markers in normal weight and obese subjects have been described in detail in the literature. Elevated fasting concentrations of glucose, triglycerides, insulin and cholesterol are some of the characteristic features found in obese subjects that reflect either adaptations undertaken to counteract the energy surplus from the diet or a

loss of regulatory control. For example, insulin resistance at the adipocyte level would impair removal of glucose and triglycerides from the bloodstream contributing to elevations in their concentrations. Furthermore the changes seen in obesity can themselves contribute to insulin resistance and metabolic complications.

The origins of insulin resistance are multifactorial and can be explained in part by the elevated NEFA concentrations in the circulation (Frayn, 2002). NEFA disrupt the main pathway of insulin signalling by reducing IRS phosphorylation and PI3K activity (Saltiel *et al.* 2001). Another pathway affected by NEFA is nitric oxide (NO) production. NO is an endothelium-derived relaxing factor, involved in blood vessel homeostasis. In humans, a lipid infusion triggers acute activation of NF-κB leading to the synthesis of ROS, which serve as NO scavengers (Triphaty *et al*. 2003). Similarly, other clinical studies have reported similar responses after a high fat meal (Mohanty *et al.* 2002; Aljada *et al*. 2004; Ghanim *et al.* 2009). These findings suggest that acute inflammatory responses induced by exogenous and endogenous circulating fatty acids have an impact in endothelium homeostasis and insulin function.

A study by Campbell *et al.* (1994) described the regulation of fatty acid metabolism in normal weight and obese subjects. Even though fasting NEFA concentrations were significantly higher in obese individuals, NEFA release per unit fat mass was lower when compared with normal weight subjects. In support of this study, a report using radiolabelled fatty acids by McQuaid *et al.* (2011) similarly demonstrated a depressed fasting NEFA secretion in obese subjects; the postprandial flow and efficiency to extract and store triglycerides from lipoproteins was diminished in adipose tissue from obese subjects. mRNA analysis revealed a down regulation of selected genes involved in the regulation of fatty acid metabolism. These included LPL, diglyceride acyltrasferase (DGAT), glycerol-3-phosphate acyltransferase (GPAM), glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (GPIHBP1), HSL and ATGL (McQuaid *et al.* 2011). Moreover, some studies have reported an inverse correlation of LPL activity with obesity (Pollare *et al*. 1991; Annuzzi *et al.* 2008). Thus, high fasting NEFA and triglyceride concentrations found in

obese subjects are a reflection of a higher total body fat mass and an inefficient energy substrate uptake by adipose tissue.

The extended residence of NEFA in the circulation, derived from either adipose tissue itself or from triglycerides from the diet, may contribute to fat accumulation in the liver and insulin resistance. These events may contribute to the increased risk of cardiovascular disease in these subjects.

In addition to the altered metabolism, obesity has been also associated with higher levels of inflammatory markers in the bloodstream than seen in normal weight individuals (Aronson *et al.* 2004; Maachi *et al.* 2004). One of the main contributors to high systemic levels of inflammation in obese subjects is the adipose tissue environment (Hotamisligil *et al.* 1995; Bastard *et al.* 2002; Maachi *et al.* 2004; Spencer *et al.* 2010). Compared with normal weight, adipose tissue from obese subjects contains a higher number of infiltrated immune cells and shows higher expression levels of inflammatory genes (Weisberg *et al.* 2003). Inflammation found in adipose tissue from obese subjects is partly explained by the active remodelling process (expansion), hypoxia and dysfunctional lipid metabolism and transport (Spencer *et al.* 2010; Bourlier *et al*. 2008). Studies in animal models and humans provide strong evidence of a negative effect of inflammatory cytokines on insulin sensitivity in adipose tissue (Hotamisligil *et al.* 1995; Lumeng *et al.* 2007).

Compared with normal weight subjects, obese subjects have an exaggerated postprandial response after meal ingestion (e.g. higher triglyceride concentrations). Studies in insulin resistant subjects demonstrated that the rise in postprandial plasma lipid concentrations is associated with an increment in pro-inflammatory cytokines (Esponsito *et al.* 2007; Nappo *et al.* 2002). This elevated postprandial metabolic and inflammatory responses seen in insulin resistant subjects were not seen in healthy controls. The differences in postprandial inflammatory response between normal weight and obese subjects have not been studied in detail.

Another factor that has been associated with postprandial inflammation is the meal fatty acid type and content. A high fat meal (HFM) challenge is related to a greater increase in plasma lipids (mainly triglycerides) than a low fat meal. The type of FA ingested also influences the course of the postprandial response. In healthy subjects, a high saturated FA (SFA) diet induced NF-κB activation while this effect was prevented in subjects receiving a high monounsaturated (MUFA) or PUFA meal (Aljada *et al.* 2004; Jimenez-Gomez *et al*. 2009; Peairs *et al.* 2011). Leukocyte TLRs are also up-regulated during the postprandial state in healthy subjects after a HFM (Ghanim *et al.* 2009; Deopurkar *et al.* 2010).

N-3 PUFA have demonstrated anti-inflammatory properties in both healthy and metabolically compromised subjects (Kew *et al.* 2003; Calder, 2004; Newens *et al.* 2011; Itariu *et al.* 2012). In the context of obesity, obese diabetic patients supplemented with n-3 PUFA presented a lower plasma concentration of MCP-1, lower adipose tissue chemokines expression (MCP-1 and MIP-α) and less macrophage infiltration in adipose tissue (Itariu *et al.* 2012, Spencer *et al.* 2013). Similarly, animal models have shown that diets containing fish oil, a rich source of n-3 PUFA, prevent insulin resistance and development of inflammation in AT (Todoric *et al.* 2006; Shi *et al*. 2006).

To summarise, obesity is a condition accompanied by different metabolic adaptations to the surplus of energy from the diet. Abnormal plasma concentrations of lipids and inflammatory molecules are a result of an overwhelmed adipose tissue that fails to manage energy influx and an inadequate immune response to components from the diet. N-3 PUFA have anti-inflammatory properties, although the effects on the context of obesity and postprandial inflammation have not been fully explored. The BIOCLAIMS study aims to explore inflammation in normal weight and obese subjects and to examine the effect of acute and chronic exposure to n-3 PUFA on fasting and postprandial metabolic and inflammatory markers in healthy normal weight and obese subjects.

3.1.2 Aim

The aim of this research is to investigate whether n-3 PUFAs improve (i.e. lower) metabolic and inflammatory responses in both normal weight and obese subjects.

3.1.3 Hypotheses

The hypotheses being tested in the work that will be described in the following chapters are that:

- obese subjects will show higher concentrations of blood lipids and related analytes (glucose, insulin)
- obese subjects will show higher concentrations of molecules related to inflammation including cytokines, adipokines, adhesion molecules and growth factors than seen in normal weight subjects;
- obese subjects will show higher postprandial metabolic and inflammatory responses to a HFM than seen in normal weight subjects;
- including n-3 PUFAs with the meal will lower the postprandial metabolic and inflammatory responses in both normal weight and obese subjects;
- chronic supplementation with n-3 PUFAs will lower blood lipids and related analytes (glucose, insulin) and markers of inflammation in both normal weight and obese subjects;
- chronic supplementation with n-3 PUFAs will lower the postprandial metabolic and inflammatory responses in normal weight and obese subjects after a HFM.

3.1.4 Objectives

The objectives of this research are:

- to evaluate the fasting concentrations of plasma triglycerides, cholesterol, non-esterified fatty acids, glucose and insulin in normal weight and obese subjects;

- to evaluate the fasting plasma concentrations of molecules related to inflammation including cytokines, adipokines, adhesion molecules and growth factors, in normal weight and obese subjects;
- to evaluate TLR-2 and -4 expression on monocytes from normal weight and obese subjects;
- to evaluate the *ex vivo* production of inflammatory cytokines in whole blood
- to evaluate the *ex vivo* intracellular production of IL-6, TNF-α and IL-1β on monocytes
- to evaluate the postprandial metabolic response to a HFM in normal weight and obese subjects;
- to evaluate TLR expression on monocytes following a HFM in normal weight and obese subjects;
- to evaluate molecules related to inflammation following a HFM in normal weight and obese subjects in plasma;
- to evaluate the effect of inclusion of n-3 PUFA with the meal on all of the above;
- to evaluate the effect of a 12 week period of n-3 PUFA supplementation prior to consumption of a HFM on all of the above.

3.2 Methods

3.2.1 Study design, inclusion and exclusion criteria, and clinic visits

3.2.1.1 Inclusion and exclusion criteria

This study is a double-blind, placebo controlled trial. Healthy men and women aged 18-65 y, not using FO supplements, with normal body weight or obesity according to body mass index (BMI) and waist circumference were recruited. The normal weight group included subjects with BMI between 18.5 and 25 kg/m^2 and the obese group subjects with BMI between 30-40 kg/m^2 and a waist circumference ≥ 94 cm for men and ≥ 80 cm for women. Subjects with diagnosed inflammatory/metabolic disease or using medication to control inflammation, blood lipids or blood pressure were excluded from the study.

Inclusion criteria

1. Male or female aged 18 to 65 years
2. BMI between 18.5 to 25 (normal weight) or 30 to 40 kg/m^2 (obese)
3. If BMI between 30 to 40 kg/m^2 waist circumference ≥ 94cm for men and ≥ 80 cm for women
4. Not consuming FO or other oil supplements
5. Not eating more than one portion of oily fish per week
6. Being able to provide written informed consent

Exclusion criteria

1. Men and woman aged < 18 or > 65 years
2. BMI < 18.5, 25.1-29.9 or > 40 kg/m^2
3. Diagnosed metabolic disease (e.g. diabetes, cardiovascular disease) or chronic gastrointestinal problems (e.g. inflammatory bowel disease, celiac disease, cancer)
4. Use of prescribed medicine to control inflammation, blood lipids or blood pressure.
5. Use of FO or other oil supplements
6. Consumption of more than one meal containing oily fish per week
7. Pregnant or planning to become pregnant within the study period

8. Participation in other clinical trial

3.2.1.2 Recruitment

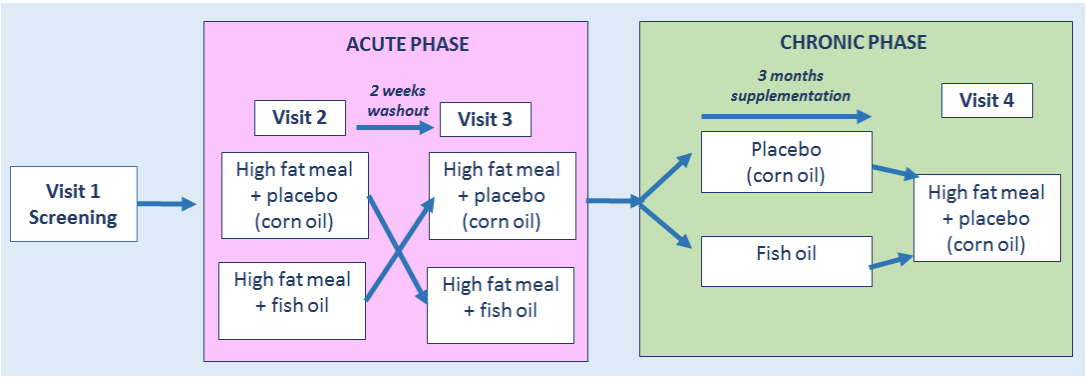
Subjects were recruited via posters, email, newspapers and radio. Subjects interested in the study were screened by telephone interview. If they appeared to meet the inclusion criteria, they received an information sheet (Appendix II) and an appointment to visit the Wellcome Trust Clinical Research Facility (WTCRF) at Southampton General Hospital was arranged.

3.2.1.3 Study design

Subjects visited the WTCRF in 4 occasions. Anthropometric measurements were made and body composition recorded at each visit, as described in section 3.2.2.1 . During visit 1 (V1), subject eligibility was confirmed and informed signed consent was obtained (Appendix II). Postprandial testing was performed for 6 hours following a HFM on 3 visits (V2, V3 and V4) (**Figure 3.1a**). **Figure 3.1b** describes subjects' clinic visits.

On the first 2 postprandial visits (V2 and V3), subjects consumed a HFM accompanied by a modified FO preparation (3 capsules of EPAX600 providing 0.36 g EPA plus 0.27 g DHA per capsule) or placebo (3 capsules of corn oil) in a random order (acute effect); all subjects underwent both HFM challenges. The meal was two croissants served with a fixed amount of butter and jam and a milkshake made with 250 mL of semi-skimmed milk, 32 g of Nesquik™ powder and 75 g of double cream. **Table 3.1** shows the weight of each meal component used and the macronutrient and energy content. The meal provided 85.8 g of fat, 76.85 g of carbohydrate and 1134 kcal of energy. Moreover, the meal was rich in saturated fatty acids, especially palmitic acid (34.8 g) (**Figure 3.2**).

a)



b)

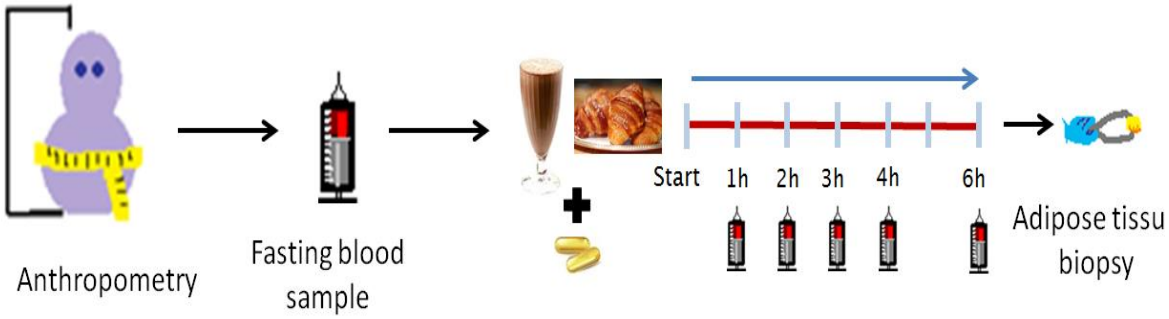


Figure 3.1 Study design (a) and clinic visits description (b)

Between the V3 and V4, subjects were randomized to a modified FO preparation called EPAX600 (3 capsules per day providing 0.36 g EPA plus 0.27 g DHA per capsule) or placebo (3 capsules per day of corn oil) for 12 weeks (chronic effect). **Table 3.3** shows the FA composition of the FO and corn oil capsules.

Ingredient	g	Fat (g)	Carbohydrate (g)	Protein (g)	Energy (kcal)	Energy (kJ)
Strawberry Jam	18.75	0.00	12.42	0.11	46.98	200.52
Apricot Jam	14.57	0.00	12.42	0.11	46.98	200.52
Semi-skim Milk	250.00	4.25	11.25	8.75	115.00	485.00
Nesquik	32.00	1.31	2.08	1.12	24.64	102.08
Butter	28.00	23.02	0.17	0.17	208.32	856.52
Croissants	86.00	16.94	37.24	7.14	320.78	1344.18
Double Cream	75.00	40.28	1.28	1.20	372.00	1530.75
TOTAL		85.80	76.85	18.59	1134.70	4719.57

Table 3.1 Composition of the HFM

		Myristic Acid	Palmitic Acid	Palmitoleic Acid	Stearic Acid	Oleic Acid	Linoleic Acid	α-Linoleic Acid	Arachidic Acid	Arachidonic Acid	Total FA
	g	14:0	16:0	16:1 n-7	18:0	18:1 n-9	18:2 n-6	18:3 n-3	20:0	20:4 n-6	Total FA
Strawberry Jam	18.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Apricot Jam	14.57	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Semi-skim Milk	250	0.55	1.69	0.09	0.55	1.15	0.11	0.02	0.01	0.01	4.18
Nesquik	32	0.00	0.30	0.00	0.38	0.39	0.20	0.02	0.01	0.00	1.30
Butter	28	3.33	9.55	0.40	2.91	5.71	0.55	0.19	0.17	0.00	22.81
Croissants	86	2.33	6.78	0.31	2.10	4.18	0.83	0.12	0.12	0.04	17.60
Double Cream	75	5.79	16.51	0.71	5.17	10.23	0.97	0.24	0.29	0.00	39.93
Total fatty acids		11.99	34.83	1.51	11.12	21.65	2.66	0.60	0.60	0.04	85.80

Table 3.2 FA composition of the HFM (g)

			Fish oil	Corn oil
Saturated fatty acids (g)		16:00	0.04	0.11
		18:00	0.04	0.02
	Total SFA		0.08	0.12
Monounsaturated (g)	n-7	16:1n-7	0.01	0.00
		18:1n-7	0.03	0.01
		Total n-7	0.04	0.01
	n-9	18:1n-9 (oleic acid)	0.08	0.27
		20:1n-9	0.02	0.00
		Total n-9	0.10	0.27
	Total MUFA	Total n-9	0.14	0.28
Polyunsaturated (g)	n-6	18:2n-6 (linoleic acid)	0.01	0.55
		20:4n-6	0.02	0.00
		Total n-6 PUFA	0.03	0.55
	n-3	18:3n-3 (α-linolenic acid)	0.02	0.01
		20:4n-3	0.02	0.00
		20:5n-3 (EPA)	0.36	0.00
		22:5n-3 (DPA)	0.05	0.00
		22:6n-3 (DHA)	0.26	0.00
		Total n-3 PUFA	0.71	0.00
	Total PUFA		0.77	0.56
Total FA			0.98	0.98

Table 3.3 FA composition of the FO and placebo capsules (g per g of oil)

3.2.1.4 Sample processing

During the post postprandial challenge, 5 mL of heparinised blood was collected at 0 h (fasting), 1 h, 2 h, 3 h, 4 h and 6 h. At each time point whole blood (400 µL) was used for cell surface marker analysis by flow cytometry. The remaining 4.6 mL of blood was centrifuged at 1000 *g* for 10 min to obtain plasma for measurement of lipids and other metabolites, insulin, FAs, lipid mediators and inflammatory markers. Subcutaneous abdominal AT biopsy samples were taken at V2 and V4; these were kept in either RNAlater or formalin or snap frozen in liquid N_2 for later analysis.

3.2.2 Anthropometry

3.2.2.1 Height

Participant height was measured using a Seca stadiometer. The participant was asked to remove his/her shoes and stand still against the stadiometer in an upright position (**Figure 3.2**). The participant was asked to take a deep breath and hold this while maintaining the body in the same position. A moveable headboard was brought to the upper point of the head making sure the hair was compressed and height recorded.

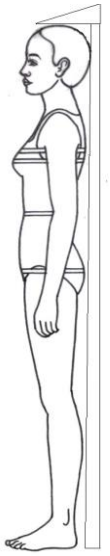


Figure 3.2 Height measurement using a stadiometer

3.2.2.2 Weight and body composition measurement

Participant weight and body composition were measured using a digital bioelectric impedance apparatus (TANITA BC-418). For this measurement, the participant was asked to remove his/her shoes, any heavy clothes and pocket contents. Participant information, including age, height and sex, was input into the TANITA system. The participant was asked to step on the TANITA platform BC-418 (metal sole plates) with bare feet making sure the heels were placed on the posterior electrodes and the front part of the feet on the anterior electrodes and to grasp the TANITA grips and stay standing still until the impedance measurement was completed. Weight, fat mass, lean mass and total body water were recorded.

3.2.2.3 Waist and hip circumference

To measure waist circumference, the participant was asked to stand upright with feet together and relax. Then, the participant was asked to lift his/her arms while a measuring tape was placed around the abdomen, between the ribs and the hip bone, and one centimetre under the umbilicus (**Figure 3.3**). The tape was kept snug and parallel to the floor; waist circumference was recorded.

To measure hip circumference, the participant was asked to stand in the same position. The measuring tape was moved down from the right side and the maximum extension of the buttocks was detected and hip circumference recorded.

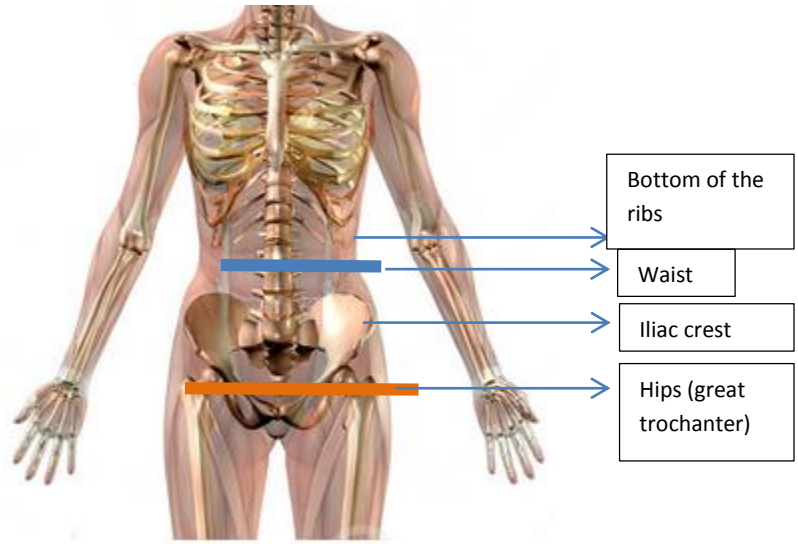


Figure 3.3 Waist and Hip circumference measurement

3.2.3 Measurement of plasma lipid and glucose concentrations

Triglyceride, cholesterol, HDL-cholesterol, NEFA and glucose concentrations were measured using an iLAB 600 clinical chemistry analyser and software (Instrumentation Laboratories, Warrington, UK) using enzyme based kits provided by Wako and Instrumentation Laboratories. 200 µL of plasma and standards were placed in inserts and analysed using the settings recommended by manufacturer for each analyte (**Figure 3.4a** and **Figure 3.4b**).

Briefly, the plasma samples were exposed to various enzymatic reactions and photometric analysis allowed the quantification of the analyte of interest. To measure

HDL cholesterol, plasma samples were incubated with β-lipoprotein antibody, which binds to all lipoproteins except HDL, and blocks their enzymatic reactions. Then, cholesterol esterase was added to hydrolyse cholesterol esters followed by cholesterol oxidase which resulted in production of a blue colour due to oxidation of cholesterol and the concomitant oxidative condensation of a chromogen. The density of the colour was measured as absorbance at 660 nm. HDL cholesterol concentration was determined by comparison with the absorbance readings produced by standards of known concentration. **Figure 3.4a** and **Figure 3.4b** describe the enzymatic reactions and wavelengths used for the measurement of glucose, triglycerides, cholesterol and NEFA.

Low density lipoprotein (LDL) was estimated using the Friedwald equation (Friedwald, 1972). This method avoids the use of preparative ultracentrifugation for the isolation of LDL. This calculation includes total cholesterol, triglyceride and HDL cholesterol concentrations (all in mmol/L):

LDL cholesterol = [Total Cholesterol] – [Triglycerides/5] – [HDL cholesterol]

Plasma glucose and lipid concentrations are expressed as mmol/L or μmol/L as appropriate.

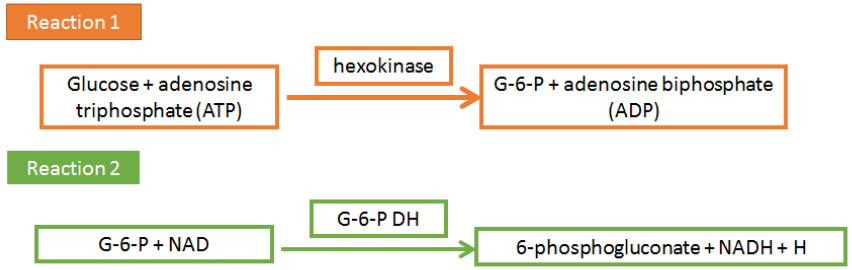
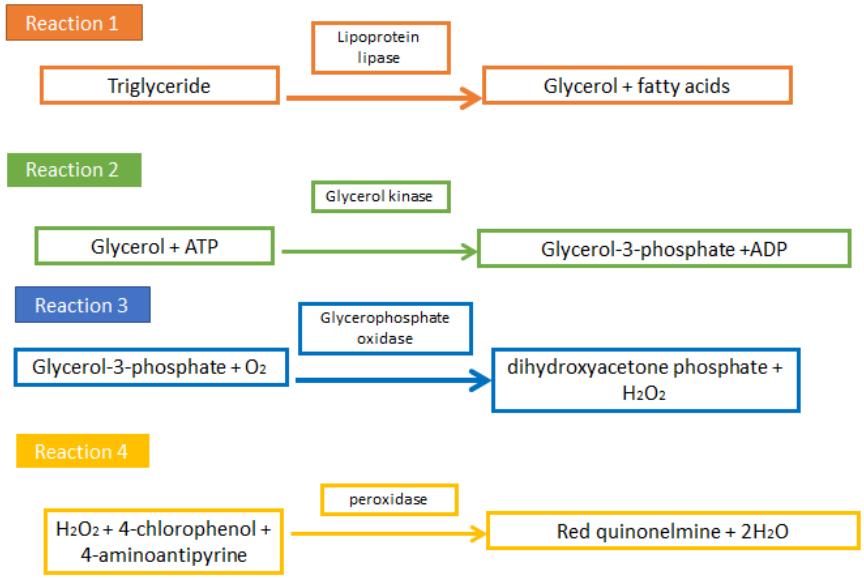
Analyte	Reactions	Outcome and absorbance
Glucose		The conversion of NAD^+ to NADH is proportional to glucose concentration; Primary wavelength: 340 nm; Blank wavelength: 370 nm
Triglycerides		Red quinonelmine is proportional to triglyceride concentration; Primary wavelength: 510 nm; Blank wavelength: 700 nm

Figure 3.4a Summary of the reactions used to determine concentrations of glucose and triglycerides

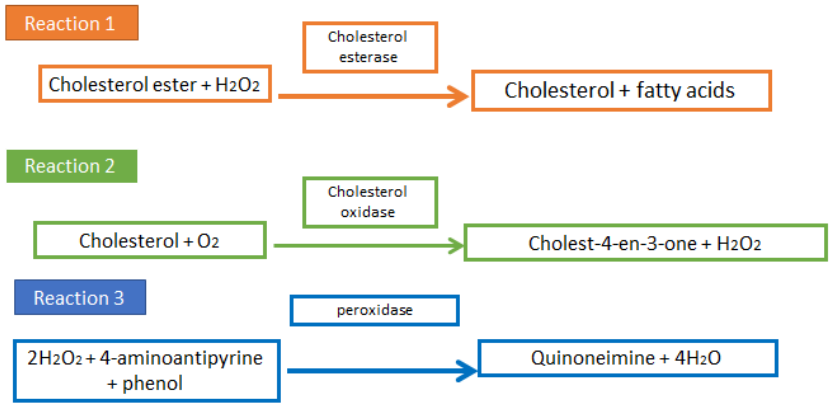
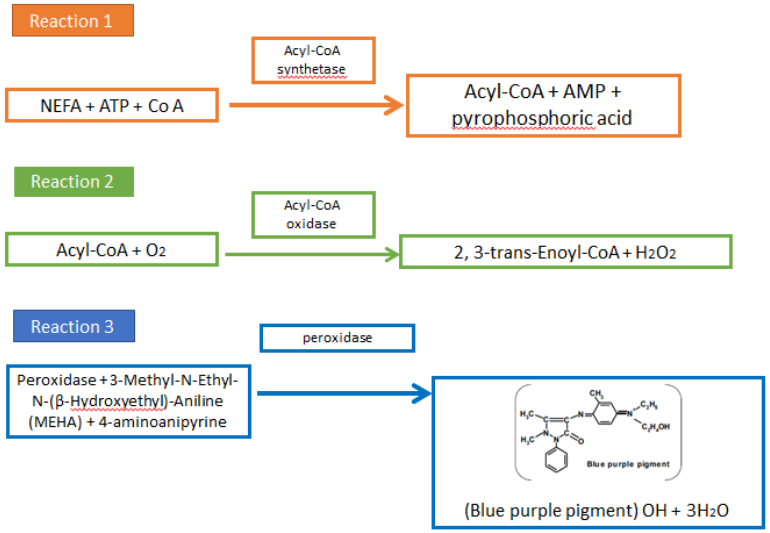
Analyte	Reactions	Outcome and absorbance
Cholesterol		Quinonelmine is proportional to cholesterol concentration; Primary wavelength: 510 nm Blank wavelength: 700 nm
NEFA		Blue purple colour is proportional to NEFA concentration Primary wavelength: 546 nm Blank wavelength: 660 nm

Figure 3.4b Summary of the reactions used to determine concentrations of cholesterol and NEFA

3.2.4 Measurement of plasma insulin concentrations

Insulin concentrations were evaluated by quantitative sandwich enzyme immunoassay (ELISA) using a commercial kit following the manufacturer's instructions (Dako). The principle of a sandwich ELISA is shown in **Figure 3.5**

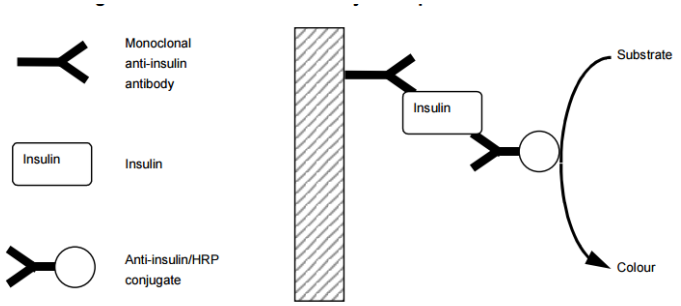


Figure 3.5 Quantitative sandwich enzyme immunoassay principle

Briefly, samples, blank and standards were placed in a microplate pre-coated with a specific anti-insulin antibody. Simultaneously, an enzyme-labelled antibody specific for insulin was added to each well and incubated for 60 min at room temperature in a shaker.

Insulin present in the sample was bound by the immobilized antibody and unbound sample and non-specifically bound molecules were removed using wash buffer. A substrate solution was added to the wells and incubated for 10 min. Colour development was stopped with "Stop solution" and the intensity was measured in a plate reader at 620 nm within 30 min. The colour developed was proportional to the amount of insulin bound in the initial step.

Insulin concentrations are expressed as µUI/mL. Beta cell function and insulin sensitivity were estimated using the homeostasis model assessment (HOMA). HOMA is a set of mathematical equations describing the functions of organs involved in glucose homeostasis and thus allowing the estimation of insulin resistance and beta cell function during homeostatic conditions (i.e. fasting state). The mathematical equations described in this method were derived from studies regarding to the glucose: insulin feedback and

comparable with hyperglycaemic, euglycaemic clamps and glucose tolerance tests (Matthews, 1985). The equations described below were reported as an alternative to the computer model and provided an approximation in this model:

HOMA-IR= [Glucose in mmol/L] x ([insulin in IU/L] / 22.5)

HOMA %B= (20 X [insulin in IU/L])/([Glucose in mmol/L]-3.5)

HOMA %S= 100/[HOMA-IR]

HOMA IR provides an estimate of insulin resistance, HOMA %B is an estimate of beta cell function (i.e. insulin secretion relative to glucose concentrations) and HOMA %S is a percentage of insulin sensitivity (i.e. capacity to uptake glucose).

In this study the updated version of HOMA (HOMA2) was employed for all calculations. HOMA2 is a computer model that accounts for glucose renal losses, allowing the estimation of insulin resistance in hyperglycaemic subjects (Levy *et al*. 1998; Wallace *et al.* 2004). Calculations were performed using HOMA software developed by Oxford University (University of Oxford, 2004).

3.2.5 Sample size and statistical analysis

The parameters considered to determine BIOCLAIMS study sample size were based on the chronic intervention and are as follows:

- typical distribution of variables of interest, e.g. IL-6 mean and distribution;
- expected response: based in previous clinical trials reporting a 20% reduction in the concentrations of some inflammatory mediators after fish oil supplementation;
- estimated loss of recruited subjects from the study: in this case 20% was considered.

A sample size of 25 volunteers per group was determined as an adequate number to obtain not less than 80% power, 5% level of significance and to allow a 20% loss of recruited subjects. Thus 50 normal weight and 50 obese subjects were recruited and 50% of them were randomly allocated to a fish oil treatment and 50% to placebo treatment for 12 weeks.

All data were managed in Microsoft Excel 2010® and all statistical tests were performed in IBM SPSS Statistics 21®. Data are expressed as mean ± standard deviation (SD).General linear model (univariate analysis) was used to compare data between normal weight and obese subjects. The contribution of age and sex were used as covariates in this analysis. The linear correlation between anthropometric and metabolic parameters was evaluated using Pearson correlation coefficient.

3.3 Results

3.3.1 Subjects' baseline subjects characteristics

3.3.1.1 Normal weight and obese subjects' anthropometric characteristics

In total 100 subjects were recruited into the study (50 normal weight and 50 obese) (**Figure 3.6**). The number of women recruited was greater than the number of men, 72% and 28% respectively. 78 subjects completed the study; 22 subjects withdrew from the study for personal reasons, because they did not wish to have a second AT biopsy, because of lack of time or loss of interest. Thus in this chapter, data are shown for those subjects for whom metabolic data from both V2 and V3 were available (n = 35 normal weight and n =40 obese).

At baseline, a number of anthropometric measurements were significantly different between normal weight and obese subjects, as expected (**Table 3.4**). Obese subjects had significantly higher BMI, waist circumference and body fat mass (all ρ<0.01), while the percentage of lean mass and W/H ratio were significantly higher in normal weight than obese subjects (ρ<0.01). Normal weight subjects were younger than the obese (ρ<0.01).

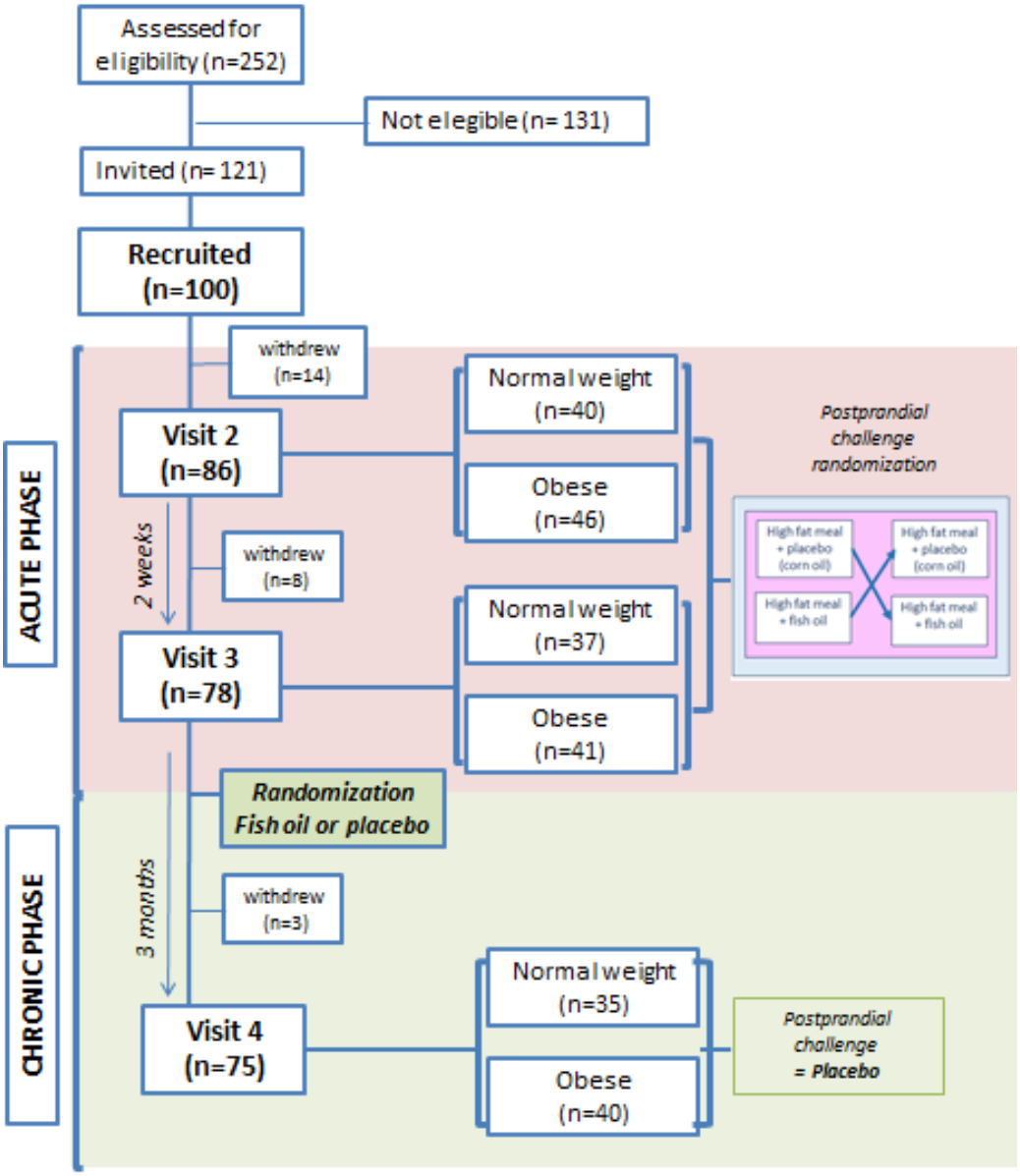


Figure 3.6 Flow of subjects through the study

	Normal weight (n=35)	Obese (n=40)	ρ-value
Age (y)	31 ± 14	44 ± 12	**0.01**
Sex (F/M)	25 / 10	29 / 11	
BMI (kg/m^2)	22.22 ± 1.83	34.71 ± 2.64	**<0.01**
Waist circumference (cm)	75.21 ± 5.86	107.31 ± 11.96	**<0.01**
W/H ratio	0.79 ± 0.06	0.92 ± 0.09	**<0.01**
Body fat (%)	22.23 ± 8.24	41.7 ± 6.36	**<0.01**
Fat mass (kg)	13.46 ± 4.75	37.30 ± 12.78	**<0.01**
Lean mass (%)	77.77 ± 8.23	58.31 ± 6.35	**0.01**
Lean mass (kg)	48.84 ± 11.23	52.61 ± 18.64	0.28

Data expressed as mean ± SD; Univariate general linear model analysis was used for comparisons between groups BMI=body mass index; W/H ratio= waist/hip ratio

Table 3.4 Anthropometric characteristics of normal weight and obese subjects at baseline

3.3.1.2 Normal weight and obese subjects' metabolic profile

At baseline, fasting plasma glucose, NEFA, triglycerides, total cholesterol and LDL cholesterol concentrations were significantly higher in obese than normal weight subjects (ρ <0.01) (**Table 4.2**). Although, the glucose and triglyceride concentrations in both groups fell within the normal range according to the NHS UK adult recommendations (NHS UK, 2013), total cholesterol and LDL cholesterol were above the normal range in obese subjects. HDL cholesterol concentration was not significantly different between groups.

Fasting insulin levels were significantly higher in obese than normal weight subjects. HOMA calculations revealed higher percentage of beta cell function in obese subjects in agreement with the higher insulin levels observed in this group. Normal weight subjects were found to have a lower insulin secretion (HOMA % B), a better insulin sensitivity (HOMA % S) and lower insulin resistance (HOMA IR) than obese subjects (**Table 3.5**). The anthropometric and metabolic differences between men and women are included in Appendix II.

	Normal weight (n=35)	Obese (n=40)	*p*-value
Glucose	4.77 ± 0.42	5.60 ± 1.05	**<0.01**
NEFA	484.48 ± 211.81	609.07 ± 215.85	**<0.01**
Triglycerides	0.79 ± 0.29	1.32 ± 0.70	**<0.01**
Total Cholesterol	4.42 ± 1.04	5.24 ± 0.92	**0.01**
HDL cholesterol	1.57 ± 0.39	1.49 ± 0.37	0.20
LDL cholesterol	2.69 ± 0.90	3.49 ± 0.78	**<0.01**
Insulin	5.75 ± 3.32	13.55 ± 7.15	**<0.01**
HOMA % B	83.91 ± 33.96	112.80 ± 45.06	**<0.01**
HOMA % S	177.55 ± 98.86	75.60 ± 48.77	**<0.01**
HOMA IR	0.71 ± 0.42	1.80 ± 0.90	**<0.01**
Data expressed as mean ± SD; all units are mmol/L, except NEFA (µmol/L) and insulin (µIU/mL); HOMA=homeostasis model assessment; HOMA IR = insulin resistance; HOMA %B= beta cell function; HOMA %S: insulin sensitivity ; Univariate general linear model analysis was used for comparisons between groups			

Table 3.5 Baseline fasting plasma glucose, insulin and lipid concentration in normal weight and obese subjects

3.3.2 Correlation between anthropometric and metabolic parameters

The association between anthropometric and metabolic parameters was assessed using Pearson correlation coefficient. As described in **Table 3.6**, all the anthropometric measurements, except lean mass (kg), were strongly correlated with the metabolic markers.

BMI, waist circumference and W/H were positively associated with plasma glucose, insulin triglycerides, cholesterol and HOMA IR. Body fat had a strong positive association with NEFA and HOMA %B (beta cell function). These results are in agreement with reports suggesting a relationship between NEFA concentrations and adipose tissue dysfunction as previously described. The positive association of HOMA %B with body fat suggests an increased requirement for insulin in obese subjects (i.e. higher beta cell function) possibly due to peripheral insulin resistance due to high systemic NEFA concentrations.

Skeletal muscle is a metabolically active tissue also involved in systemic glucose and fatty acids uptake. The inverse association of % lean mass with glucose, insulin and HOMA IR suggest that the lower % of lean mass in obese subjects is also detrimental for metabolic homeostasis.

	Age	BMI	Waist (cm)	W/H ratio	% Body Fat	Fat Mass (kg)	% Lean Mass	Lean Mass (kg)
Glucose	.384**	**.537****	**.605****	**.534****	.116	.247*	**-.500****	-.107
Insulin	.154	**.628****	**.599****	**.452****	**.406****	**.511****	**-.441****	.057
Triglycerides	.373**	**.512****	**.585****	**.507****	.253*	.391**	-.379**	.039
NEFA	.384**	.391**	.376**	.267*	**.425****	**.435****	-.336**	-.076
Total Cholesterol	.223	**.682****	**.667****	**.516****	**.479****	**.584****	**-.515****	-.044
HDL Cholesterol	**.597****	.396**	**.428****	.375**	.301**	.352**	-.368**	-.061
LDL Cholesterol	.138	-.180	-.238*	-.219	.085	-.062	-.034	-.258*
HOMA %B	-.126	.350**	.274*	.142	**.405****	**.428****	-.187	.111
HOMA %S	-.180	**-.620****	**-.602****	**-.451****	**-.469****	**-.556****	**.484****	-.027
HOMA IR	.162	**.638****	**.613****	**.463****	.393**	**.500****	**-.467****	.034

*BMI=body mass index; W/H=waist/hip ratio; TG=triglycerides; NEFA=non esterified fatty acids, HOMA=homeostasis model assessment; HOMA IR = insulin resistance; HOMA %B= beta cell function; HOMA %S: insulin sensitivity;*ρ-value<0.05, ** ρ-value<0.001*

Table 3.6 Pearson correlations between anthropometric and metabolic parameters

3.4 Discussion

The current chapter aimed to describe the study design and the characteristics of the subjects recruited into the BIOCLAIMS study. In addition, the effect of obesity on the metabolic profile was described. Based on their medical history, only healthy normal weight and obese subjects were recruited for this trial. Obesity was determined using the combination of BMI and waist circumference. These criteria allowed the selection of a population of healthy obese subjects with a higher risk of developing metabolic diseases to be identified and recruited.

In agreement with the anthropometric data, the metabolic profile in the obese subjects was altered when compared with normal weight subjects. This agrees with previous reports which found altered metabolic markers in obese subjects when compared with normal weight subjects (Frayn, 2010). In particular, obese subjects had higher triglycerides, total cholesterol, LDL-cholesterol, glucose, NEFA and insulin concentrations compared with normal weight subjects. Moreover, strong associations between anthropometric and metabolic parameters were detected. Particularly waist circumference and W/H ratio had a strong positive association with most of the metabolic markers, thus confirming the detrimental effect of abdominal obesity in metabolic homeostasis.

In this study, the homeostasis model assessment (HOMA) was used to determine beta cell function, insulin sensitivity and insulin resistance. HOMA scores are based on glucose and insulin concentrations. HOMA IR scores in obese subjects were within range suggested by various authors for the diagnosis of insulin resistance (1.7-2.6) (Ascaso *et al*. 2003; Estegamati *et al.* 2010; Qu *et al.* 2011; Yamada *et al.* 2012), and the insulin sensitivity estimate (HOMA % S) was significantly lower in obese subjects than normal weight subjects. The elevated beta cell function and low insulin effectiveness found in obese subjects may reflect the early stages of insulin resistance as suggested by the HOMA IR scores. These results agree with previous clinical and epidemiological studies reporting a better insulin sensitivity in subjects with a lower BMI (Abassi *et al*. 2002; Esser *et al.* 2013a). The HOMA IR values of the healthy obese subjects in this study are comparable to those reported in

previous studies (values between 2.0-3.4) in obese non-diabetic subjects (Phillips *et al.* 2013; Esser *et al.* 2013a). Taking this evidence together, the obese population recruited in this study had some degree of insulin resistance.

Even though special attention was paid to obtaining a representative and evenly matched population in terms of age and sex, the majority of the subjects in this study (in both groups) were female, and the obese subjects recruited were slightly but significantly older than the normal weight subjects. It is known that age has an effect on some physiological processes, including metabolism and the immune system (Grundy *et al.* 1985; Weiskopt *et al.* 2009). Nevertheless, the majority of the volunteers in both groups were aged between 20 and 40 years (60% normal weight and 50% obese). Although the chronological age of the subjects covered a range of around ten years. This may mitigate the potential for age bias in the results, but for completeness age and sex were still considered in the statistical analysis.

3.5 Conclusions

The obese and normal weight subjects recruited for this study were different based on anthropometric and metabolic markers. Abdominal obesity has a negative effect on the overall lipid and glucose metabolism.

CHAPTER 4

Fasting hormones and immune system markers in healthy normal weight and obese subjects

CHAPTER 4

Fasting hormones and immune system markers in healthy normal weight and obese subjects

4.1 Introduction, objective, hypothesis, and aims

4.1.1 Introduction

The list of the elements involved in the metabolic regulation and pathogenesis of cardiometabolic diseases is growing. From inflammatory mediators to newly characterized molecules derived from adipose tissue, emerging research is providing evidence of the coordination of systems that were thought to be independent in metabolic homeostasis regulation. In this chapter the roles of different cytokines, adhesion molecules, growth factors and adipose tissue derived molecules in metabolic regulation are reviewed.

4.1.1.1 Elements of the inflammatory cascade: surface receptors and cytokines

The toll-like receptors (TLR) are a family of transmembrane proteins found on the surface of innate immune cells. TLR have been linked to insulin resistance. In a mice model, deletion of the TLR4 gene results in an improvement in insulin sensitivity and a reduction of inflammatory markers (Shi, 2006). Similarly, studies in humans have found higher numbers of monocytes expressing TLR4 and TLR2 in diabetic than in healthy subjects (Devaraj *et al.* 2008; Dasu *et al.* 2010). The downstream signals generated after TLR activation lead to NF-κB translocation to the nucleus and activation of the synthesis of inflammatory cytokines. TNF-α, IL-6, IL-1β and IL-10 are within the group of molecules produced in response to TLRs activation and that have been intensely studied in the context of inflammation and metabolic health.

TNF-α, IL-6 and IL-1β are important pro-inflammatory cytokines actively produced by immune cells during inflammation. TNF-α and IL-6 are also synthesized in human adipose tissue, and mainly in the omental compartment (Mohamed *et al.* 1997; Bastard *et al.* 2002; Kern *et al*. 2003). These cytokines are found in higher concentrations in the plasma of obese and diabetic subjects than in healthy controls (Derosa *et al.* 2013) and they have been found

to play a role in glucose and fatty acid metabolism (Hotamisligil *et al.* 1995; Stephens *et al.* 1997; Rotter *et al.* 2003; Klover *et al*. 2005).

TNF-α affects energy homeostasis, interfering with glucose homeostasis, lipolysis and lipogenesis in adipocytes. *In vitro* TNF-α disrupts various proteins involved in insulin signalling and lipoprotein lipase regulation (Kawaskami, 1987; Stephens *et al.* 1997; Ruan et al. 2002). Furthermore, TNF-α antagonizes PPAR-Y partly by inducing the transcription factor NF-κB (Yuan *et al.* 2001; Ruan *et al.* 2002). In addition, the feedback loop induced by TNF-α inhibits the production of insulin sensitizing molecules, such as adiponectin and increases pro-atherogenic molecules, such as PAI-1 (Ouchi *et al.* 2000; Swiatkwoska *et al.* 2005). Human studies showed a reduction of fasting glucose concentrations but not an improvement in insulin sensitivity or inflammatory parameters after chronic treatment with an anti-TNF antibody (Stanley *et al.* 2011). Thus, TNF-α plays a role on systemic glucose concentration regulation, although other factors are also involved.

IL-6 is a multifaceted cytokine that has been found to have negative and positive effects on human metabolism. Studies of *ex vivo* adipose tissue cultures showed a positive correlation between insulin resistance and adipose tissue IL-6 concentrations (Bastard *et al.* 2002; Kern *et al.* 2003). In agreement with these reports, prospective studies suggested an association between IL-6 and CRP concentrations and risk of type 2 diabetes (Pradhan *et al.* 2001). However, recent studies have challenged the idea of IL-6 as a pro-diabetogenic molecule. In humans, intravenous infusion of a high and low dose of IL-6 did not alter glucose metabolism (Steensberg *et al.* 2003). Lynso reported an increased IL-6 output from adipose tissue after exercise and suggested a role of this molecule in lipolysis and fatty acid mobilization (Lynso *et al.* 2002). In agreement with this report, Ellingaasgard *et al*. 2011, reported an increase in systemic IL-6 concentrations after exercise, these changes were followed by an increment of GLP-1 secretion, promoting insulin secretion. These findings together agree that adipose tissue produces IL-6 in response to different physiological conditions and that the acute or chronic exposure to IL-6 in different tissues produces a particular outcome that can be detrimental or beneficial for glucose homeostasis.

IL-1β, in a metabolic context, inhibits the function and proliferation of beta cells (Dinarello *et al*. 2011). The deletion of IL-1β in mice ameliorates diet-induced infiltration of macrophages into adipose tissue, up regulates the expression of adipogenic genes in adipose tissue (PPAR-Y, CCAAT/enhancer biding protein (CEBPA), fatty acid binding protein (FABP4)) and prompts its capacity for expandability (Nov *et al.* 2012). In agreement with this report, a study in IL-1R KO mice or a model where IL-1β was agonised reported a defect in lipid accumulation (Matsuki *et al.* 2003). *In vitro* work with human adipocytes has shown a down regulation of PPAR-Y and insulin signalling downstream by this cytokine (Legathu *et al.* 2006; Jager *et al.* 2007). Thus, IL-1β has negative effects in insulin regulation by directly affecting beta cell function. Additionally, the findings in IL-1β KO mice suggest that IL-1β has an effect on pathways related to adipose tissue plasticity that once affected, prompt tissue atrophy, further recruitment of inflammatory cells and ectopic fat accumulation.

A remarkable beneficial effect of IL-1β antagonism was reported by Larsen and colleagues (2007). This randomized clinical trial described the long-term effect of anakinra, an IL-1β antagonist, on glucose homeostasis in type 2 diabetic patients (Larsen *et al.* 2007). According to this report, beta cell function (based in proinsulin/insulin ratio and C-peptide), fasting glucose and glycated haemoglobin were improved after treatment, while inflammatory markers were reduced. Although, insulin sensitivity and the expression of GLUT4 were not affected after treatment. Hence, these findings are in agreement with *in vitro* and *in vivo* reports suggesting that IL-1β plays a role in insulin resistance.

IL-10 is generally recognized as an anti-inflammatory cytokine limiting inflammation (Ouyang *et al.* 2011). In human monocytes IL-10 inhibits NF-κB in a dose dependent fashion without affecting other transcription factors, such as AP-1 (Wang *et al.* 1995). IL-10 treatment improved insulin sensitivity in muscle and liver in wild-type mice while the overexpression of this protein in transgenic mice intensified insulin signalling in muscle (Hong *et al.* 2009). IL-10 has been positively associated with insulin sensitivity in healthy middle aged subjects after adjusting data for other factors contributing to insulin homeostasis (Straczkowski *et al.* 2005). Similarly, a report by Esponsito *et al* (2002) documented significantly lower concentrations of IL-10 in patients with metabolic

syndrome compared with controls in a population of obese and normal weight subjects (Esponsito *et al.* 2002). Moreover, IL-10 has been found at lower concentrations in plasma from women with android obesity when compared with normal weight or gynoid obesity (Manigrasso *et al.* 2005). Taking all the evidence together, IL-10 is reduced in the presence of some metabolic syndrome features; the accumulation of adipose tissue is accompanied by an increment in inflammatory cytokine production (e.g. TNF-α) (Hotamisligil *et al.* 1994) that may antagonize IL-10 synthesis thus affecting insulin regulation.

Granulocyte macrophage-colony stimulating factor (GM-CSF) is a cytokine governing the functions of granulocytes and macrophage lineage populations. GM-CSF primes the e*x vivo* production of pro inflammatory cytokines (Hamilton *et al.* 2002). Recently GM-CSF has been studied in relation to metabolism and cardiovascular disease. In animal models, GM-CSF mutation reduced expression of IL-1β, TNF-α and MIP-1α in adipose tissue and enhanced peripheral uptake and use of glucose (Kim *et al.* 2008). In relation to atherosclerosis, a report using a similar mouse model found a reduction of PPAR-y expression and an increased infiltration of macrophages into atherosclerotic lesions (Ditiatkovski *et al*. 2006). In clinical studies, prediabetic (glycated haemoglobin = >5.7 and <6.5%) and hypertensive patients present increased concentrations of GM-CSF when compared with controls (Parissis *et al.* 2002; Lucas *et al.* 2013). Thus, GM-CSF antagonism may be beneficial as it alters mononuclear cell proliferation and migration to metabolic tissues although the role of GM-CSF in cardio-metabolic diseases remains inconclusive and requires more mechanistic evidence.

4.1.1.2 Chemokines and adhesion molecules

Chemokines are a family of cytokines regulating cell trafficking. These peptides induce the infiltration of monocytes, neutrophils and lymphocytes through the vascular endothelium; they act via G-protein coupled receptors (Deshnane *et al.* 2009). Within this family, MCP-1 and IL-8 have been studied in detail in terms of obesity and cardiovascular disease.

IL-8 is a neutrophil chemoattractant that has been strongly associated with cardiovascular risk. Reports from large prospective studies revealed a strong association between IL-8

concentrations in plasma, risk of cardiovascular events and subsequent survival (Boekholdt *et al*. 2004; Inonue *et al.* 2008). Like other cytokines, IL-8 is produced in large amounts by stromal cells from adipose tissue and its synthesis is enhanced in obese subjects (Bruun *et al.* 2003). In agreement with these findings, systemic concentrations of IL-8 are found in higher concentrations in obese subjects (Kim *et al*. 2006).

Monocyte Chemoattractant Protein-1 (MCP-1) is a chemoattractant produced in response to inflammatory cytokines and insulin (Sartipy *et al*. 2002; Westerbacka *et al.* 2008). According to studies in mice, MCP-1 is upregulated in adipose tissue during an obesity inducing high fat diet and it is a determinant for mononuclear cell infiltration into this tissue (Xu *et al.* 2003; Kanda *et al.* 2006; Chen *et al.* 2012). In humans, MCP-1 release from visceral adipose tissue is 7-fold higher in obese than normal weight persons and this is reflected in its systemic concentrations (Bruun *et al.* 2005; Christiansen *et al.* 2005; Kim *et al.* 2006). Upon a euglycemic insulin clamp, MCP-1 gene and protein concentrations increase significantly in adipose tissue from insulin resistant but not control subjects. MCP-1 may not directly interfere with metabolic pathways but it contributes to the infiltration of inflammatory cells in adipose tissue.

Cell adhesion molecules are a family of proteins involved in the initial rolling, attachment and transendothelial migration of leukocytes during inflammation. The immunoglobulin superfamily, selectins, cadherins and integrins are included in this family. Adhesion molecules have an important role in atherosclerosis as leukocyte binding to the vascular wall appears to be one of the initial steps of the formation of the atheroma. Clinical studies reported high expression of E-selectin, ICAM-1 and vascular adhesion molecule (VCAM-1) in coronary artery lesions and neovascular endothelium (Wood *et al.* 1993; O'Brian *et al*. 1996). Moreover, prospective data indicates a strong association between ICAM-1 and E-selectin concentrations and the risk of myocardial infarction (Hwang *et al*. 1997; Lemos *et al.* 2000). Similarly to proinflammatory cytokines and adipokines, concentrations of adhesion molecules have been found higher in obese than normal weight subjects (Zicarddi *et al.* 2002; Bosanska *et al.* 2010). The elevation of proinflammatory cytokines found in

obese subjects may trigger the release of cellular adhesion molecules (CAMs) from the endothelium and contribute to atherosclerosis development in these subjects.

4.1.1.3 Adipose tissue derived molecules: adipokines

In the recent years, the role of adipose tissue derived molecules in the regulation of human metabolism and inflammation has been intensively investigated. Adipokines are a family of molecules with cytokine-like structures, derived from adipose tissue. They have endocrine functions and some of them are suggested to act as immune-modulatory agents (Lago *et al.* 2007; Deng and Scherer, 2010).

Ex vivo studies with human adipose tissue have demonstrated that the physiology and endocrine capacity of adipose tissue is determined by its location. For example, visceral fat has been associated with metabolic risk while peripheral fat depots are known to be protective. In this context, leptin is found in higher concentrations in subcutaneous adipose tissue than omental (van Harmelen *et al.* 2002) while adiponectin concentrations are higher in omental than subcutaneous (Motoshima *et al.*2002). As adiponectin plays a role in insulin homeostasis, its production by omental adipose tissue may be a natural protective mechanism to trigger fatty acid oxidation and prevent ectopic accumulation of fat in vital organs such as the liver and pancreas.

Changes in adipose tissue found in obese subjects are followed by a switch in the production of adipokines and other metabolic molecules. In this section the adipokines that have been shown to play a pivotal role in metabolic homeostasis, based on *in vivo*, clinical trials and epidemiological studies will be discussed in detail. Additionally, some emerging molecules will be described.

Leptin was one of the first adipose tissue derived molecules to be elucidated. Leptin plays a role in the regulation of food intake and energy regulation. Its concentrations are increased in obese subjects when compared with normal weight subjects (Considine *et al*. 1996; Silha *et al.* 2003). In the context of cardiovascular disease, leptin has been found to enhance platelet aggregation and angiogenesis and to trigger PAI-1 production (Konstantinides *et al.* 2001).

Leptin has been shown to play a role in the regulation of the Th1 and Th2 phenotype. *In vivo* studies on T-lymphocyte responses showed that leptin increases a Th1 phenotype and suppresses Th2 cytokine production triggering the progression of autoimmune diseases (Matarese *et al.* 2001). Moreover, leptin restored Th1 responses in immunosuppressed experimental animals (Lord *et al*. 1998). Clinical studies have reported high systemic concentrations of leptin in various autoimmune disorders, including lupus, rheumatoid arthritis and multiple sclerosis. Leptin administration to leptin deficient patients normalized their cytokine concentrations (Farroqui *et al.* 2002). Together, these results suggest that leptin is involved in both metabolism and immune system regulation.

Adiponectin is an anti-inflammatory adipokine largely produced in adipose tissue that belongs to the soluble defence collagen superfamily. Paradoxically, systemic concentrations of adiponectin are reduced in obese and insulin resistant subjects (Arita *et al.* 1999; Engeli *et al*. 2003). The prevalence of metabolic syndrome and visceral adiposity has been negatively associated with systemic adiponectin concentrations (Ryo *et al*. 2004). In animal models, adiponectin stimulated glucose utilization and fatty acid oxidation via activation of 5' adenosine monophosphate-activated protein kinase (AMPK) (Yamaguch *et al*. 2002). Additionally, in similar animal models adiponectin alleviated hyperinsulinemia and hyperglycemia (Maeda *et al.* 2001; Lihn *et al.* 2004).

Another protective feature of adiponectin is its effect on lipoprotein lipase activity. Von Eynatten *et al.* 2004 observed a strong correlation between lipoprotein lipase and adiponectin concentrations. According to this study adiponectin contributed to more than 20% of lipoprotein lipase variation. Studies by Ouchi *et al.* 2001 and Kumada *et al.* 2004 provided evidence of the possible cardioprotective role of adiponectin. Adiponectin prevented droplet formation by suppressing class-A macrophage scavenger receptor (MSR) on human monocyte-derived macrophages (Ouchi *et al.* 2001). Furthermore, adiponectin regulated the TIMP metalloproteinase inhibitor 1 (TIMP-1)/metalloproteinase-9 (MMP-9) ratio, a role in preventing plaque rupture, by upregulating the expression of IL-10 in monocytes. Taken all the evidence together, adiponectin may play a role in insulin homeostasis and prevention of atherosclerosis.

Adipsin or complement D is an adipokine produced by adipose tissue involved in the regulation of the alternative pathway of complement activation. Similar to adiponectin, adipsin paradoxically decreases in animal models of obesity and diabetes. In animal models, deletion of adipsin resulted in a reduction of insulin secretion in response to glucose (Lo *et al.* 2014). Furthermore adipsin concentrations was associated with beta cell function. According to this study, within a population with type 2 diabetes, those with insufficient beta cell function (i.e. requiring treatment with insulin) present with lower concentrations of adipsin than those subjects with sufficient beta cell function (i.e. not requiring insulin but treated with metformin). Adiponectin and leptin concentrations were not different between these groups (Lo *et al*. 2014). Observational studies comparing adipsin concentrations in normal weight and obese subjects have shown contradictory results (Pomeroy *et al.* 1997; Maslowska *et al.* 1999; Derosa *et al.* 2013). Thus, the role of adipsin in metabolic regulation requires further assessment.

CRP is an acute phase protein indicator of systemic inflammation and athero-thrombosis. CRP is mainly produced in the liver and some by adipose tissue (Peydin-Biroulet *et al.* 2012; Ouchi *et al.* 2003). CRP is positively correlated with BMI and it is a well-recognized as a risk factor for cardiovascular disease (Engeli *et al.* 2003; Derosa *et al.* 2013). According to a 12-14 years follow up study from the Framingham cohort, men and women in the highest CRP quartiles were more likely to have an ischemic stroke (Rost *et al.* 2001). CRP has been also among the strongest predictors of coronary heart disease (CHD) incidence and mortality in other epidemiological studies (Cushman *et al.* 2005; Matthijs *et al.* 2006).

Plasminogen activator inhibitor-1 (PAI-1) is an inducible molecule produced by adipose tissue and the liver. The detrimental effects of PAI-1 on cardiovascular health reside in its antagonistic regulation of plasminogen activators and urokinase type plasminogen activator, a family of molecules involved in plasma fibrin clearance (Loskutoff *et al.* 1998). Excessive deposition of fibrin on the vascular wall enhances atherogenesis and the risk of cardiovascular events. Similar to other inflammatory molecules, PAI-1 is found at high concentrations in plasma in obese subjects compared with normal weight subject (Landin *et al.* 1990; Skurk *et al.* 2004). TNF-α is an important PAI-1 inducer, thereby the increment

in TNF-α associated with obesity may also explain the changes in PAI-1 (Swiatkowska *et al.* 2005; Cao *et al.* 2008).

Resistin is another protein derived from adipose tissue discovered by Steppan and colleagues (2001). In the initial *in vivo* studies, resistin was related to the development of insulin resistance and inflammation (Steppan *et al.* 2001; Qatanani *et al.* 2009). Following these observations, studies in humans reported high concentrations of this molecule in plasma from diabetic and hypertensive patients in comparison with healthy controls (McTernan *et al.* 2003; Zhang *et al.* 2003). Additionally a study reported associations between plasma concentrations of resistin and CRP and risk of coronary atherosclerosis (Reilly *et al.* 2005). In contrast, studies in normal weight and obese subjects found no difference in the circulating concentrations of this adipokine (Bo *et al.* 2005). Even though these studies have found some associations between resistin and diabetes, the evidence suggests that in humans the changes in circulating concentrations of resistin appear as a consequence of, but not a key determinant for diabetes.

4.1.1.4 Growth factors in the context of metabolic health

Vascular endothelial growth factor (VEGF) is a key angiogenic factor implicated in vascular endothelial cell growth and apoptosis. Adipose tissue, especially omental, expresses high concentrations of VEGF and is believed to be the main source of this peptide (Cao *et al*. 2007). VEGF is upregulated in the presence of hypoxia inducible factor , a key mediator of hypoxic responses (Ferrara *et al.* 2004), and IL-6 (Rega *et al.* 2007). *In vivo* VEGF has been found to be essential for regulating appropriate adipogenesis and preventing hypoxia in adipose tissue (Sung *et al.* 2013). Some of the components of the VEGF family are also involved in the development of pancreatic islets (Lammert *et al.* 2003). Hence, during obesity development, the influx of inflammatory and hypoxic signals trigger VEGF synthesis. This adaptation may promote adipose tissue and pancreatic islet growth in order to adjust to the increased insulin demands and storage capacity.

The evidence reviewed in this section gives insight into the role of different components of the immune system and other molecules involved in homeostatic regulation in the

pathogenesis of cardio-metabolic diseases. Thus, in this study we aim to study in more detail the effect of obesity on the systemic concentrations of these molecules and how they interact with classic metabolic markers.

4.1.2 Aim

The aim of this research is to investigate the differences between obese and normal weight subjects in circulating inflammatory markers and other molecules involved in metabolic regulation.

4.1.3 Hypothesis

The hypothesis being tested in the work that will be described in this chapter is that:

- obese subjects will show higher concentrations of molecules related to inflammation including cytokines, adipokines, adhesion molecules and growth factors than seen in normal weight subjects;

4.1.4 Objectives

The objectives of this research are:

- to evaluate the fasting plasma concentrations of molecules related to inflammation including cytokines, adipokines, adhesion molecules and growth factors, in normal weight and obese subjects;
- to evaluate TLR-2 and -4 expression on monocytes from normal weight and obese subjects;
- to evaluate the *ex vivo* production of inflammatory cytokines in whole blood
- to evaluate the *ex vivo* intracellular production of IL-6, TNF-α and IL-1β on monocytes.

4.2 Methods

4.2.1 TLR analysis

TLR 2 and 4 expression on $CD14^+$ monocytes was measured by flow cytometry. Whole blood was withdrawn into lithium heparin at each time point (0 h, 1 h, 2 h, 3 h, 4 h and 6 h) of the study described in sections 3.2.1.3 and 3.2.1.4. Aliquots of 100 µL of whole blood were stained using 20 µL of FITC conjugated anti-human CD14 and 10 µL or 5 µL respectively of phycoerythrin (PE) conjugated anti-human TLR2 or TLR4 for 30 min at 4°C. An unstained blood sample (100 µL) at each time point was used as a negative control.

After incubation, red blood cells were lysed using FACS Lysing buffer for 10 min at 4°C. Samples were centrifuged for 5 min at 600 *g* at room temperature and washed with 2 mL of FACS Cell Wash buffer.

The centrifugation step was repeated, supernatant was discarded and samples were resuspended in 200 µL of FACS Flow buffer and analysed within 20 min using a FACS Calibur flow cytometer with CellQuest software.

CellQuest software was configured as follows. First, a template was designed to collect data of interest. A side/forward scatter (SSC/FSC) plot was drawn to identify and gate monocytes based in their size and granularity. Voltage and fluorescence channels were set to obtain a clear cell population pattern.

To correct fluorescence spillover, lysed whole blood was stained with either PE or FITC fluorochrome. Compensation wizard was used to adjust detectors and voltage to correct signal overlap. Once instrument settings and compensation was set up, a histogram including cell counts and fluorescence was drawn. As monocytes represent 5-10% of the mononuclear cell population, at least 1000 events of the monocyte population were collected to obtain relevant data.

4.2.2 Intracellular cytokine concentrations in CD14+ monocytes

Intracellular staining, a flow cytometry based assay, was used to detect the cytokine production by $CD14^+$ monocytes For this assay the cytokines produced in the basal state

and during LPS stimulation were arrested in the cell cytoplasm using the protein transport inhibitor brefeldin A. Monocytes were then stained with an antibody to the surface marker $CD14^+$ and permeabilized with a detergent to create pores in the cell membrane to allow the labelling of intracellular cytokines.

Briefly, fasting whole blood was diluted with an equal volume of RPMI medium supplemented with penicillin (50 U/mL), streptomycin (50 µg/mL) and L-glutamine (2 mM) and treated with either lipopolysacharide (10 µg/mL) and brefeldin A or brefeldin A alone (1 µL/mL) and incubated at 37°C with 5% CO_2 for 6 h.

After the incubation period, red blood cells were lyzed with 1x BD Pharmalyse buffer for 10 min. Samples were centrifuged for 6 min at 800 *g* (low brake), and the supernatant containing lyzed RBC was discarded. The pellet containing mononuclear cells was washed with 2.5 mL of staining buffer, centrifuged for 6 min at 800 *g* and the supernant was discarded. Cells were resuspended in 100 µL of staining buffer containing 20 µL of fluorescein isothiocyanate (FITC) conjugated anti-human CD14 and incubated for 15 min in the dark at room temperature.

Following $CD14^+$ staining, unbound antibody was removed by washing cells with staining buffer as described previously. Subsequently, cells were fixed and permeabilized with 500 µL of Fix/Perm solution for 20 min in the dark. Samples were centrifuged and the supernatant was discarded. Following this step, samples were incubated with 2 mL of BD Perm/Wash buffer for 10 min at room temperature. Following permeabilization steps, samples were centrifuged for 6 min at 800 *g* and the supernanant was discarded.

Cells were resuspended in 100 µL of Perm/Wash buffer containing phycoerythrin (PE) conjugated anti-human for IL-6 (1.3 µL), IL-1β (5 µL) or TNF-α (2.5 µL) and incubated for 30 min at room temperature in the dark. Finally samples were washed with 2 mL of Perm/Wash buffer to remove unspecific staining, centrifuged for 6 min at 800 *g* and resuspended in 200 µL of Perm/Wash buffer. Quantification of positive cells and fluorescence intensity was performed using a FACS Calibur flow cytometer with CellQuest software. Calibration and instrument setting were set up as described in **Appendix II**.

4.2.3 *Ex-vivo* production of cytokines from whole blood with or without LPS stimulation

The aim of this experiment was to explore the production of a range of cytokines in fasting whole blood in the basal state and during LPS stimulation. This assay provides information about the potential contribution of the whole blood leukocytes to the systemic concentrations of cytokines found in normal weight and obese subjects.

Fasting whole blood was diluted in 5 volumes of RPMI medium supplemented with penicillin (50U/mL), streptomycin (50µg/mL) and L-glutamine (2mM) (1:5). The diluted blood (1 mL) was transfered to a 24--well plate and treated with or without LPS (final concentration = 15 µg/mL), samples were gently mixed and incubated at 37°C with 5% CO_2 for 24 h.

After 24 hours the 24--well plate containing the cultures was centrifuged at 8°C for 5 min at 800 *g*. Supernatant was collected and stored at -20°C for cytokine analysis.

The cytokine concentrations were measured by flow cytometry as described in **Chapter 2.**

4.2.4 Plasma cytokine, adhesion molecule and obesity biomarker concentrations

Luminex Performance Assay multiplex kits from R&D were used to simultaneously assess the concentrations of various inflammatory and obesity biomarkers. Luminex kits contain colour-coded microparticles pre-coated with antibodies specific to different analytes. Immobilized antibodies on each particle bind to the analyte of interest. A biotinylated antibody cocktail specific to a specific analyte is added to each sample to amplify the signal. Finally the microparticles are incubated with a streptavidin-phycoerythrin conjugate which binds to the captured biotinylated detection antibodies. The microparticles are resuspended in buffer and read using a Luminex platform, which determines the magnitude of the phycoerythrin-derived signal which is in direct proportion to the amount of analyte bound. The procedures to perform this assay are the following.

Reagents, standards and samples dilutions were prepared as recommended by manufacturer. A filter-bottomed microplate was prewet with 100 µL of wash buffer, liquid was removed using a vacuum manifold fitted for a microplate. Microparticle cocktail (100 µL) and 100 µL of samples or standards 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 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 on the Biorad Luminex 200 employing the Bio-Plex Manager ™ 6.1.

4.2.5 Statistical analysis

Flow cytometry data are expressed as % of positive population (e.g. $CD14^+$ $TLR2^+$ or $CD14^+$ $IL\text{-}6^+$) and median fluorescence intensity (MFI). Plasma concetrations of cytokines, adhesion molecules and obesity-related molecules were calculated using Luminex BioRad software. Cytokines concentrations from 24 h *ex vivo* culture of whole blood were calculated using FlowCytomix Pro software from eBioscience.

All databases were managed in Microsoft Excel 2013® and all statistical tests were performed in IBM SPSS Statistics 22®. Data are expressed as median and 25^{th} and 75^{th} percentiles, as dara for most of the parameters measured were not normally distributed.

For baseline comparison between normal weight and obese subjects, the mean of the fasting concentrations of the variables of interest at visit 2 and 3 were considered. All data were log base 10 transformed to obtain a symmetrical data distribution before proceeding with data analysis. Univariate linear regression was employed to study the differences between normal weight and obese subjects, as it allows the study of the interactions of two independent variables on a dependent variable. In this case, the model allowed exploration of the differences between normal weight and obese subjects considering their age and sex. In other words, this model allows to control for variables that may contribute to the fluctuation of the variables of interest. Correlations between metabolic and inflammatory molecules were performed using Pearson correlation test.

4.3 Results

4.3.1 Fasting cytokine, adhesion molecule and obesity-related molecule concentrations in plasma at baseline in normal weight and obese subjects

Univariate regression model analysis revealed that age and sex contributed to the variation of the analytes discussed in this section, including adiponectin and adipsin. After the data were adjusted for age and sex, fasting plasma concentrations of IL-6 and VEGF were found to be significantly higher in obese compared with normal weight subjects (ρ=0.02 and ρ=0.01, respectively). No statistically significant differences between groups were found for fasting plasma IL-8, GM-CSF, TNF-α or IL-10 concentrations (**Table 4.1**).

Table 4.2 shows the fasting plasma concentrations of adhesion molecules. Plasma VCAM-1 concentration was significantly higher in the normal weight compared with the obese subjects ($\rho<0.01$). P-selectin, ICAM and E-Selectin concentrations in the fasting state lay within a similar range in both groups, with no significant differences between them.

	Normal weight (n=36)	**Obese (n=41)**	**ρ-value**
IL-6 *(pg/mL)*	1.90 (0.80-2.77)	2.30 (1.48-3.31)	**<0.05**
IL-8 *(pg/mL)*	4.12 (2.69-6.15)	4.58 (3.33-5.93)	0.21
GM-CSF *(pg/mL)*	1.23 (0.82-2.25)	1.77 (0.77-3.04)	0.27
TNF-α *(pg/mL)*	8.12 (5.40-9.63)	8.50 (6.02-10.74)	0.59
VEGF *(pg/mL)*	11.93 (8.72-19.07)	18.39 (11.74-30.79)	**0.01**
IL-10 *(pg/mL)*	0.70 (0.47-1.07)	0.63 (0.36-0.89)	0.12
All data were adjusted for age and sex and were log transformed for multiple linear regression analysis. Data expressed as median and percentiles 25 and 75			

Table 4.1 Fasting plasma cytokine concentrations in normal weight and obese subjects.

	Normal weight (n=36)	Obese (n=41)	ρ-value
P-Selectin *(ng/mL)*	58.49 (44.09-73.88)	54.91 (48.76-66.78)	0.29
ICAM-1 *(ng/mL)*	238.26 (185.88-305.95)	261.08 (190.65-297.24)	0.11
VCAM-1 *(ng/mL)*	780.80 (599.83-933.69)	448.75 (384.46-560.50)	**<0.01**
E-Selectin *(ng/mL)*	34.65 (26.77-46.19)	36.66 (26.50-46.48)	0.79
All data were adjusted for age and sex and were log transformed for multiple linear regression analysis. Data expressed as median and percentiles 25 and 75			

Table 4.2 Fasting plasma adhesion molecule concentrations in normal weight and obese subjects.

The normal weight and obese groups showed significant differences in the fasting concentrations of obesity related molecules (**Table 4.3**). Adiponectin, adipsin and MCP-1 were found at significantly lower concentrations in obese than nomal weight subjects (ρ<0.001). In contrast CRP, leptin and PAI-1 were found at significantly higher concentrations in obese compared with normal weight subjects (ρ<0.05).

	Normal weight (n=36)	Obese (n=41)	ρ-value
Adiponectin *(µg/mL)*	7.90 (5.87-11.32)	4.83 (3.76-7.06)	**<0.01**
CRP *(µg/mL)*	0.65 (0.26-1.50)	2.60 (1.51-5.19)	**<0.01**
Adipsin *(µg/mL)*	2.87 (2.22-3.63)	2.53 (2.04-2.97)	**0.01**
MCP-1 *(pg/mL)*	168.10 (134.53-196.94)	152.51 (126.09-183.49)	**0.05**
Leptin *(ng/mL)*	10.70 (5.59-17.23)	42.87 (30.28-67.78)	**<0.01**
Resistin *(ng/mL)*	5.76 (4.78-7.31)	5.78 (4.47-7.70)	0.80
PAI-1 *(ng/mL)*	19.05 (14.29-27.02)	25.87 (20.73-35.01)	**<0.01**

All data were adjusted for age and sex and were log transformed for multiple linear regression analysis. Data expressed as median and percentiles 25 and 75

Table 4.3 Fasting plasma obesity related molecule concentrations in normal weight and obese subjects.

4.3.2 TLR expression on $CD14^+$ monocytes at baseline in normal weight and obese subjects

Cells from whole blood were stained with FITC conjugated anti-CD14 and gated to discriminate between monocytes (defined as being $CD14^+$) and other cell populations and debris. The $CD14^+$ cell population was double stained for either TLR2 or TLR4 to detect the levels of expression of these molecules on the CD14 cell surface.

After adjustment for age and sex, the percentage of TLR4 expressing monocytes was significantly higher (more than 2-fold) in obese than in normal weight subjects (ρ<0.01) (**Table 4.4**). In contrast, the percentage of TLR2 positive monocytes was lower (by about 30%) in obese subjects than normal weight (ρ<0.03), and the level of expression (i.e. MFI) was lower too (ρ<0.001) (**Table 4.4**).

		Normal weight (n=27)	Obese (n=30)	*ρ*-value
$CD14^+$ $TLR2^+$				
	% gated	23.25 (11.44-44.96)	16.52 (8.34-27.91)	**<0.05**
	MFI	61.29 (58.59-75.34)	58.21 (57.02-60.40)	**<0.01**
$CD14^+$ $TLR4^+$				
	% gated	1.80 (0.36-6.51)	6.91 (4.43-13.30)	**<0.01**
	MFI	149.71 (68.95-279.20)	174.53 (72.87-478.81)	0.11
All data were adjusted for age and sex and were log transformed for multiple linear regression analysis. Data expressed as median and percentiles 25 and 75; MFI= media fluorescence intensity				

Table 4.4 TLR4 and TLR2 expression on $CD14^+$ monocytes at baseline from obese and normal weight subjects.

4.3.3 *Ex-vivo* production of TNF-α, IL-1β and IL-6 by $CD14^+$ monocytes from obese and normal weight subjects

Intracellular staining was performed to detect IL-6, TNF-α and IL-1β in the $CD14^+$ monocyte population. The percentage of monocytes expressing $CD14^+IL\text{-}6^+$, $CD14^+TNF\text{-}\alpha^+$ and $CD14^+IL\text{-}1\beta^+$ and their fluorescence intensity after samples were cultured with or without LPS for 6 h at 37°C is presented in **Table 4.5**.

After samples were incubated for 6 h without LPS, the percentage of $CD14^+TNF\text{-}\alpha^+$ cells was significantly higher in obese than normal weight subjects (ρ<0.01) (**Table 4.5**). LPS treatment resulted in a marked increase in $CD14^+IL\text{-}6^+$, $CD14^+TNF\text{-}\alpha^+$ and $CD14^+IL\text{-}1\beta^+$ cells and their expression intensity (**Table 4.5**).

When samples were treated with LPS, obese subjects had a significantly lower number of $CD14^+IL\text{-}6^+$, $CD14^+TNF\text{-}\alpha^+$ and $CD14^+IL\text{-}1\beta^+$ cells when compared with normal weight subjects (ρ<0.01). This suggest that monocytes from obese subjects are less responsive to acute inflammatory challenges than those from normal weight subjects.

		Basal			LPS challenge		
		Normal weight (n=16)	**Obese (n=28)**	**ρ-value**	**Normal weight (n=16)**	**Obese (n=28)**	**ρ-value**
$CD14^+IL\text{-}6^+$	**% gated**	2.19 (1.04-5.40)	2.53 (1.11-3.69)	0.46	79.83 (58.16-86.98)	44.87 (15.15-78.50)	**0.01**
	MFI	38.62 (31.03-50.21)	47.41 (32.19-78.82)	0.33	78.64 (63.67-138.69)	96.79 (69.53-122.15)	0.29
$CD14^+TNF\text{-}\alpha^+$	**% gated**	3.85 (3.65-4.90)	7.23 (5.28-11.77)	**0.01**	80.19 (61.69-95.70)	63.92 (47.77-89.96)	**0.01**
	MFI	79.23 (64.74-114.50)	88.55 (52.97-161.16)	0.78	728.75 (455.14-832.24)	495.96 (257.11-665.36)	0.06
$CD14^+IL\text{-}1\beta^+$	**% gated**	1.75 (1.08-2.80)	2.21 (1.13-6.29)	0.45	83.37 (59.83-94.96)	61.54 (28.45-86.72)	**0.01**
	MFI	210.40 (181.21-223.05)	204.26 (162.46-233.60)	0.06	558.63 (455.40-623.76)	648.84 (455.29-767.53)	0.17

All data were adjusted for age and sex and were log transformed for multiple linear regression analysis. Data expressed as median and percentiles 25 and 75; MFI= media fluorescence intensity

Table 4.5 Intracellular cytokine production by $CD14^+$ monocytes from whole blood cultured for 6 h with or without LPS from obese and normal weight subjects.

4.3.4 *Ex-vivo* production of cytokines from fasting whole blood samples.

The concentrations of cytokines during the basal state and after an inflammatory challenge (LPS) were determined in supernatants from whole blood cultured for 24 h.

Cytokine concentrations were typically very low after whole blood was incubated in the absence of LPS for 24 h (**Table 4.6**). IL-4 and IL-2 were found at significantly lower concentrations in cultures of blood from obese compared with normal weight subjects. (**Table 4.6**).

Concentrations of all cytokines except IL-2 and IL-4 were markedly increased after LPS challenge compared to the basal state (**Table 4.6**). IL-10 was present at higher concentrations in whole blood cultures from obese subjects. IL-2 and IL-4 concentrations were lower in whole blood from obese than normal weight subjects after LPS stimulation, but these concentrations were similar to those found in the basal state. Thus the production of these two cytokines were not a result of the TLR-4 downstream signalling pathway following activation after LPS stimulation or affected by the changes in pro-inflammatory cytokines. IL-12, IL-5 and TNF-β were measured in supernatants but data will not be reported as the concentrations of these analytes were very low or not detectable by the assay

	Basal			LPS Challenge		
	Normal weight (n=20)	**Obese (n=38)**	**ρ-value**	**Normal weight (n=20)**	**Obese (n=38)**	**ρ-value**
IL-2	84.31 (0.00-122.41)	0.00 (0.00-0.00)	**0.01**	0.00 (0.00-173.67)	0.00 (0.00-0.00)	**0.01**
IL-10	0.00 (0.00-0.00)	0.00 (0.00-4.45)	0.10	214.78 (186.92-458.53)	454.42 (244.31-805.34)	**<0.05**
IL-8	0.00 (0.00-27.94)	27.56 (0.00-181.23)	0.77	6176.01 (5537.34-6149.35)	5266.34 (4517.95-6032.46)	0.94
IL-6	0.00 (0.00-0.00)	3.23 (0.00-16.15)	0.20	6152.66 (5169.67-7653.80)	6517.00 (5218.04-7716.53)	0.40
IL-4	12.19 (0.00-46.93)	0.00 (0.00-0.00)	**<0.01**	11.72 (0.00-52.05)	0.00 (0.00-0.00)	**<0.01**
IL-1β	0.00 (0.00-0.00)	0.00 (0.00-12.42)	0.09	2310.91 (2046.17-2489.81)	2077.66 (1747.31-2234.88)	0.10
TNF-α	0.00 (0.00-0.00)	0.00 (0.00-11.38)	0.15	2874.21 (1471.04-5856.84)	2859.88 (2163.91-3918.17)	0.23

All data were adjusted for age and sex and were log transformed for multiple linear regression analysis. Data expressed as median and percentiles 25 and 75

Table 4.6 Ex-vivo production of cytokines from fasting whole blood with or without LPS stimulation for 24 h from normal weight and obese subjects

4.3.5 Correlation between metabolic and inflammatory markers

Since metabolism and inflammation interact, the association between metabolic variables and inflammatory variables was explored in detail.

As previously described in the literature, BMI and W/H ratio had a positive correlation with CRP and leptin concentrations and with the % of TLR4 positive monocytes, while this association was negative for VCAM-1 and adiponectin concentrations and with the % of TLR2 positive monocytes. Fasting leptin concentrations were strongly associated with fat mass. VCAM-1 had a strong negative association with fasting triglyceride concentrations (**Table 4.7a**).

HOMA was another important determinant for many of the variables measured. VCAM-1 and adiponectin had a strong positive association with HOMA %S (insulin sensitivity) while this correlation was negative and significant for leptin and $CD4^{+}TLR4^{+}$ (**Table 4.7a and 4.7b**).. Conversely, HOMA IR (insulin resistance) had an inverse association with VCAM-1 and adiponectin and a positive association with CRP, leptin, PAI-1 and $CD14^{+}TLR4^{+}$ (**Table 4.7a and 4.7b**).

IL-6 and VEGF were among the cytokines with the strongest positive correlation with BMI. IL-6 was associated with insulin resistance (HOMA-IR) and VEGF was inversely associated with insulin sensitivity (HOMA %S).

The percentage of TLR4 and TNF-α positive monocytes during the basal state were inversely associated with lean mass and HOMA %S while having a positive correlation with triglyceride, BMI and HOMA IR (**Table 4.7b**). Moreover TLR2 expression on monocytes was inversely correlated with BMI.

	BMI	W/H	% Body Fat	% Lean Mass	TG	NEFA	HOMA %B	HOMA %S	HOMA IR
Cytokines									
IL-6	**.368****	**.299***	.241*	-.177	.196	.013	.192	-.184	**.280***
IL-8	.228	.223	.068	.000	.097	.143	-.015	-.030	.119
GM-CSF	.078	.035	.066	-.026	.017	-.167	.153	.089	-.070
TNF-α	.086	.090	.042	-.035	.147	.084	-.066	.116	-.063
VEGF	**.248***	.149	.074	**-.239***	.100	.134	.140	**-.233***	.160
IL-10	-.147	-.022	-.120	.057	-.163	-.152	-.140	**.238***	-.222
Adhesion Molecules									
P-Selectin	.035	.096	-.010	.118	-.019	-.001	-.050	-.003	.034
ICAM-1	-.059	.102	-.164	.055	-.016	.066	-.248*	.173	-.209
VCAM-1	**-.599****	**-.453****	**-.469****	**.447****	**-.416****	-.263*	**-.411****	**.598****	**-.526****
E-Selectin	.132	.167	-.154	-.117	.118	-.023	-.028	-.086	.211
Adipokines									
Adiponectin	**-.486****	**-.412****	-.208	.197	**-.357****	.012	**-.412****	**.521****	**-.547****
CRP	**.478****	**.403****	.292*	**-.382****	.276*	.256*	.176	**-.363****	**.378****
Adipsin	-.204	-.110	-.166	.094	-.026	.000	**-.338****	.263*	-.269*
MCP-1	-.117	.069	**-.354****	.128	.026	-.213	-.217	.301**	-.183
Leptin	**.666****	.269*	**.719****	**-.650****	**.336****	**.425****	**.441****	**-.577****	**.515****
Resistin	-.017	-.024	.101	.060	-.126	-.045	.136	-.031	-.042
PAI-1	**.340****	.245*	.237*	-.195	.252*	.230*	.148	**-.345****	**.373****

*BMI=body mass index; W / H = ; TG=triglycerides; NEFA=non esterified fatty acids, HOMA=homeostasis model assessment; HOMA IR = insulin resistance; HOMA %B= beta cell function; HOMA %S: insulin sensitivity;. All the parameters were log transformed for statistical analysis;*p-value<0.05, ** p-value<0.001*

Table 4.7a Pearson correlations between metabolic, anthropometric and inflammatory markers

	BMI	W/H ratio	% Body Fat	% Lean Mass	TG	NEFA	HOMA %B	HOMA %S	HOMA IR
Fasting levels									
% $CD14^+TLR4^+$	**.533****	**.412***	.265*	**-.324****	**.383***	.241	.204	**-.350****	**.429***
MFI $CD14^+TLR4^+$	.162	.193	-.099	-.236	.293*	-.051	.262*	-.229	.174
% $CD14^+TLR2^+$	-.202	-.248*	-.091	.191	-.112	-.075	.182	-.043	.153
MFI $CD14^+TLR2^+$	**-.346****	-.139	-.171	.255*	.013	.013	-.163	.209	-.217
Basal (*ex-vivo* 6h)									
% $CD14^+IL1\beta^+$	.197	.027	.093	-.187	-.063	-.123	.196	-.142	.145
MFI $CD14^+L1\beta^+$	.021	.140	-.063	.046	.220	.018	.065	.086	-.026
% $CD14^+TNF\text{-}\alpha^+$	.254	.263	-.070	**-.369***	**.543***	.091	-.084	**-.318***	**.313**
MFI $CD14^+TNF\text{-}\alpha^+$	.207	.174	-.122	-.164	.226	.020	-.080	-.012	.118
% $CD14^+IL\text{-}6^+$	.002	.023	-.061	.120	.141	-.242	-.067	.091	-.085
MFI $CD14^+IL\text{-}6^+$	.229	.104	-.023	-.211	-.007	-.031	.063	-.092	-.069
LPS challenge (*ex-vivo* 6h)									
% $CD14^+IL1\beta^+$	-.266	-.080	**-.315***	.231	-.120	-.284	-.144	.227	-.186
MFI $CD14^+L1\beta^+$	.052	.093	-.123	.019	-.016	-.020	.088	-.053	.155
% $CD14^+TNF\text{-}\alpha^+$	-.259	-.131	-.294	.180	-.178	-.233	-.059	.215	-.161
MFI $CD14^+TNF\text{-}\alpha^+$	-.292	-.032	**-.396***	.292	-.263	**-.333***	-.010	.216	-.171
% $CD14^+IL\text{-}6^+$	-.260	-.055	-.342*	.209	-.178	-.395*	-.124	.228	-.157
MFI $CD14^+IL\text{-}6^+$	.140	.219	-.102	-.049	-.089	-.213	.232	-.022	.165

*BMI=body mass index; W / H = ; TG=triglycerides; NEFA=non esterified fatty acids, HOMA=homeostasis model assessment; HOMA IR = insulin resistance; HOMA %B= beta cell function; HOMA %S: insulin sensitivity;. All the parameters were log transformed for statistical analysis;*p-value<0.05, ** p-value<0.001*

Table 4.7b Pearson correlations between metabolic, anthropometric and inflammatory markers

4.4 Discussion

Obesity has been related to chronic low levels of inflammation. Studies in healthy and metabolically compromised subjects have correlated the concentrations of inflammatory markers with insulin resistance. Circulating inflammatory molecules have also been postulated as predictors of future cardiac events. Thus, inflammation found in these subjects increases their risk of developing chronic degenerative diseases.

Some studies have suggested AT (cytokines) and the gut (bacteria debris) as the main sources of pro-inflammatory molecules (Weisberg *et al.* 2003; Erridge *et al.* 2007; Ghanim *et al*. 2009). The results presented in this chapter described in detail different markers of inflammation in the context of obesity and their association with metabolic parameters.

A number of significant differences were found between normal weight and obese subjects, as summarised in **Table 4.8**.

Higher in normal weight subjects	Higher in obese subjects	Not different between normal weight and obese subjects
Plasma: • Adiponectin • Adipsin • MCP-1 • VCAM-1 TLR expression on monocyte • % of $CD14^+$ cells: TLR-2 • MFI of $CD14^+$ cells: TLR-2 Intracellular cytokines • % of $CD14^+$ cells (LPS): IL-6, IL-1β and TNF-α. Supernatant cytokines (24 h) • Basal and LPS: IL-2 and IL-4	Plasma: • IL-6 • VEGF • CRP • Leptin • PAI-1 TLR expression on monocyte • % of $CD14^+$ cells: TLR-4 Intracellular cytokines • % of $CD14^+$ cells (basal): TNF-α Supernatant cytokines (24 h) • LPS: IL-10	Plasma: • IL-8 • GM-CSF • TNF-α • IL-1β • P-Selectin • ICAM-1 • E-Selectin TLR expression on monocyte • MFI of $CD14^+$ cells: TLR-4 Intracellular cytokines • % of $CD14^+$ cells (basal): IL-1 β and IL-6 • MFI: all Supernatants cytokine (24 h) • Basal and LPS: IL-8, IL-6, IL-1β, TNF-α, • Basal: IL-10

Table 4.8 Summary of findings from this chapter

As previously described, TLRs are pattern recognition receptors playing a role in innate immune responses and providing a link with adaptive immunity (**Chapter 1**). In *in vivo* studies in transgenic mice, deletion of the TLR4 gene was protective against HFD-induced insulin resistance and adipose tissue inflammation (Shi *et al.* 2006, Saberi *et al.* 2009). This suggests a pathogenic role of TLR4. These findings are in agreement with human studies, where fasting TLR2 and TLR4 were found to be highly expressed on monocytes in diabetic individuals when compared with healthy controls (Devaraj *et al.* 2008; Dasu *et al.* 2010).

In the current study, the expression of TLR2 and TLR4 were explored on monocytes in healthy normal weight and obese subjects at the protein level. The observations presented in this study are similar to those reported by Ahmad *et al.* 2012. In this study, TLR4 gene expression was upregulated in obese and diabetic compared with normal weight (Ahmad *et al.* 2012). Similar to previous reports in obese diabetic patients, TLR4 was found to be more highly expressed on monocytes from obese individuals when compared with normal weight (Dasu *et al.* 2010; Devaraj *et al.* 2008). In addition, the presence of $TLR4^{+}$ monocytes had a positive correlation with insulin resistance as evidenced by HOMA-IR.

In contrast to the higher TLR4 expression, TLR2 levels were lower on monocytes from obese than normal weight subjects. This finding is different from previous reports, where monocytes expressing TLR2 were found at higher levels in healthy and diabetic obese patients than in healthy normal weight patients (Devaraj *et al.* 2008; Dasu *et al.* 2010; Ahmad *et al.* 2012). The low levels of TLR2 but higher TLR4 in obese subjects may reflect a switch from a regulatory to an inflammatory state. There might be a link between monocyte TLR2 expression and insulin sensitivity, although no study has explored this possibility. Another aspect to be considered is the age difference between normal weight and obese groups, although previous reports failed to show significant differences in levels of TLR2 between young and old populations (Boehmer *et al.* 2005; Panda *et al.* 2010). In addition in this study the subjects' age was controlled for in the statistical analysis, thus the group differences described in this report are independent to variations regarding to subjects age.

T cells are a specialized cell subset involved in adaptive immune response. A T cell subpopulation, FoxP3 T reg, is involved in the suppression of innappropriate immune responses. Animal studies found a linear reduction of T reg population in AT during the development of obesity and AT inflammation (Feuerer *et al*. 2009). In humans, FoxP3 gene expression is downregulated in obese when compared with normal weight subjects (Feuerer *et al*. 2009). Interestingly, recent reports have linked TLR-2 expression with Treg cell responses (Iwasaki *et al.* 2004; Imanishi *et al.* 2007). In addition, the concentration of IL-2 is related to Treg proliferation (Malek *et al.* 2008). In the present study, TLR-2 was found to be down-regulated in obese subjects and these subjects presented lower plasma concentrations of IL-2 and IL-4 when compared with the normal weight subjects. Taken this evidence together, normal weight subjects seems to be in an "environment optimal for T-cell development" while the opposite is seen in obesity.

The observations made in the current study confirm an upregulation of inflammation in the moderately obese subjects studied as seen by higher plasma CRP, IL-6 and VEGF concentrations in plasma and greater expression of TLR4 on blood monocytes. However, some inflammatory markers previously reported to be higher in obese subjects (e.g. MCP-1 and VCAM-1) were not elevated in the obese subjects studied here, perhaps because these subjects were at the lower end of the obese range of BMI and they were not taking medications to control cardiometabolic disease (Maachi *et al.* 2004). Thus these subjects may be relatively "healthy obese".

CRP is an acute phase protein synthesised in the liver, and recently found to be expressed in AT (Ouchi et al. 2003). It is considered to be a good marker of inflammation and is an independent predictor of future cardiovascular risk and events (Ridker *et al.* 1998). CRP concentrations are under transcriptional control by different inflammatory molecules, mainly IL-6 (Castell *et al.* 1990). Obese, diabetic and metabolic syndrome patients have higher blood concentrations of CRP and some inflammatory cytokines (e.g. IL-6, TNF-α, IL-8) (Bastard *et al*. 2000, Aronson *et al.* 2004). As CRP has been shown to be associated with BMI and insulin sensitivity, AT inflammation levels may partially explain the difference in plasma CRP concentrations between obese and diabetic subjects and healthy subjects. When compared with previous studies exploring cardiovascular disease risk, the plasma CRP concentrations of the obese subjects in this

study were in the middle quartiles, suggesting a modest increase in cardiovascular risk and diabetes (Pradhan *et al.* 2001; Matthiis *et al.* 2006).

Consistent with these reports, obese subjects were also found to have higher plasma concentrations of IL-6 than normal weight. As described in previous sections, high systemic IL-6 concentrations have been found in insulin resistance subjects and this cytokine has a negative effect on insulin signals in adipose tissue. The results presented in this chapter showed high concentrations of IL-6 in the obese subjects and this molecule had a positive correlation with HOMA-IR. Thus the obese subjects in this study present a significantly higher grade of inflammation than normal weight, as evidenced by CRP and IL-6 systemic concentrations.

During the basal state, obese subjects had a higher % of TNF-α producing monocytes than normal weight subjects. However, during an *ex vivo* acute inflammatory response, monocytes from obese subjects were found to express lower intracellular concentrations of TNF-α, IL-6 and IL-1β than normal weight subjects. Thus, monocytes from obese subjects responded more weakly *ex vivo* than those from normal weight subjects, which contrasts with the higher inflammatory state of the obese subjects. One explanation to this could be that, *in vivo* the cells are in a state of chronic low level activation seen here as a higher proportion of $TLR4^+$ and $TNF\text{-}\alpha^+$ monocytes, but that when they experience an *ex vivo* re-stimulation they respond sluggishly because they are "exhausted" by their *in vivo* experience. There is some support for this concept of immune exhaustion. *Ex vivo* preincubation of PBMCs with an inflammatory stimulus (e.g. IL-6) reduced the production of TNF-α and IL-1β in response to LPS stimulation (Schindler *et al.* 1990). Similarly, when healthy subjects were treated sequentially with intravenous endotoxin, the magnitude of *ex vivo* production of inflammatory cytokines by mononuclear cells was reduced (Granowitz *et al*. 1993). Similarly studies in septic patients observed that cultured cells collected early in sepsis had an impaired *ex vivo* inflammatory cytokine response (Barbosa *et al*. 2010). As circulating cytokines concentrations declined over time, leukocyte's *ex vivo* cytokine response was improved. The implication of such immune exhaustion is that an immune stimulation of cells that have received a chronic low grade inflammatory stimulus may not initiate a good protective response. Indeed, some studies have associated obesity with impaired

host defence responses (Jain *et al.* 2011; Hedge *et al.* 2013) including poor response to vaccination (Sheridan *et al.* 2012). Thus a chronic exposure to inflammatory cytokines may impair acute inflammatory responses in obese subjects. Alternatively other factors like adipokines may play a role in cell responsiveness. Moreover, whole blood cultures from obese subjects were found to produce significantly less IL-4 and IL-2. These results suggest that there is a difference in immune cell phenotype from these subjects and this diversity may impact the overall immune responses in these individuals. Importantly, these differences in cell phenotype and basal state may influence metabolic homeostasis as both, TLR4 and TNF-α expression on monocytes, had a strong positive correlation with HOMA-IR and triglyceride levels (**Table 4.7b**).

As previously described, TNF-α synthesis was higher in monocytes from obese compared with normal weight and this was posively associated with plasma PAI-1 (correlation coefficient= 0.310; ρ=0.01). These results suggest that TNF-α synthesis may partly explain the higher levels of PAI-1 found in obese subjects. In addition, adipose tissue may also contribute to this difference, as it is a paracrine organ involved in TNF-α and PAI-1 synthesis (Mohamed *et al.* 1998; Cao *et al.* 2008).

The group differences in plasma adhesion molecule concentrations found in this study were in disagreemenet with previous studies reporting higher concentrations of these molecules in obese than healthy normal weight subjects (Ziccardi *et al.* 2002), even though, the study population presented in this study is similar to that reported by Ziccardi *et al.* (2002) (health status, age, BMI, cytokine concentrations). Intriguingly in this study VCAM-1 was found in lower concentrations in plasma from obese than normal weight subjects.

VCAM-1 is synthesized in response to inflammatory signals and it has been related to atherosclerosis development. Generally, VCAM-1 is found at higher concentrations in middle aged, obese (Ziccardi *et al.* 2002) and elderly (Richter *et al*. 2003) subjects and those at greater risk of cardiovascular disease (Blakenberg *et al.* 2001). Some other studies have reported that ICAM-1 but not VCAM-1 is the major predictor of coronary heart disease (Hwang *et al*. 1997; Lemos *et al.* 2000; Malik *et al.* 2001). Thus, the results obtained in this study are unexpected and It is possible that other factors may affect VCAM-1.

The plasma adipokines profile was markedly different between normal weight and obese subjects. In accordance with previous reports, the plasma concentrations of adiponectin were found to be higher in normal weight than obese subjects. Additionally, adiponectin had a strong negative correlation with BMI, W/H and HOMA-IR and a positive correlation with insulin sensitivity (HOMA %S). This finding agrees with the reports suggesting a role of adiponectin in promoting insulin sensitivity (Ryo *et al.* 2004; Lihn *et al.* 2005). Moreover, obese subjects were found to have much higher concentrations of leptin than normal weight subjects and this switch was found to be detrimental for insulin sensitivity as evidenced by HOMA-IR. These findings suggest that the loss of equilibrium between these hormones may have an impact on insulin action.

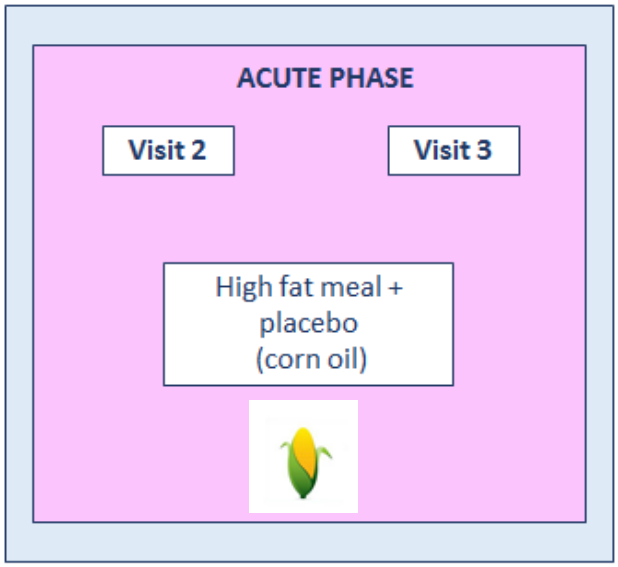
Adipsin is an adipokine that has been found at higher concentrations in obese than normnal weight subjects (Maslowska *et al.* 1999; Derosa *et al.* 2013). Furthermore, recent studies in diabetic patients have associated this molecule with impaired beta cell function (Lo *et al.* 2014). Here a modestly significantly lower concentration of adipsin was found in obese compared with normal weight subjects. This finding is in contrast with the existing literature. Some of the differences between studies may be related to the method employed for adipsin analysis and the subjects HOMA-IR. Compared with Derosa *et al. (2013)* in this study normal weight and obese subjects had a lower HOMA-IR and higher concentrations of adipsin, thus the subjects in this study were more insulin sensitive. In addition, the mean waist circumference of the normal weight subjects was greater in the study by Derosa *et al* when compared with this study. Insulin sensitivity and waist circumference were not reported by Maslowska *et al* (1999). Previous studies have reported variability in the absolute concentrations of analytes when samples are analysed by different methods (e.g. ELISA or multiplex kits), although overall trends are similar (Elshal *et al.* 2006). Thus, the metabolic status and technical factors may partly explain the discrepancies between studies.

VEGF concentrations were also found at higher concentrations in obese subjects. VEGF is associated with tissue growth and is activated during hypoxia. Studies in obese subjects have reported differences in the phenotype of adipose tissue resident cells and concentrations of tissue oxygenation in subjects with a BMI higher than 30 (Kern *et al.* 1994; Mohamed *et al.* 1997; Bastard *et al.* 2000).

Moreover, observational studies have reported high concentrations of VEGF in obese and overweight patients and a positive correlation with body mass index (Miyazawa-Hoshimoto *et al*. 2003; Silha *et al.* 2005). The VEGF family has also been shown to be Involved in the development of pancreas islets (Lammert *et al.* 2003). Thus, the systemic concentrations of VEGF observed in the obese population may reflect this physiological process activated in order to trigger adipose tissue and pancreas growth to prime energy storage and to cope with the insulin demands due to the energy surplus.

4.5 Conclusion

Overall, the results presented in this chapter confirm the findings by other authors suggesting the presence of elevated levels of inflammation in obese subjects compared with normal weight subjects. Furthermore, the immune system of obese and normal weight subjects were different, in terms of the concentrations of cytokines produced and the phenotype of the mononuclear cell population in the basal state and during an inflammatory challenge.


ACUTE PHASE
Visit 2
Visit 3
High fat meal +
placebo
(corn oil)

CHAPTER 5

Postprandial metabolic and inflammatory responses to a high fat meal in normal weight and obese subjects

CHAPTER 5

Postprandial metabolic and inflammatory responses to a high fat meal in normal weight and obese subjects

5.1 Introduction, objective, hypothesis and aims

5.1.1 Introduction

5.1.1.1 Postprandial lipid metabolism

As described in previous sections (**Chapter 1.3.2.2**), the postprandial response is a series of events occurring after a meal to provide energy to different tissues or to store it in the form of triglycerides.

The postprandial response in healthy and diabetic subjects has been described in detail in the literature (Frayn, 2010). Overall, studies have found higher glucose, triglyceride and insulin concentrations during this period in diabetic and obese subjects compared with normal weight subjects (Phillips *et al.* 2013; Esser *et al.* 2013a).

Lipoproteins, the complex molecules involved in transporting triglycerides and other lipids in the bloodstream, are intimately involved in postprandial changes and are influenced by metabolic status and body mass index. The response of chylomicrons, the main transporters of fatty acids (as triglyceride) in the fed state, is higher in diabetic than healthy subjects (Annuzzi *et al.* 2008). Fasting and postprandial VLDL (lipoproteins derived from the liver) are observed at high concentrations in obese and diabetic compared with normal weight subjects (Eriksson *et al.* 2003; Annuzzi *et al.* 2008). Altered LPL activity has been associated with these alterations. Clinical studies have reported higher LPL activity in muscle and adipose tissue from healthy subjects compared with diabetic subjects (Pollare *et al*. 1991; Annuzzi *et al.* 2008). Thus, the impaired hydrolysis of TAG fatty acids in the peripheral tissues by LPL plays a role in the lipid profile alterations found in metabolically compromised subjects. These changes also impact liver function, increasing the fatty acid metabolism demands due to defective fatty acid uptake by other tissues.

The overall postprandial lipaemia and glucose homeostasis is altered in subjects with metabolic alterations (e.g. hypertriglyceridemia), and these changes are a result of an dysregulation of systems involved in energy uptake in the periphery.

5.1.1.2 Postprandial changes in inflammatory cell populations and activation state

The postprandial response has been related to recruitment of immune cells into the circulation and changes in endothelial function. In healthy subjects, a high fat meal (HFM) induced increased numbers of leukocytes, neutrophils and platelets into circulation at early time points after ingestion of a meal (van Oostrom *et al.* 2004; Esser *et al.* 2013a).

These changes are concomitant with modifications in immune cell activation status (van Oostrom *et al.* 2004) with an increment of $CD11a^+$ expression on neutrophils and monocytes after an HFM. The percentage of $CD11a^+$ and $CD11b^+$ cells was correlated to postprandial triglyceride concentrations. These findings were confirmed by Esser *et al.* (2013a) who also concluded that cell phenotype and percentage of circulating immune cells are affected by the type of fat consumed.

Water and free-fat meal challenges seem to have an effect on leukocyte and neutrophil concentrations at late time points (van Oostrom *et al*. 2003). This phenomenon can be explained by the physiological FFA rise during the transition from the fed to starved state. Circulating FAs, either from the diet or endogenous sources, may be an important determinant of immune cell activation state and endothelial function.

Furthermore, a study by Ghanim and colleagues reported an increment in toll-like receptor (TLR) 2 and TLR4 expression on mononuclear cells during a HFM challenge (Ghanim *et al.* 2009). Transcriptional changes induced by a HFM are accompanied by a significant increase in NF-κB expression in PBMCs (Bellido *et al.* 2004; Aljada *et al.* 2004). Thus these studies suggest an active mobilization and activation of cells from the immune system during the postprandial response and suggest a role of dietary and endogenous fatty acids in this process.

5.1.1.3 Postprandial changes in inflammatory and adhesion molecules

Accumulation of immune cells in the bloodstream during the fed state is accompanied by production of inflammatory and chemotactic molecules. In the fasting state IL-6, TNF-α and adhesion molecule concentrations were influenced by age, BMI and metabolic status (Nappo *et al.* 2002; van Oostrom *et al.* 2003; Ghanim *et al.* 2004; Esposito *et al.* 2007; Lundman *et al.* 2007; Poppitt *et al.* 2008).

At baseline and under a high SFA challenge, obese subjects exhibited increased NF-κB binding activity within PBMCs when compared with those from normal weight subjects (Ghanim *et al.* 2004; Patel *et al.* 2007). Fasting CRP and serum amyloid A (SAA) concentrations were also higher in obese subjects (Esser *et al.* 2013a).

The postprandial response in healthy subjects is followed by a modest and delayed production of IL-6, while these responses are maximized and found at early time points in insulin-resistant subjects (Nappo *et al.* 2002; Blackburn *et al.* 2006; Esser *et al*. 2013a). Patients with a cardiovascular condition have similar postprandial IL-6 concentrations when compared with healthy controls (Lundman P, 2007).

Postprandial TNF-α concentrations are determined by metabolic status. Several studies have reported an elevated concentration of TNF-α in insulin resistant and cardiac patients after a HFM, while TNF-α concentration does not seem to be altered in healthy subjects (Nappo *et al.* 2002; Blackburn *et al.* 2006; Esposito *et al.* 2007; Peairs *et al.* 2011; van Dijk *et al.* 2012, Esser *et al.* 2013b). TNF-α expression in adipose tissue has been negatively associated with insulin sensitivity in AT (Hotamisligil *et al.* 1995). Circulating and postprandial TNF-α concentrations may reflect AT homeostasis and insulin sensitivity in metabolic tissues.

Changes in cell activation and cytokine concentrations are accompanied by fluctuations in adhesion molecule concentrations and ROS production. Various studies observed alterations in plasma ICAM-1, VCAM-1 and selectins concentrations after a HFM challenge in healthy subjects (Nappo *et al.* 2002, Peairs *et al.* 2011; Esser *et al.* 2013a; Esser *et al*. 2013b) this effect was exaggerated by diabetes and obesity (Nappo *et al.* 2002; Esser *et al*. 2013b). No differences in postprandial adhesion molecule

concentrations in plasma between healthy obese and normal weight subjects were reported (Esser *et al.* 2013a).

5.1.1.4 Postprandial changes in adipokines

Some adipokines such as leptin and adiponectin have been studied during the postprandial period. Plasma leptin concentrations increased after a HFM in normal weight subjects, and this response was found to be higher in women than men (Kennedy *et al.* 1997; Imbeautl *et al.* 2001). Studies exploring the postprandial modulation of adiponectin have reported conflicting results. While some studies reported higher net postprandial adiponectin response in healthy normal weight subjects compared with diabetic and obese subjects (Annuzi *et al.* 2010; Phillips *et al.* 2013), others found no significant changes in this response (Poppit *et al.* 2008). The differences in the protocols, subjects' characteristics (sex and age) and sample size are some of the factors that may explain these discrepancies.

Various human studies have reported a decrease in concentration of PAI-1 antigens in the fed state (Jellerma *et al.* 2004; Bladbierg *et al.* 2014); simultaneously an increment in total plasminogen activator activity was observed (Tholstrup *et al.* 1999). As PAI-1 has been considered a pro-atherogenic agent, these changes appear as protective mechanisms activated during the postprandial state in healthy subjects.

Postprandial MCP-1 and resistin concentrations have been found to be modest in the presence or absence of metabolic dysfunction (Gruendel *et al.* 2006; Meneses *et al.* 2011; CruzTeno *et al.* 2012). MCP-1 gene expression was up-regulated in adipose tissue during the postprandial state in parallel to an increment on IL-6 and IL-1β expression in patients with metabolic syndrome. Moreover, various studies have reported an active secretion of IL-6 and TNF-α from adipose tissue (Hotamisligil *et al.* 1995; Mohamed *et al.* 1997). These observations suggest that the local secretion of cytokines in adipose tissue after a meal might be responsible for MCP-1 activation. MCP-1 may serve as an intermittent signal triggering a sustained chemoattraction of immune cells to this tissue, an event similar to that found in cardiovascular disease development but with slightly different key players.

These observations suggest an acute response initiated postprandially that not only involves changes in plasma glucose and lipids but a modulation of cells and mediators of the immune system and adipose tissue derived molecules. Some of these appear to be protective for the host, and others an inadequate adaptation to the energy surplus.

Some elements of the metabolic and inflammatory response have been studied independently and in the context of metabolic disease. Furthermore, emerging adipokines and other molecules have not been studied in the context of postprandial metabolism.

This chapter aims to describe in detail the postprandial metabolic and inflammatory responses in normal weight and obese subjects and to provide novel evidence about the role of newly characterized adipokines in metabolic homeostasis.

5.1.2 Aim

The aim of this research is to investigate the differences between obese and normal weight subjects in postprandial metabolic and inflammatory responses.

5.1.3 Hypothesis

- Obese subjects will show higher postprandial metabolic and inflammatory responses to a HFM than seen in normal weight subjects.

5.1.4 Objectives

- to evaluate the postprandial metabolic response to a HFM in normal weight and obese subjects;
- to evaluate TLR expression on monocytes following a HFM in normal weight and obese subjects;
- to evaluate molecules related to inflammation and adipokines following a HFM in normal weight and obese subjects.

5.2 Methods

5.2.1 Postprandial metabolic and inflammatory profile

The results reported in this chapter describe the postprandial metabolic and inflammatory response to a HFM and placebo (3 capsules containing corn oil as described in Chapter 3.2) in normal weight and obese subjects. Subject characteristics, the nature of the HFM challenge, the blood collection protocol, and the methods employed for the analysis of the metabolic profile, inflammatory molecules, adipokines and TLR expression are described in **Chapter 3.2 and 4.2**.

5.2.2 Statistical analysis

Glucose and plasma lipid data are expressed as mmol/L or µmol/L as appropriate. For flow cytometry data, the $CD14^+$ monocyte population (%) and its median fluorescence intensity (MFI) were considered. Cytokines, adhesion molecules and adipokines units (pg/mL, µg/mL and ng/mL) are indicated on each individual figure or table. All databases were managed in Microsoft Excel 2010®. All statistical tests were performed in IBM SPSS Statistics 21®. Area under the curve (AUC) and incremental area under the curve (iAUC = baseline adjusted AUC) were calculated using GraphPad Prism 6® software.

AUC and iAUC were calculated to evaluate the differences between the postprandial responses in different groups. They allow an integrated measurement of the fluctuation of a variable over a period of time. GraphPad Prism 6® software calculations are based on the trapezoid model (**Figure 5.1d**). The net postprandial response including the baseline differences were considered for AUC (**Figure 5.1.a**), while for iAUC analysis, variables were normalized using baseline concentrations (time 0 h) (**Figure 5.1.b**). Only values over the baseline were considered for this analysis. Changes in NEFA in the fed state were evaluated using the model shown in **Figure 5.1.c** (described by Jackson *et al* (2005)). Additionally, % NEFA suppression was calculated (% NEFA suppression = ([minimum NEFA concentrations]-[fasting NEFA])/[fasting NEFA]*100).

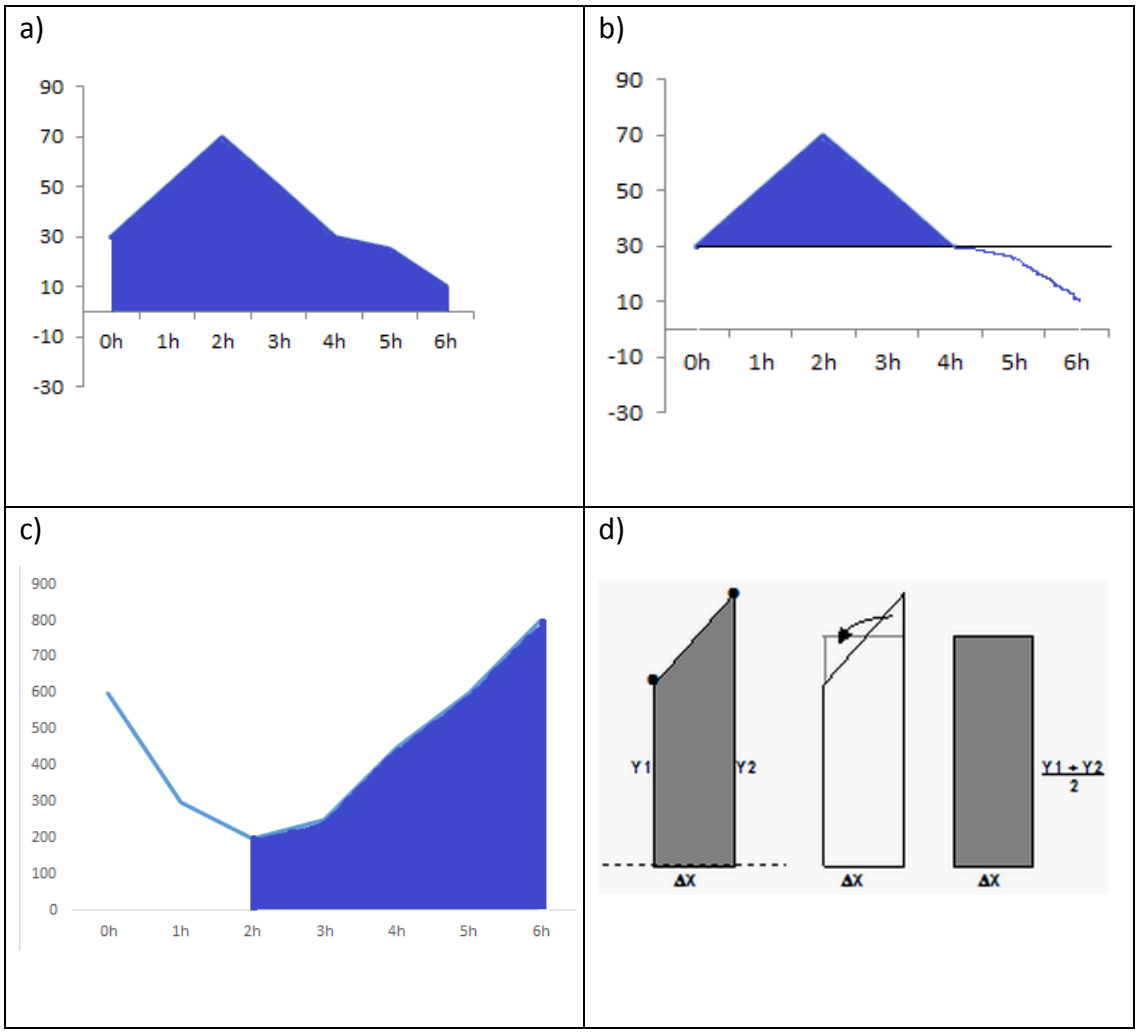


Figure 5.1 Postprandial response analysis. a) and b) show in blue the area considered for AUC and iAUC for calculations; for NEFA calculations peak after 2 h was considered as shown in figure c); and d) shows GraphPad® trapezoid model calculations.

Due to the skewed distribution of the TLR expression data and data for inflammatory cytokines and other mediators, the data were transformed using log10 before statistical analysis. Repetitive measures ANOVA (RMA) was used to evaluate the time effect on the variables and the overall postprandial differences between the obese and normal weight groups (data fixed for age). The time point differences between groups were tested using one way ANOVA, if the data were normally distributed or using a Mann Whitney test if data were not normally distributed. All data except for metabolic profile are expressed as median and percentiles.

5.3 Results

5.3.1 Normal weight and obese subjects postprandial metabolic response after a HFM

The HFM challenge was followed by an alteration in the postprandial plasma lipid, insulin and glucose concentrations. Over this period, there was a a steady increase of triglyceride concentrations in both groups (**Figure 5.2a**). In normal weight subjects, triglyceride concentrations peaked at 3 h, while in obese subjects, triglyceride concentrations continued to rise until 4 h after the HFM. Triglyceride concentrations were significanlty higher at baseline and at all postprandial time points following the HFM challenge in obese compared with normal weight subjects (ρ<0.05) (**Figure 5.2a**).

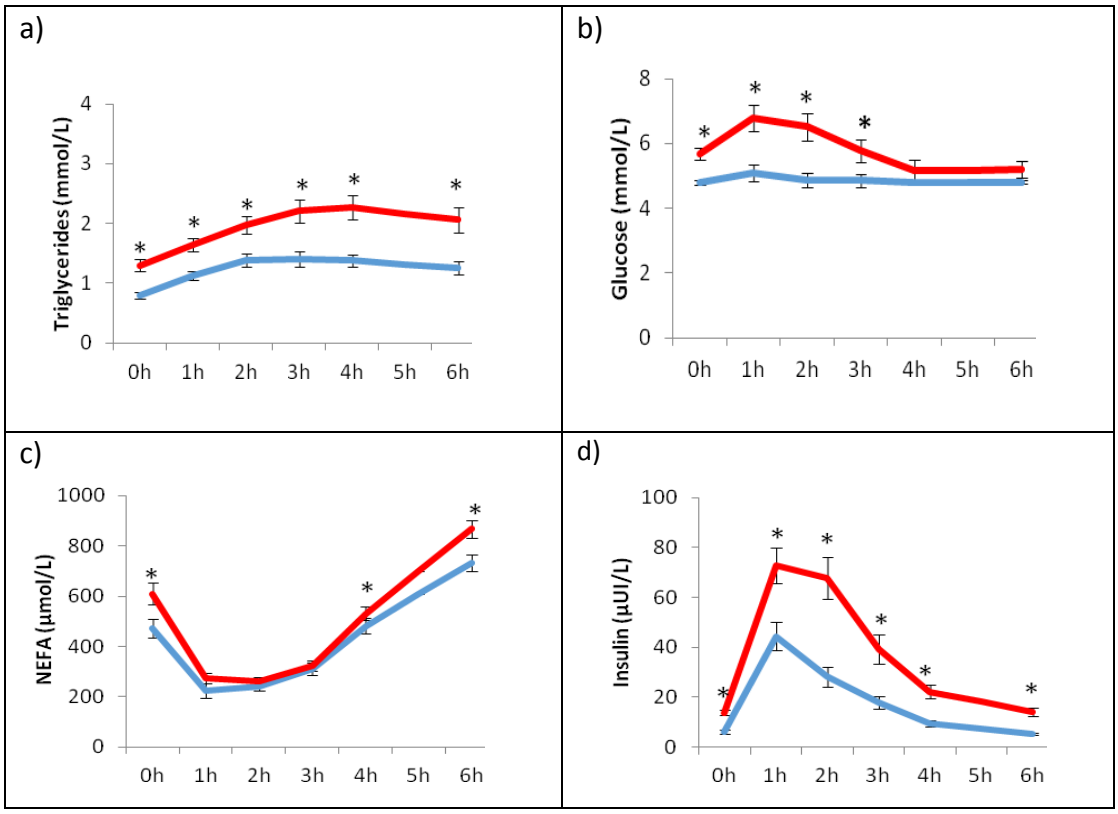


Figure 5.2 Metabolic response to a HFM challenge in normal weight and obese subjects. Plasma a) Triglycerides, b) glucose, c) NEFA and d) insulin concentrations of normal weight (blue) and obese (red) subjects. Data expressed as mean concentration and SE bars; *p-value <0.05, comparison between normal weight and obese subjects time points (normal weight n=37; obese n=41).

Plasma glucose concentrations were significanlty different at 0 h, 1 h, 2 h and 3 h between obese and normal weight subjects (ρ<0.05) (**Figure 5.2b**). After a mixed meal glucose enters the circulation rapidly peaking after approximately 30 min (Terronen, 2012; Juntunen, 2002). Thus, some of the early fluctuations in glucose were not captured in this study. NEFA concentrations decreased sharply during the first hours after the HFM challenge, reaching their lowest point after 2 h and returning to baseline concentrations at 4 h (**Figure 5.2c**). Plasma NEFA concentrations were significantly higher in obese subjects than normal weight at 0 h, 4 h and 6 h (ρ<0.05).

The metabolic changes induced after the HFM in both groups were quantified using AUC and iAUC calculations as described in the section 5.2.2. In response to the HFM, AUC and iAUC for plasma triglycerides and insulin were almost doubled in obese compared with normal weight subjects (ρ<0.001) (**Table 5.1**). Moreover, overall plasma NEFA

concentrations were maintained at significantly higher concentrations in obese than lean subjects during the postprandial challenge (ρ<0.01). The similarity in the % NEFA suppression in both groups suggest a similar capacity to adapt to substrate influx. Although the fasting concentrations of glucose were higher in obese than normal weight subjects, no significant differences between AUC were detected between groups (**Table 5.1**).

		Normal weight (n=37)	Obese (n=41)	ρ-value
Triglycerides				
(0 h-6 h)	**AUC**	7.62 ± 3.06	11.93 ± 6.03	**<0.01**
	iAUC	2.84 ± 1.71	4.15 ± 2.62	**<0.01**
Glucose				
(0 h-6 h)	**AUC**	29.17 ± 3.58	34.84 ± 11.08	0.13
	iAUC	2.02 ± 1.93	3.24 ± 2.31	0.93
Insulin				
(0 h-6 h)	**AUC**	112.86 ± 62.07	233.91 ± 128.86	**<0.01**
	iAUC	79.53 ± 58.59	153.29 ± 96.19	**<0.01**
NEFA				
(2 h-6 h)	% NEFA suppression	-42.36 ± 28.37	-37.52 ± 17.95	0.18
	AUC	1883.63 ± 440.93	2119.12 ± 464.98	**<0.01**
	iAUC	479.16 ± 443.95	335.16 ± 302.90	0.89
AUC= area under the curve; iAUC=incremental area under the curve (peak area); NEFA= non-esterified fatty acids. Data expressed as mean and standard deviation. Data were analysed by univariate linear regression model(fixed for age and sex)				

Table 5.1 AUC and iAUC of plasma lipid, glucose and insulin concentration following a HFM challenge in normal weight and obese subjects.

5.3.2 Normal weight and obese subjects postprandial TLR2 and TLR4 response after a HFM

The postprandial response involved an alteration in the % of monocytes expressing $TLR4^+$ and $TLR2^+$. **Figures 5.3a** and **Figure 5.3b** show the fluctuation in $TLR2^+$ and $TLR4^+$ during the postprandial period in normal weight (blue) and obese subjects (red). The postprandial % $TLR2^+$ monocytes response was significantly different between groups based in RMA analysis (ρ=0.003) (**Figure 5.3a**). Postprandial $TLR2^+$ MFI in monocytes was different at 0 h and 6 h (**Figure 5.3a**).

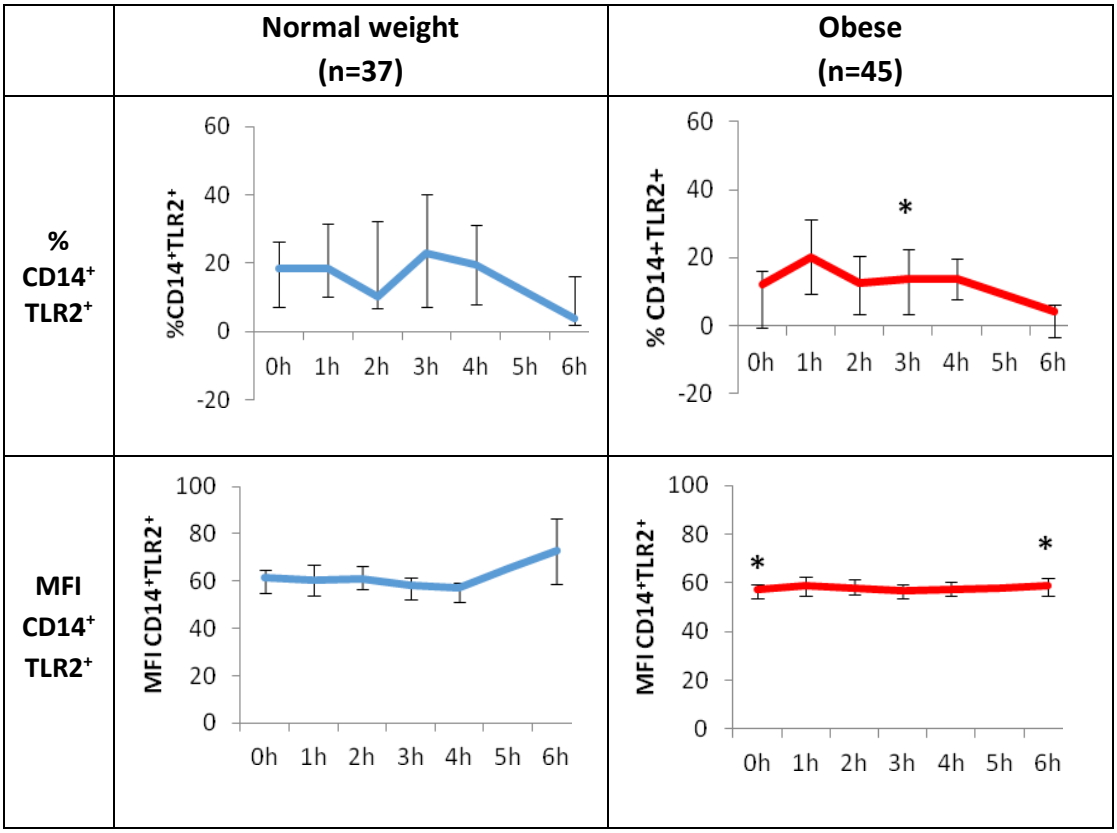


Figure 5.3a Postprandial changes in CD14+ TLR2+ monocytes after a HFM in normal weight and obese subjects. Changes in the % and MFI of CD14+TLR2+ in normal weight (blue) and obese (red) subjects. Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the overall difference between the group's responses and time effect was analysed by repetitive measures ANOVA. Time point differences between groups were evaluated using a univariate linear regression model (*ρ<0.05).

The postprandial %TLR4^{+} monocytes was higher in obese than normal subjects at all time points (ρ<0.001) (**Figure 5.3b**). The cumulative change in MFI during the postprandial period was significantly higher in obese than normal weight subjects based on RMA analysis (ρ=0.005) (**Figure 5.3b**).

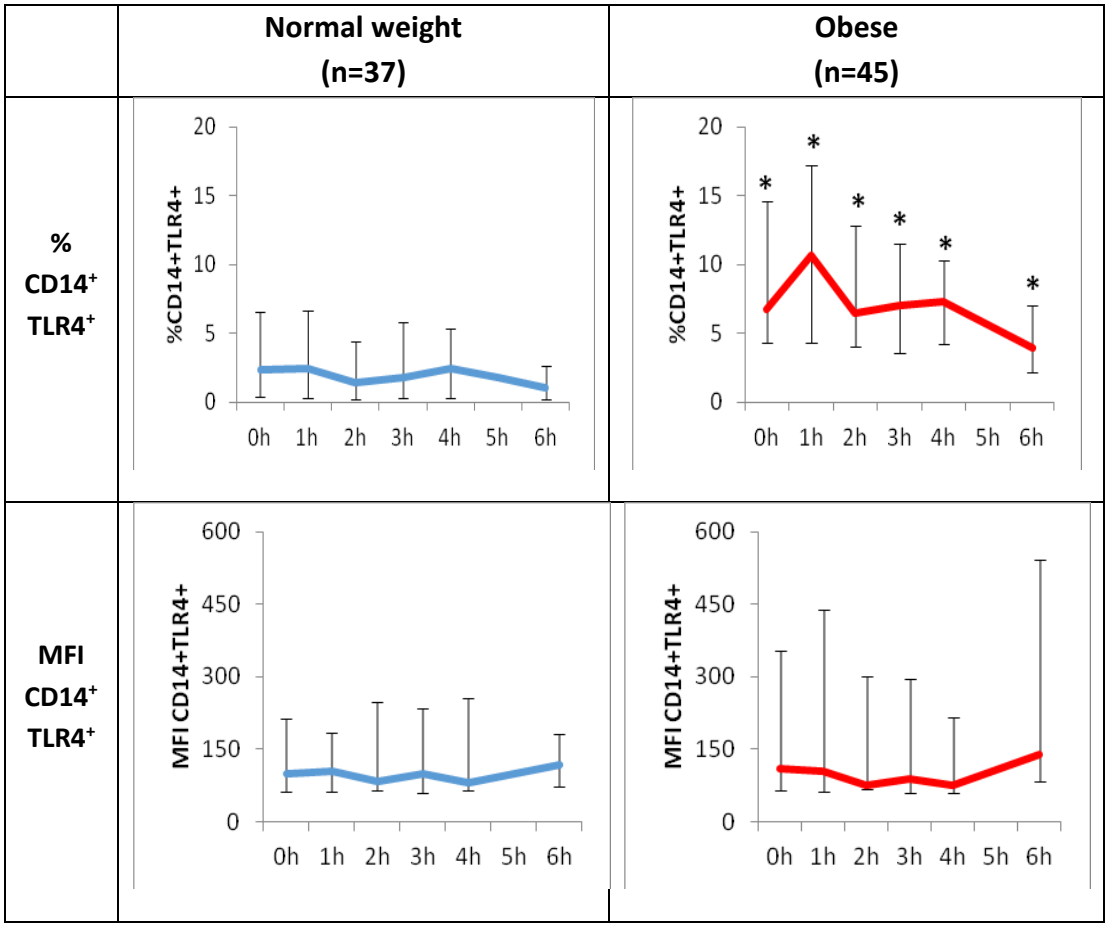


Figure 5.3b Postprandial changes in CD14+ TLR4+ monocytes after a HFM in normal weight and obese subjects. Changes in the % and MFI of $CD14^{+}TLR4^{+}$ in normal weight (blue) and obese (red) subjects. Time points expressed as median and error bars as 25^{TH} and 75^{TH} percentiles. Data were log10 transformed and the overall difference between the groups responses and time effect was analysed by repetitive measures ANOVA. Time point differences between groups were evaluated using a univariate linear regression model (*$\rho<0.05$)

In agreement with the postprandial time points and RMA analysis, AUC and iAUC for TLR expression on monocytes was different between the obese and normal weight subjects (**Table 5.2**). The AUC and iAUC for the percentage of $CD14^{+}TLR4^{+}$ monocytes was more than 4 times higher in obese than normal weight subjects ($\rho<0.001$). In contrast, AUC for TLR2 expression (MFI) on $CD14^{+}$ monocytes after the HFM meal was higher in normal weight than obese ($\rho<0.001$).

			Normal weight (n=37)	Obese (n=45)	ρ-value
$CD14^+TLR4^+$	**% gated**	**AUC**	8.41 (1.46-26.55)	47.91 (25.73-80.67)	**< 0.01***
		iAUC	0.43 (0.00-2.09)	5.76 (0.72-12.51)	**0.01***
	MFI	**AUC**	560.35 (454.05-2259.50)	840.45 (406.20-1819.25)	0.88
		iAUC	83.42 (0.00-453.62)	56.66 (8.05-369.23)	0.34
$CD14^+TLR2^+$	**% gated**	**AUC**	101.25 (54.97-217.92)	75.16 (45.45-135.00)	0.18
		iAUC	9.88 (1.28-31.45)	7.99 (1.73-28.38)	0.64
	MFI	**AUC**	383.2 (355.77-429.87)	353.8 (333.50-365.20)	**< 0.01***
		iAUC	6.12 (0.00-22.56)	9.76 (0.08-27.10)	0.07
Data expressed as median and percentile 25^{TH} and 75^{TH}; *Data were log10 transformed and analysed by univariate linear regression model (fixed for age and sex); MFI= median fluorescence intensity*					

Table 5.2 AUC and iAUC of $CD14^+TLR2^+$ and $CD14^+TLR4^+$ cells following a HFM challenge in normal weight and obese subjects.

5.3.3 Normal weight and obese subjects' postprandial cytokine response after a HFM

In addition to the changes in the metabolic profile and the phenotype of monocytes, the HFM was followed by a significant time-dependent modulation in the plasma concentrations of IL-6, IL-8 and VEGF (ρ<0.01) based on RMA analysis. **Figure 5.4a** and **Figure 5.4b** illustrate the changes in the concentrations of different cytokines after a HFM in normal weight and obese subjects. In both groups, plasma IL-6 concentrations increased steadily and peaked at 6 h. IL-8 and VEGF followed a similar pattern during the HFM challenge (ρ<0.05) (**Figure 5.4a** and **Figure 5.4b**).

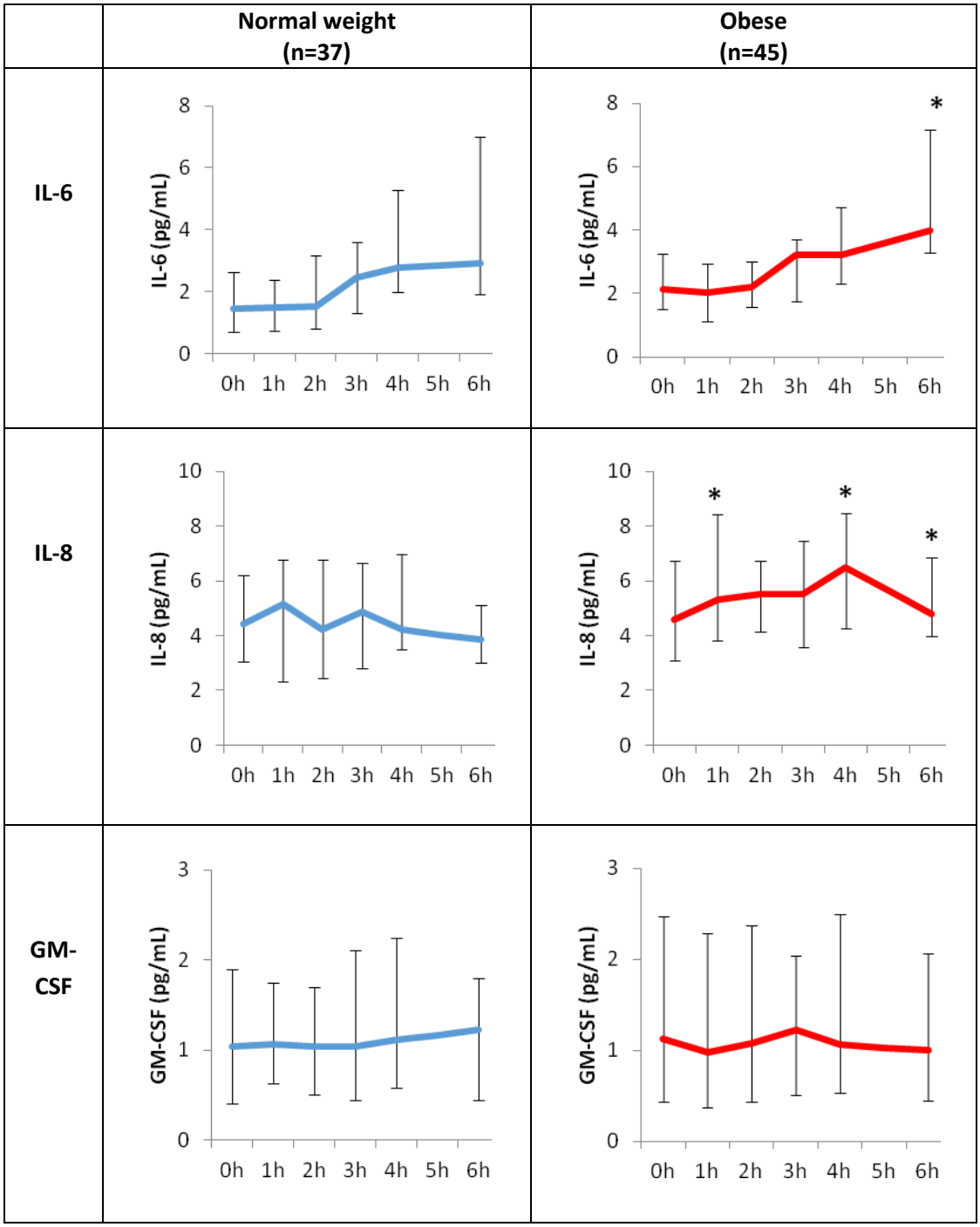


Figure 5.4a. Postprandial changes in plasma cytokines after a HFM in normal weight and obese subjects. Changes in different cytokines in normal weight (blue) and obese (red) subjects. Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the overall difference between the groups' responses and time effect was analysed using repetitive measures ANOVA. Time point differences between groups were evaluated using a univariate linear regression model (*$p<0.05$) (normal weight n=37; obese n=45).

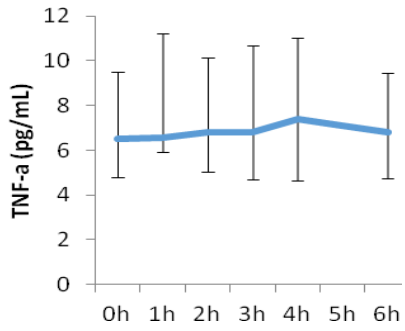
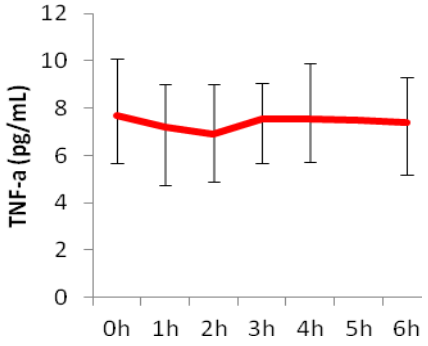
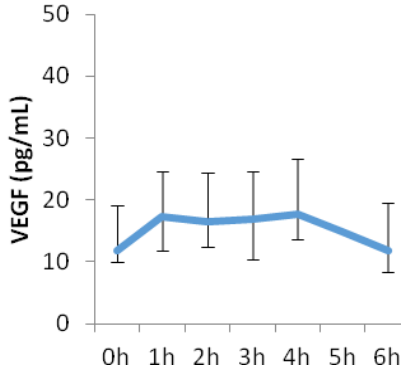
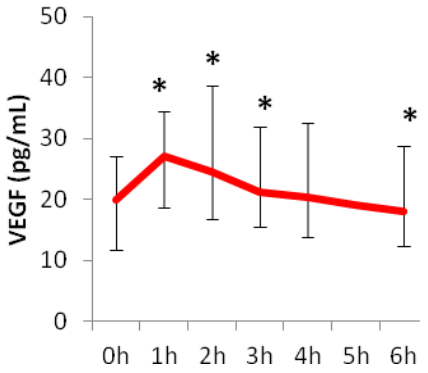
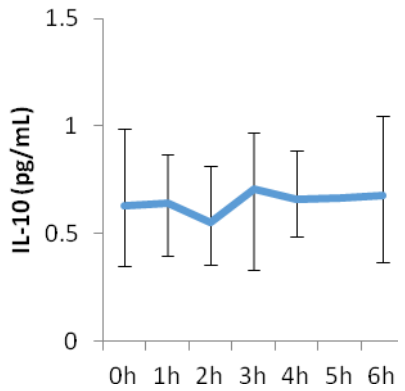
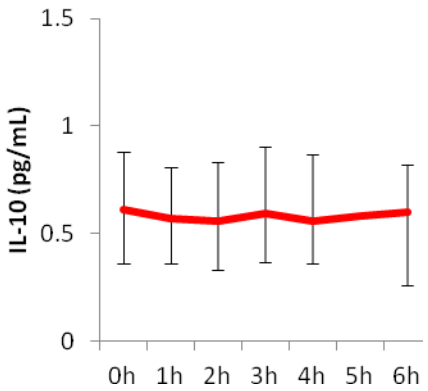
	Normal weight (n=37)	Obese (n=45)
TNF-α		
VEGF		
IL-10		

Figure 5.4b. Postprandial changes in plasma cytokines after a HFM in normal weight and obese subjects. Changes in different cytokines in normal weight (blue) and obese (red) subjects. Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the overall difference between the groups responses and time effect was analysed using repetitive measures ANOVA. Time point differences between groups were evaluated using a univariate linear regression model (*p<0.05)

AUC calculations revealed no significant differences in the net postprandial plasma cytokine response between the normal weight and obese subjects (**Table 5.3**).

When baseline was subtracted from the postprandial values (iAUC), VEGF iAUC was significantly higher in obese than normal weight subjects (ρ<0.05). The IL-6, IL-8 and VEGF postprandial plasma concentrations were affected by the HFM while the other cytokines remained at similar concentrations following the challenge.

		Normal weight (n=37)	Obese (n=45)	ρ-value
IL-6				
	AUC	15.90 (10.68-25.06)	18.94 (13.78-24.62)	0.11
	iAUC	5.12 (1.13-14.12)	4.62 (1.22-14.40)	0.95
IL-8				
	AUC	28.79 (20.36-36.99)	35.45 (25.64-45.49)	0.21
	iAUC	2.42 (0.35-6.32)	6.00 (1.45-11.22)	0.06
GM-CSF				
	AUC	7.17 (3.58-11.40)	7.19 (2.57-13.43)	0.74
	iAUC	0.73 (0.00-2.36)	0.39 (0.00-1.00)	0.21
TNF-α				
	AUC	45.88 (31.27-59.35)	45.23 (33.27-56.06)	0.14
	iAUC	1.72 (0.00-4.81)	0.80 (0.10-2.80)	0.45
VEGF				
	AUC	104.00 (71.36-130.32)	134.80 (102.70-190.00)	0.06
	iAUC	12.41 (5.58-30.12)	32.89 (11.95-57.39)	**<0.05**
IL-10				
	AUC	4.05 (2.64-5.30)	3.59 (2.19-5.13)	0.06
	iAUC	0.25 (0.01-0.84)	0.13 (0.01-0.39)	0.22
Data expressed as median and percentile 25TH and 75TH; Data was log10 transformed and analysed by univariate linear regression model (fixed for age and sex)				

Table 5.3 AUC and iAUC of plasma cytokines after a HFM challenge in normal weight and obese subjects.

5.3.4 Normal weight and obese subjects' postprandial adhesion molecules response after a HFM

The adhesion molecules changes in response to the HFM were modest; only VCAM-1 concentrations were significantly different between groups ($\rho<0.05$) (**Figure 5.5**). Normal weight subjects displayed higher plasma concentrations of VCAM-1 at all times during the postprandial response when compared with obese subjects. Nevertheless these differences were due to differences in the baseline plasma VCAM-1 concentrations and not a time or meal effect.

The VCAM-1 response considering baseline (AUC), was significantly higher in normal weight than obese subjects ($\rho=0.001$) (**Table 5.4**). Similar results were observed when iAUC was calculated, although the difference did not reach a significant level ($\rho=0.06$).

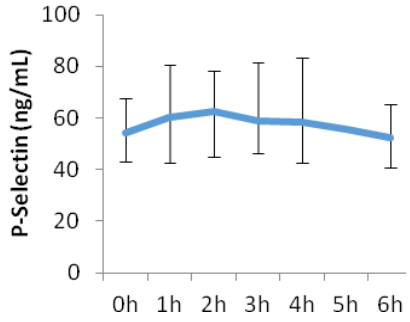
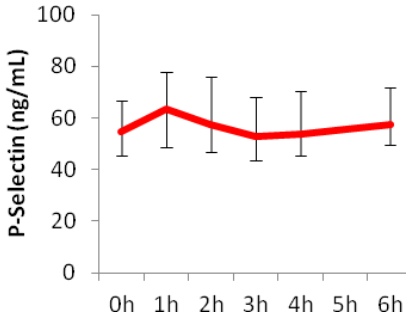
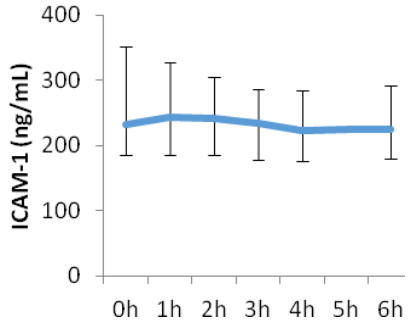
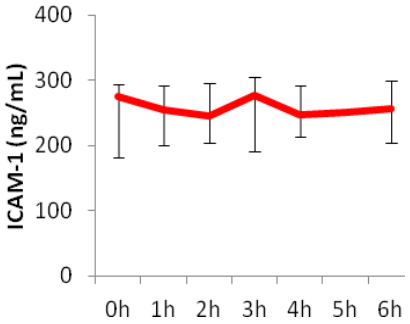
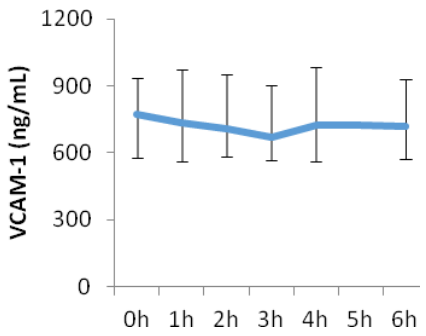
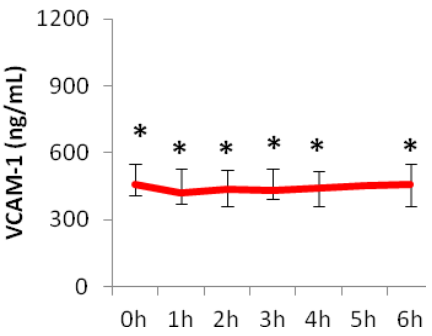
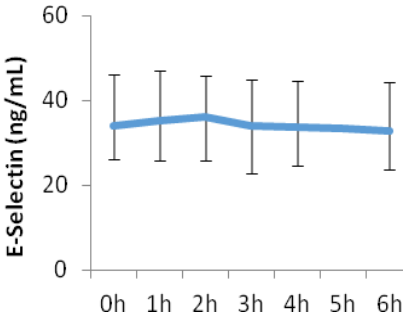
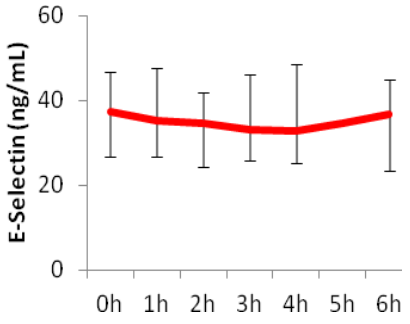
AUC	Normal weight (n=37)	Obese (n=45)
P-Selectin		
ICAM-1		
VCAM-1		
E-Selectin		

Figure 5.5. Postprandial changes in plasma adhesion molecules after a HFM in normal weight (blue) and obese (red) subjects. Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the overall difference between the groups' responses and time effect was analysed using repetitive measures ANOVA. Time point differences between groups were evaluated using a univariate linear regression model (*ρ<0.05).

		Normal weight (n=37)	Obese (n=45)	ρ-value
P-Selectin				
	AUC	35.58 (26.82-46.93)	32.92 (30.05-42.89)	0.31
	iAUC	1.62 (0.32-5.29)	2.11 (0.62-4.61)	0.61
ICAM-1				
	AUC	140.80 (109.25-183.70)	148.65 (120.80-177.07)	0.16
	iAUC	1.48 (0.11-7.89)	2.62 (0.00-9.22)	0.34
VCAM-1				
	AUC	449.90 (348.05-579.65)	264.15 (222.15-307.9)	**<0.01**
	iAUC	1.81 (0.00-22.43)	0.46 (0.00-9.21)	0.06
E-Selectin				
	AUC	20.36 (14.28-27.33)	20.88 (15.01-27.39)	0.65
	iAUC	0.12 (0.00-1.10)	0.15 (0.00-0.97)	0.63
*Data expressed as median and percentile 25TH and 75TH; *ρ-value <0.001. Data were log10 transformed and analysed by univariate linear regression model (fixed for age and sex)*				

Table 5.4 AUC and iAUC of plasma adhesion molecule concentrations after a HFM challenge in normal weight and obese subjects.

5.3.5 Normal weight and obese subjects' postprandial adipokine response after a high fat meal

In addition to inflammatory molecules, the adipose tissue derived molecules response after a HFM was monitored. According to RMA analysis, only plasma resistin and MCP-1 were significantly affected by time (ρ<0.05). Fasting and postprandial plasma concentrations of adiponectin, CRP and leptin were different among groups (ρ<0.05) (**Figure 5.6a and Figure 5.6b**). Plasma adiponectin concentrations were significantly lower in obese compared with normal weight subjects. Conversely, plasma CRP and leptin concentrations were significantly higher at all time points in obese than normal weight subjects.

Following the HFM challenge, PAI-1 concentrations were significantly higher in obese than normal weight subjects at 3 h and 6 h (ρ<0.05) (**Figure 5.6b**).

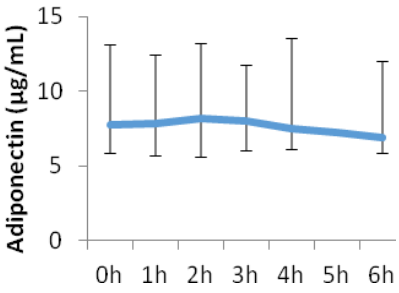
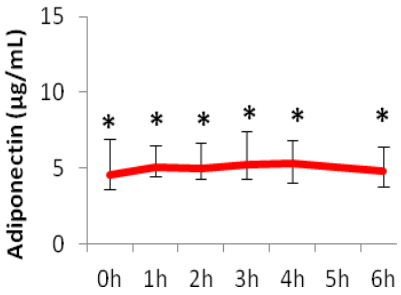
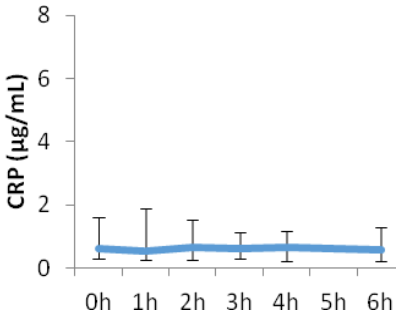
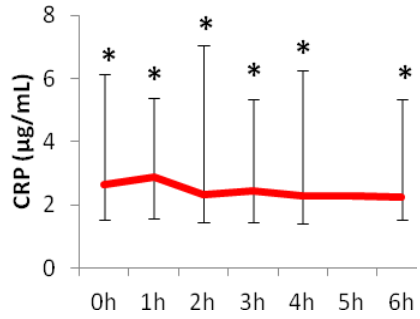
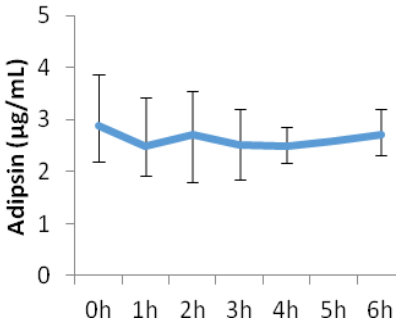
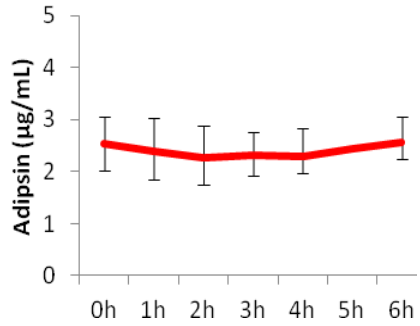
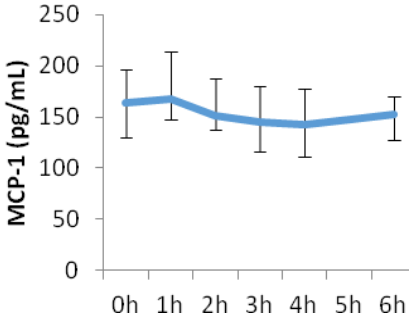
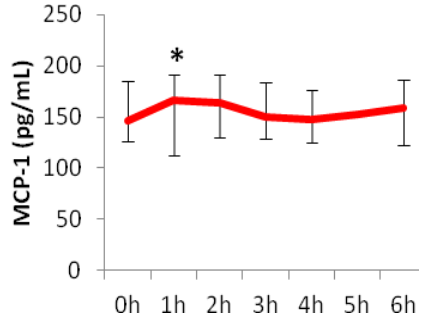
	Normal weight (n=36)	Obese (n=41)
Adiponectin		
CRP		
Adipsin		
MCP-1		

Figure 5.6a. Postprandial changes in plasma adipokines after a HFM in normal weight (blue) and obese (red) subjects. Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the overall difference between the groups' responses and time effect was analysed using repetitive measures ANOVA. Time point differences between groups were evaluated using a univariate linear regression model (*ρ<0.05) (normal weight n=37; obese n=45).

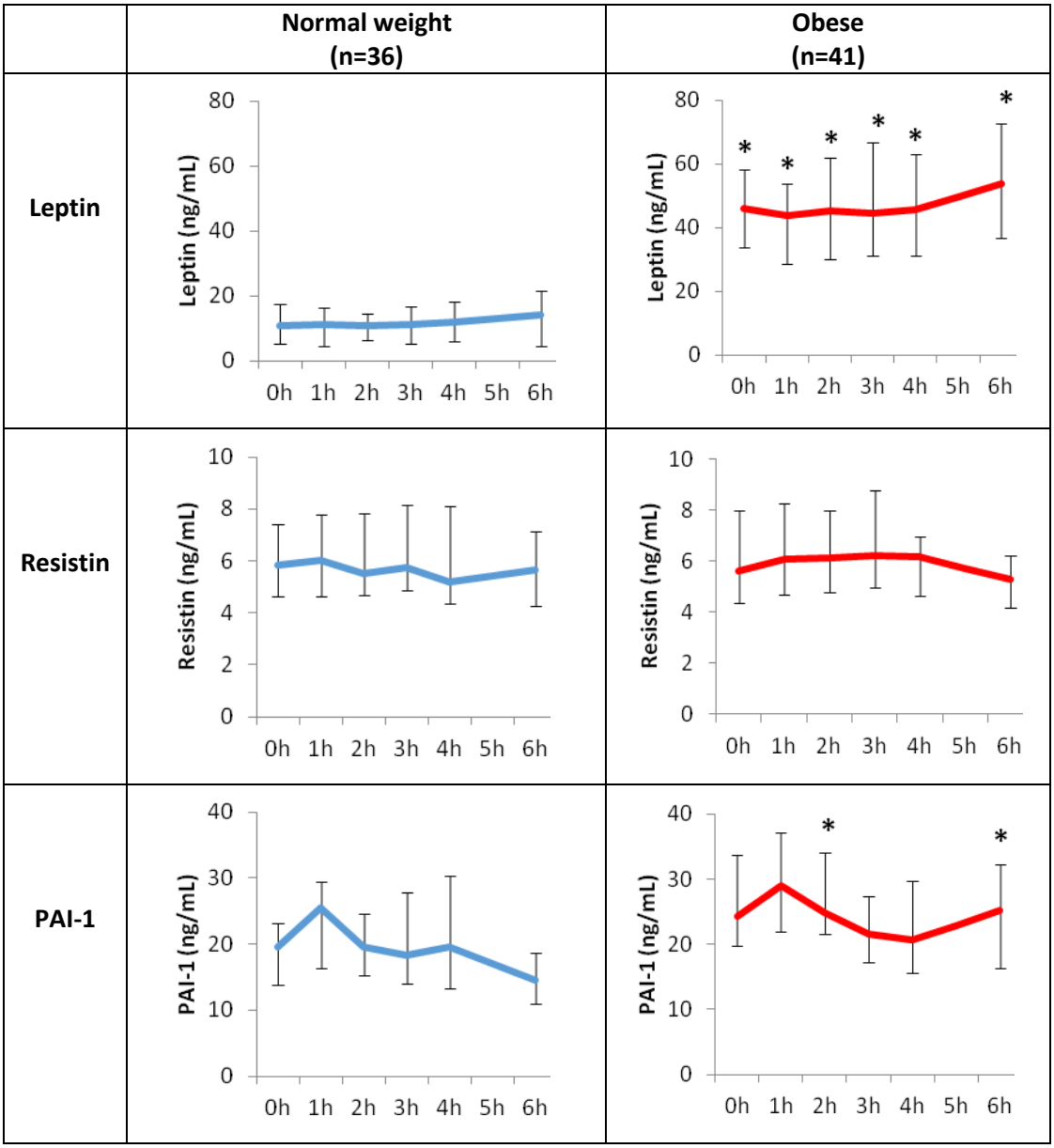


Figure 5.6b. Postprandial changes in plasma adipokines after a HFM in normal weight (blue) and obese (red) subjects. Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the overall difference between the groups' responses and time effect was analysed using repetitive measures ANOVA. Time point differences between groups were evaluated using a univariate linear regression model (*ρ<0.05) (normal weight n=37; obese n=45).

The overall postprandial adipokine response was different between normal weight and obese subjects according to AUC and iAUC analysis (**Table 5.4**). As described in **Table 5.4** the net CRP, leptin and PAI-1 response to the HFM was significantly different between groups (ρ<0.01). The differences in CRP and leptin remained after iAUC analysis

(ρ<0.01). In contrast, the net adiponectin response (AUC) was higher in normal weight than obese subjects (ρ<0.01), although this difference was not significant when the baseline was subtracted (iAUC) (**Table 5.5**). These results suggest that from the molecules analysed some of the differences observed between groups during the postprandial state are explained by differences in the fasting concentrations. In contrast other adipokines, are actively synthesized during the postprandial state and may play a role in the events related to energy metabolism.

		Normal weight (n=35)	Obese (n=40)	*p-value*
Adiponectin				
	AUC	4.55 (3.72-8.02)	3.02 (2.55-3.95)	**<0.01**
	iAUC	0.21 (0.00-0.56)	0.27 (0.00-0.57)	0.52
CRP				
	AUC	0.38 (0.15-0.91)	1.45 (0.88-3.61)	**<0.01**
	iAUC	0.00 (0.00-0.04)	0.12 (0.00-0.39)	**<0.01**
Adipsin				
	AUC	1.63 (1.27-2.00)	1.47 (1.24-1.71)	0.06
	iAUC	0.00 (0.00-0.15)	0.03 (0.00-0.08)	0.98
MCP-1				
	AUC	89.45 (79.14-107.45)	90.85 (78.28-111.00)	0.14
	iAUC	1.10 (0.00-7.42)	2.77 (0.41-8.33)	0.08
Leptin				
	AUC	77.93 (43.33-108.24)	272.71 (187.94-385.97)	**<0.01**
	iAUC	6.54 (0.51-13.31)	19.08 (1.63-31.06)	**<0.01**
Resistin				
	AUC	34.19 (27.63-47.18)	36.02 (28.08-45.84)	0.38
	iAUC	0.77 (0.25-3.51)	2.09 (0.29-5.95)	0.68
PAI-1				
	AUC	12.26 (8.84-15.63)	15.34 (11.58-20.79)	**<0.01**
	iAUC	1.11 (0.21-3.07)	0.63 (0.00-3.29)	0.31

Data expressed as median and percentile 25^{TH} and 75^{TH};
Data were log10 transformed and analysed by univariate linear regression model (fixed for age and sex)

Table 5.5 Plasma adipokines AUC and iAUC after a high fat meal challenge in normal weight and obese subjects.

5.4 Discussion

The main objective of the work described in this chapter was to examine the differences in the postprandial metabolic and inflammatory response between obese and normal weight subjects. Both groups had a standardized breakfast containing a high content of fat, saturated fatty acids and simple carbohydrates in order to induce a high postprandial metabolic response. Some differences between groups were observed after the HFM, as summarized in **Table 5.6**.

Higher in normal weight subjects	Higher in obese subjects	No differences between groups
Metabolism		
-	**AUC**: Triglycerides, Insulin and NEFA **iAUC**: Triglycerides and Insulin **Time points**: Triglycerides and insulin (all time points), glucose (0 h-3 h) & NEFA (0 h, 4 h & 6 h)	**AUC**: Glucose **iAUC**: NEFA and glucose, % NEFA suppression
TLR expression on monocytes		
AUC: MFI $CD14^+TLR2^+$	**AUC**: % $CD14^+TLR4^+$ **iAUC**: % $CD14^+TLR4^+$	**AUC**: % $CD14^+TLR2^+$, MFI $CD14^+TLR4^+$ **iAUC**: % $CD14^+TLR2^+$, MFI $CD14^+TLR2^+$, MFI $CD14^+TLR4^+$
Plasma		
Cytokines		
-	**iAUC:** VEGF **Time points:** IL-6 (6 h), IL-8 (1 h, 4 h & 6 h) and VEGF (1 h, 2 h, 3 h, 6 h)	**AUC**: all cytokines **iAUC**: all except VEGF
Adhesion Molecules		
AUC: VCAM-1 **Time points:** VCAM (al time points)	-	**AUC**: all except VCAM-1 **iAUC**: all adhesion molecules
Adipokines		
AUC: Adiponectin **Time points:** Adiponectin (all time points) & MCP-1 (1 h).	**AUC**: CRP, leptin, PAI-1 **iAUC**: CRP and leptin **Time points:** CRP & Leptin (all tie points; PAI-1 (2 h & 6 h)	**AUC**: Resistin, MCP-1 and adipsin **iAUC**: Adiponectin, resistin, MCP-1, adipsin and PAI-1

Table 5.6 Summary of the main differences between normal weight and obese subjects in of the postprandial response to a HFM

Various methods have been described to determine the magnitude of the postprandial response (Wolever *et al.* 1986; FAO, 1997; Wolever *et al.* 2003). Among them AUC has been the most widely used as it provides a net measurement of the magnitude of the postprandial response, although it does not exclude any baseline differences between subjects. The net iAUC and iAUC are alternative methods that provide information about this response excluding baseline. iAUC describes the positive peaks area during a time response, while net iAUC provides information about the positive peaks considering also negative events. Given the nature of the data and aims of this research, AUC and iAUC were considered appropriate measurements to evaluate the metabolic and inflammatory response. Additionally, statistical analysis provided information about the magnitude and the kinetics of the postprandial response in these subjects.

In the fed state, dietary triglycerides are absorbed by enterocytes and transported in chylomicrons to be taken up by tissues via LPL-insulin action. Clinical studies have reported differences in lipid transport and uptake between subjects with a wide range of body fat (Deprs *et al.* 1990). LPL activity and expression in different tissues was negatively correlated with total body fat (Depres *et al.* 1990; Pollare *et al.* 1991; Eriksson *et al.* 2003). Furthermore, low LPL activity was associated with higher triglyceride concentrations, suggesting that elevated TAG result from impaired LPL activity. In this study the postprandial TAG response was significantly higher in obese subjects. This reflects reduced uptake of lipid into peripheral tissues, which extends the findings of previous clinical trials reporting an elevated postprandial plasma triglyceride concentrations in obese compared with normal weight subjects (Philips *et al.* 2013; Esser *et al.* 2013).

After a meal, glucose enters the circulation via the hepatic portal vein to be phosphorylated in the liver. More than 30% of dietary glucose is absorbed by the liver minimizing the glycemia changes induced during the fed and fasting state. During this process, glucose which is not taken up by the liver enters the circulation and is taken up mainly by skeletal muscle, brain and red blood cells, and in small amounts by adipose tissue via insulin action. In this study, insulin and glucose responses were higher in obese than normal weight subjects. Previous studies have reported similar results. The high insulin demands and high postprandial glucose concentrations observed in obese

subjects indicate a degree of insulin resistance or a lack of tissue responsiveness to insulin signals (Philips *et al.* 2013). Additionally, it may reveal a reduced capacity of the liver to deal with large glucose loads.

The postprandial response has been associated with the increment in the circulating numbers of inflammatory cells into the bloodstream and production of inflammatory mediators. Additionally, recent reports have related postprandial inflammation with infiltration of bacterial debris from the gut to the circulation (Erridge *et al.* 2007). These events are affected by the type of meal ingested (nutrient composition) and the subjects' metabolic status. TLRs play a role in innate immune responses although their effect on postprandial inflammation and their relationship with metabolic status have not yet been explored. In the present study the difference in postprandial TLR 2 and TLR4 expression in normal weight and obese subjects was investigated for the first time.

The postprandial TLR response has been described previously in normal weight subjects (Ghanim *et al*. 2009). In agreement with that report, postprandial TLR2 levels were up regulated in normal weight subjects, although the postprandial TLR4 response was modest. These differences can be explained by the techniques used and the cell population targeted for TLR analysis. In this study TLR expression on monocytes was assessed by flow cytometry while Ghanim *et al* (2009) evaluated the whole mononuclear cell population expression by Western Blotting. Flow cytometry analysis offers the advantage of studying the expression of molecules on specific cell populations; compared with Western Blotting, flow cytometry offers more detailed and quantitative information.

In obese subjects the postprandial state was associated with an increment in TLR4 expression and in the % of TLR4 positive monocytes. The findings reported herein uncover a novel difference between the postprandial inflammatory response (TLR4) between obese and normal weight subjects. According to previous reports, when compared with normal weight subjects, obese and diabetic individuals present a higher basal lipid response accompanied by endotoxemia (Erridge *et al.* 2007; Harte *et al*. 2011). Additionally, their AT has been reported to be in a more inflammatory state (Hotamisligil *et al.* 1995). Thereby, the levels of inflammation in AT plus basal

inflammation may account to the higher levels of TLR4 activation found in obese subjects.

One of the downstream events of TLR activation is NF-κB activation and in consequence the production of cytokines. Thus, the activation of TLRs suggests a possible release of cytokines from mononuclear cells. To study the inflammatory events following a metabolic response several molecules involved in inflammation were evaluated.

Time dependent changes of plasma IL-6 were noted during the postprandial response and this response was similar in both normal weight and obese subjects. These results agree with previous studies reporting a steady increment over time in systemic concentrations of this cytokine in response to a mixed meal or an oral lipid test (Oostrom *et al.* 2003; Blackburn *et al.* 2006; Poppit *et al.* 2008; Phillips *et al.* 2013; Esser, 2013b). Moreover, the fluctuation of this cytokine has been partly attributed to its active production by adipose tissue in the fed state (Mohamed *et al.* 1997). The half-life of IL-6 is approximately 3 min, thus adipose tissue, in addition to mononuclear cells, could be an important contributor to the plasma IL-6 concentrations observed in normal weight and obese subjects (Mohamed *et al.* 1997). The higher concentrations of IL-6 observed in obese at certain times during the postprandial state are consistent with studies reporting higher release from adipose tissue in these subjects (Kern *et al.* 2001).

Intriguingly, a study reported by Philips *et al* (2013) observed an equally steady increase in plasma IL-6 in both normal weight and obese subjects after a mixed meal or water. Type-2 diabetic patients had a similar response to the mixed meal, although their plasma IL-6 concentrations remained unchanged after water. Van Hall *et al* (2013) reported higher lipolysis rates, without triglyceridemia or insulin and glucagon changes, during an infusion of IL-6 and radiolabelled fatty acids in healthy subjects. Thus these studies suggest that in healthy subjects IL-6 may have a housekeeping role in energy regulation. IL-6 may serve as a signaling molecule involved in energy generation in response to energy deprivation, by inducing lipolysis (van Hall *et al.* 2013) and energy production (Ruderman *et al.* 2006). In mice and rat models of infection and cerebral ischemia, IL-6 played an important role in temperature regulation, energy regulation and neutroprotection (Schobitz *et al*. 1995; Herrmann *et al.* 2003; Erta *et al.* 2012). Nevertheless the concomitant exposure to high concentrations of this cytokine found in

obesity may have detrimental effects on insulin signaling (Vicennati *et al.* 2002; Lagathu *et al.* 2003). Together, high concentrations of IL-6 and other inflammatory mediators (e.g. IL-8, PAI-1) have been related to cardiovascular disease. They are also involved in leukocyte recruitment to the vascular wall, one of the main events in plaque formation and endothelial dysfunction. Previous studies have reported an increment in plasma IL-8 and MCP-1 concentrations during the postprandial state in healthy and diabetic subjects (Esponsito *et al.* 2003; VanWiyk *et al.* 2006; Esser *et al.* 2013b).

Some reports have observed reductions of PAI-1 in response to a mixed meal in healthy normal weight subjects (Jellerma *et al.* 2004; Bladbjerg *et al.* 2013), although this response has not been explored in healthy obese subjects. In this study PAI-1 concentrations were higher in obese compared with normal weight after a HFM challenge at some time points. In parallel, monocytes expressing TLR4 were more abundant in obese subjects than normal weight. IL-8, PAI-1 and MCP-1 peaked after around one or two hours and these events coincided with changes in $TLR4^{+}$ cells. These observations suggest that during the fed state there is an acute response of monocytes that may be involved in the elevation of these molecules, and this response is much higher in obese subjects than normal weight. These cytokines have been related to recruitment of inflammatory cells to different tissues (Baggiolini *et al.* 1997; Kanda *et al.* 2006). Thus, the postprandial fluctuation of these cytokines in response to a HFM may contribute to premature cardiovascular disease development.

Recently VEGF has been studied in relation to metabolism. Animal model studies have reported contradictory results. While some reported improvement in metabolic markers following VEGF deletion, others attribute a beneficial effect of this cytokine on insulin sensitivity and adipose tissue vascularization (Elias *et al.* 2012; Hagberg *et al.* 2012; Sung *et al.* 2013). In general VEGF effects, either positive or negative, appear to be tissue and age-dependent in mice (Honek *et al.* 2014).

In human adipose tissue hypoxia and VEGF mRNA levels are higher in middle aged obese than normal weight subjects (Pasarica *et al.* 2009). These findings are consistent with studies reporting higher plasma concentrations of VEGF in obese and diabetic than normal weight subjects (Schlich *et al.* 2013). Previously, only one human study has

reported the kinetics of VEGF concentrations in response to glucose and insulin (Dandona *et al.* 2003). No changes in concentrations of circulating VEGF in response to glucose were reported, although a reduction of this molecule was observed in the presence of circulating insulin. In this study, an increment in VEGF was observed in obese subjects but not in normal weight subjects in response to a HFM. When compared with the studies previously described, these results suggest that the increase in plasma VEGF concentrations may be an acute protective pro-angiogenic response, probably in response to episodes of hypoxia in adipose tissue. These changes may be seen to a greater extent in obese subjects due to their higher baseline inflammation and hypoxic status. On the other hand, a study reported a role of VEGF in the facilitation of human monocyte transmigration through the endothelial monolayer (Heil *et al.* 2000). This study used VEGF concentrations higher than those observed in human plasma. Moreover, VEGF has been found expressed in human endothelial cells and macrophages from coronary atherosclerotic plaques (Inoue *et al.* 1998). Taking all the evidence together, these studies suggest that postprandial VEGF kinesis may play a role in atherosclerosis although this associations require further investigation.

In parallel to the kinetics of the systemic concentrations of inflammatory cytokines following the HFM, other molecules were also modulated during this process.

CRP is a sensitive systemic marker of inflammation and tissue damage. CRP is rapidly synthesized *de novo* by hepatocytes in response to cytokines, mainly IL-6 (Pepys and Hirschfield, 2003). In previous studies, no significant changes in CRP concentrations after a meal were observed in healthy subjects (Blackburn *et al*. 2006; Poppit *et al.* 2008; Payette *et al.* 2009). Moreover, no comparisons between normal weight and obese subjects are reported in the literature. In this study CRP net postprandial response (AUC and iAUC) was higher in obese than normal weight subjects. The postprandial CRP differences between groups may be partly explained by the higher fasting concentrations of CRP and IL-6 found in the obese. An acute inflammatory response in the liver may be also a possible explanation of this effect.

As with CRP, leptin was found at high concentrations during the postprandial response in obese subjects. In terms of metabolism, leptin has been implicated in the regulation of energy homeostasis by interacting with the hypothalamus and reducing neuropeptide

Y, a protein involved in satiety regulation (Wang *et al.* 1997; Klok *et al.* 2007). In ideal conditions leptin serves as an orexigenic peptide, although this effect is deficient in obese subjects (Yang *et al.* 2007). As previously discussed, leptin is produced in adipose tissue, thus the differences between groups may be related to adipose tissue mass and its responsiveness (higher demand).

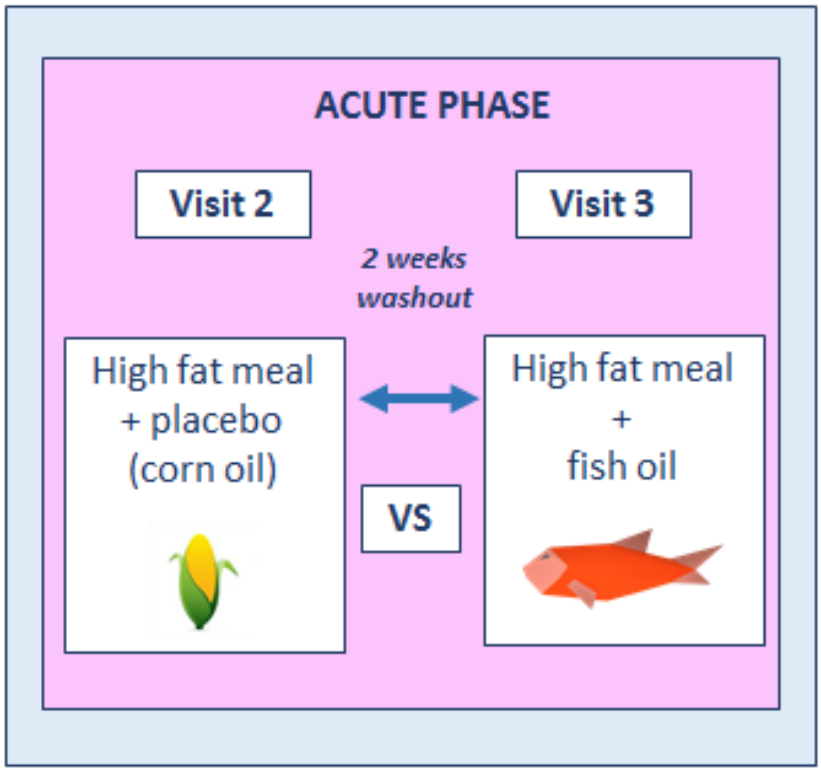
When the fasting concentrations were considered to calculate the net postprandial response, adiponectin concentrations were significantly higher in the normal weight than obese subjects. These differences were relative to fasting concentrations of adiponectin as iAUC analysis did not reveal group differences, and this is consistent with previous reports (Philips *et al.* 2013). Thus, adiponectin is modestly affected during the postprandial response. Adiponectin has anti-inflammatory and insulin sensitizing properties, thus its presence in circulation during in the fasting and fed state may be beneficial to maintain insulin sensitivity.

Previous studies have reported contradictory results regarding the postprandial modulation of adhesion molecules (Nappo *et al*. 2002; Peairs *et al.* 2011; Esser *et al.* 2013**b**). My findings are similar to those reported by Esser *et al* (2013), where no significant changes in plasma E-Selectin and P-selectin were found after a mixed meal in middle age subjects. In contrast to Nappo *et al* (2002), this study and others observed modest or no significant changes in postprandial plasma ICAM-1 (Peairs *et al.* 2011; Esser *et al.* 2013a). VCAM-1 is expressed on activated endothelial cells and higher plasma concentrations has been suggested a marker for cardiovascular risk (Lemos *et al.* 2000; Ley *et al.* 2001). Fasting and postprandial response of VCAM-1 were higher in normal weight than obese subjects. The response to the meal challenge was attributed to the fasting concentrations of this molecule based on iAUC analysis. Nevertheless, these results suggest a greater cardiovascular risk in normal weight subjects or the presence of an inflammatory process yet to be explored as hypothesized in **Chapter 4**.

5.5 Conclusion

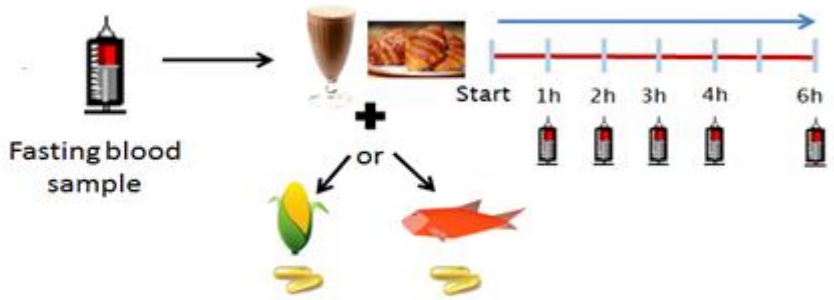
To summarize, the overall postprandial response was different between normal weight and obese subjects. An efficient postprandial metabolic response was observed in

normal weight subjects when compared with obese subjects. In parallel, the postprandial concentrations of some proatherogenic and inflammatory markers were present at higher concentrations in obese subjects than normal weight during this response.


ACUTE PHASE
Visit 2
Visit 3
2 weeks
washout
High fat meal
+ placebo
(corn oil)
VS
High fat meal
+
fish oil

CHAPTER 6

Acute effect of fish oil on the postprandial metabolic and inflammatory responses in normal weight and obese subjects



CHAPTER 6

Acute effect of fish oil on the postprandial metabolic and inflammatory responses in normal weight and obese subjects

6.1 Introduction, objective, hypothesis and aims

6.1.1 Introduction

The beneficial effects of n-3 PUFA on human health were first described by epidemiological studies on populations with high consumption of marine mammals (seal, whale) and/or oily fish (both rich in n-3 PUFA). These studies demonstrated that higher oily fish or n-3 PUFA consumption was associated with a reduction of cardiovascular risk (Kromhout *et al.* 1985; GISSI, 1999; Yokoyama *et al.* 2007). This suggested that those subjects with a diet high in n-3 PUFA were protected against cardiovascular events through one or more mechanisms that included a beneficial modulation of lipid metabolism. These findings raise the question of whether acute exposure to a meal containing these fatty acids, rather than chronic exposure through the diet, could influence the magnitude of the postprandial metabolic and inflammatory responses.

Postprandial studies reported a modest effect of acute exposure to fish oil on lipid metabolism. While some reports have found no difference in the postprandial triglyceride and NEFA concentrations after a standard meal and one containing fish oil (Harris *et al.* 1988; Burdge *et al*. 2009; Newens *et al.* 2011), others found a significant reduction of these metabolic markers (Williams *et al.* 1997; Chong *et al.* 2010). In obese and normal weight subjects aged between 45-70 years, an n-3 PUFA-rich milkshake induced a lower triglyceride response than a MUFA-rich shake (van Dijk *et al*. 2012a; Esser *et al.* 2013b). Subjects who consumed an n-3 PUFA shake also presented a less pronounced increase of insulin concentrations compared with SFA and MUFA rich shakes (van Dijk *et al*. 2012a). The differences in fish oil concentrations used in these studies and the subjects' characteristics are some of the elements that may account for the discrepancies between studies.

DHA and EPA (from fish oil) are rapidly incorporated into lipoprotein fractions during the postprandial response and alter their metabolism (Heath *et al.* 2003; Burdge *et al.* 2009). A single meal rich in EPA and DHA increases postprandial VLDL concentrations and reduces the size of these lipoproteins compared with a meal rich in saturated fatty acids (Burdge *et al.* 2009). Some of the mechanisms involved in these effects have been suggested to be attributed to the interaction of fish oil with different transcription factors in the liver (PPAR-Y and SREBP-1), or changes in fatty acids hydrolysis rate (LPL action) (Burdge *et al.* 2009). Overall, these studies suggest an acute effect of n-3 PUFA on postprandial lipid metabolism.

In parallel to metabolic markers, the acute effect of fish oil on some elements of inflammation, an important determinant of metabolic dysfunction, has been explored. Cytokines are differentially modulated by n-3 PUFA and other dietary fatty acids in the postprandial state. A milkshake containing n-3 PUFA, SFA and MUFA induced increased plasma IL-8, ICAM-1, and VCAM-1 in middle age subjects (van Dijk *et al.* 2012; Esser *et al.* 2013a). SFA consumption increased P-selectin concentrations significantly after 2 h. Studies in young and elderly normal weight subjects reported increases in plasma IL-6 concentrations after a meal rich in SFA, n-6 PUFA and MUFA at 6 h (Jiménez-Gómez *et al.* 2009; Esser *et al.* 2013a, Esser *et al.* 2013b).

The activation of the transcription factor NF-κB has also been reported in response to different fatty acids during the postprandial response. Ingestion of a SFA rich meal was accompanied by a significant increase in NF-κB expression in PBMC (Bellido *et al.* 2004, Aljada *et al.* 2004). MUFA and n-6 PUFA rich meals had a lesser effect on NF-κB binding and IL-6 mRNA levels than a SFA rich meal (Bellido *et al.* 2004, Jiménez-Gómez *et al.* 2009). Transcriptional changes induced by SFA were accompanied by an up-regulation of total TLR2 and TLR4 and oxidative stress (Ghanim *et al.* 2009).

Peairs *et al* (2011) reported activation of NF-κB after an n-3 PUFA rich meal but no effect after an SFA meal. However, NF-κB activation induced by n-3 PUFA did not up-regulate TNF-α or adhesion molecule concentrations. Additionally, basal NF-κB activity was higher in the n-3 PUFA group than the others and the changes of other proteins involved in the regulation of NF-κB signals were not reported.

These studies provide some descriptive information about the acute effects of n-3 PUFA and other fatty acids on postprandial inflammation. However, the interaction of these fatty acids with other markers of inflammation, such as adipokines, has not been explored and require further investigation.

Overall, according to these studies the acute exposure to fish oil has a modest effect on human metabolism. Although some studies have reported the role of n-3 PUFA in postprandial inflammation, more evidence is necessary to understand the acute effect of these fatty acids in the postprandial state.

6.1.2 Aim

The aim of the experiments described in this chapter is to determine the acute effect of fish oil on the postprandial metabolic and inflammatory responses.

6.1.3 Hypothesis

- including n-3 PUFAs with the meal will lower the postprandial metabolic and inflammatory responses in both normal weight and obese subjects;

6.1.4 Objectives

- to evaluate the effect of inclusion of n-3 PUFA in the postprandial metabolic and inflammatory responses in normal weight and obese subjects.

6.2 Methods

6.2.1 Postprandial metabolic and inflammatory profile

The results reported in this chapter describe the postprandial metabolic and inflammatory response (composition described in **Chapter 3.2**). Subject characteristics, the nature of the HFM challenge, the blood collection protocol, and the methods employed for the analysis of the metabolic profile, inflammatory molecules, adipokines and TLR expression are described in **Chapter 3.2 and 4.2**.

6.2.2 Statistical analysis

AUC and iAUC were calculated as described in **Chapter 5.2.**

Comparisons of intra-individual responses to the HFM containing fish oil or placebo were made using a paired t-test. Metabolic data are expressed as mean and standard deviation. Due to the skewed distribution of the TLR data and other inflammatory variables, these data were log10 transformed before statistical analysis and data are expressed as median and percentiles. The time point differences between fish oil or placebo were tested using paired t-test per time point, if the data were normally distributed, or using a Wilcoxon test if data were non normally distributed or negative.

Comparisons of the postprandial metabolic and inflammatory responses to the different treatments (fish oil or placebo) and the group differences were evaluated using 2-way-ANOVA (fix for age and sex).

6.3 Results

6.3.1 The acute effect of fish oil on the postprandial metabolic response in normal weight and obese subjects

As illustrated in **Figure 6.1,** the postprandial metabolic response was similarly affected by the HFM containing fish oil or placebo. In normal weight subjects, the postprandial triglyceride concentrations were significantly lower at 2 h and 6 h after the HFM containing fish oil than the HFM containing placebo (ρ<0.05). Similarly, the magnitude of the net triglyceride postprandial response (AUC) and the iAUC were both lower when normal weight subjects consumed the HFM with fish oil capsules (**Table 6.1a and Table 6.1b**).

The main effects of group, treatment and their interaction on the postprandial metabolic response are shown in **Table 6.1a** and **Table 6.1b**. The consumption of a HFM (fish oil and placebo) was associated with a higher increase in triglycerides, glucose, insulin and NEFA (AUC) in obese subjects compared with normal weight (**Table 6.1a**). The same effect was observed in the iAUC for triglycerides and insulin (**Table 6.1b**).

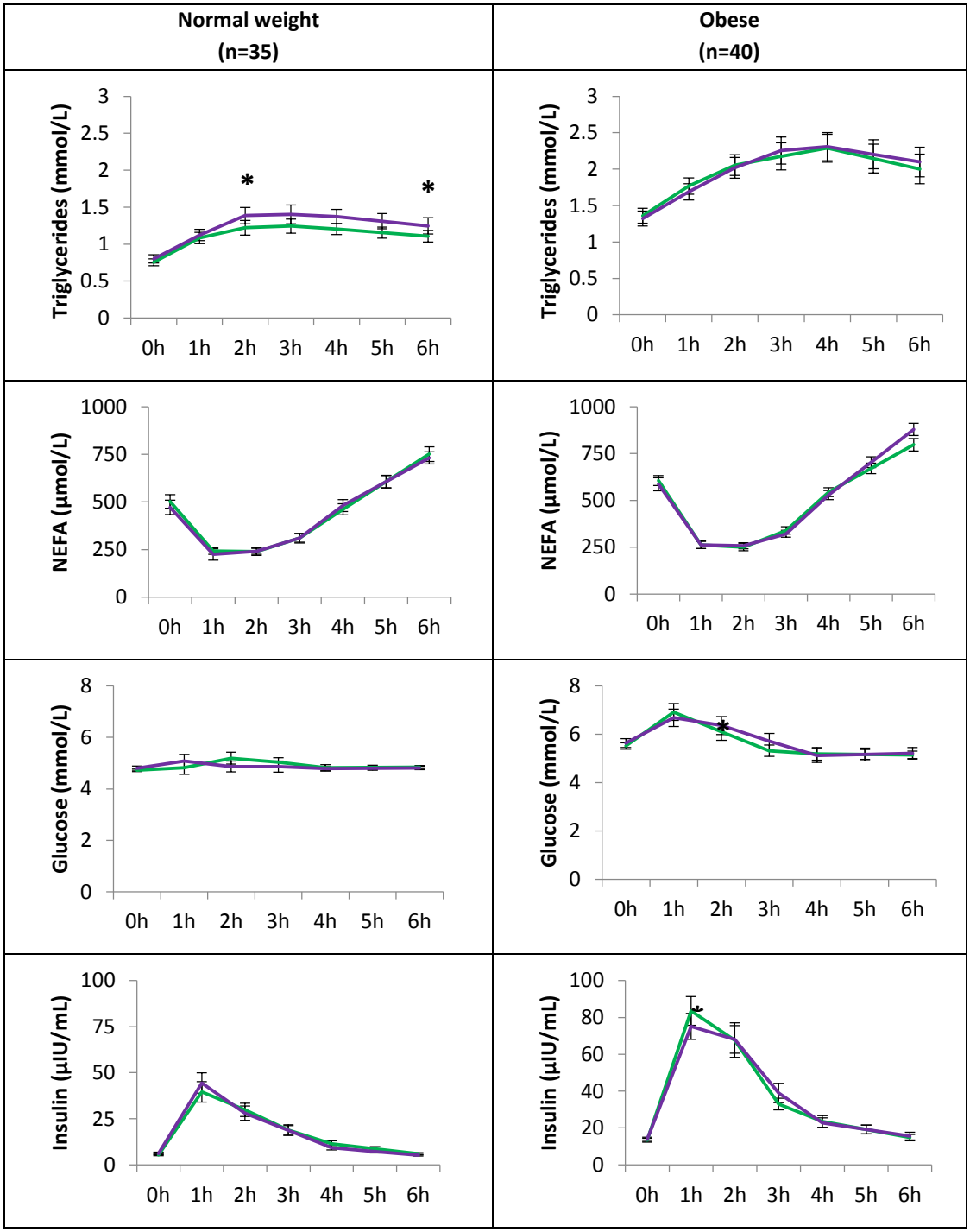


Figure 6.1 Metabolic response after HFM challenge including placebo or fish oil in normal weight and obese subjects. Normal weight subjects (left) and obese (right) response; fish oil (-green-) or placebo (-purple-). Data are expressed as mean concentration and SE bars; *ρ-value <0.05, comparison between fish oil and placebo time point

				Fish oil vs Placebo ρ-value	Main Effects ρ-value		Interaction ρ-value
		Fish oil	**Placebo**		*group*	*treatment*	*group*treatment*
Triglycerides							
	Normal weight	6.85 ± 2.47	7.91 ± 3.50	**<0.05**	**<0.01**	0.55	0.49
	Obese	12.29 ± 6.60	12.14 ± 6.25	0.27			
NEFA							
	Normal weight	1873.12 ± 481.55	1890.61 ± 436.63	0.85	**<0.01**	0.72	0.91
	Obese	2092.26 ± 417.56	2125.38 ± 455.26	0.42			
Glucose							
	Normal weight	29.53 ± 2.97	29.21 ± 3.54	0.53	**<0.01**	0.87	0.67
	Obese	33.97 ± 8.58	34.69 ± 10.85	0.33			
Insulin							
	Normal weight	112.59 ± 66.86	112.83 ± 60.77	0.97	**<0.01**	0.86	0.85
	Obese	243.07 ± 152.22	236.73 ± 128.01	0.66			
% NEFA suppression							
	Normal weight	-42.57 ± 38.26	-37.35 ± 40.51	0.53	0.18	0.39	0.92
	Obese	-54.04 ± 29.58	-49.81 ± 27.62	0.47			

*Data expressed as mean and standard deviation ;*Difference in the postprandial response between FO and placebo response per group was analysed by paired t test; The main effects of fish oil and placebo were analysed using 2 way ANOVA (data fixed for age and sex)

Table 6.1a AUC of plasma lipids, glucose and insulin during HFM challenge containing fish oil or placebo in normal weight (n=35) and obese subjects (n=40).

				Fish oil vs Placebo ρ-value	Main Effects ρ-value		Interaction ρ-value
		Fish oil	Placebo		*group*	*treatment*	*group*treatment*
Triglycerides							
	Normal weight	2.16 ± 1.53	2.61 ± 1.83	**0.01**	**<0.01**	0.21	0.93
	Obese	3.47 ± 2.92	3.97 ± 2.88	0.20			
NEFA							
	Normal weight	872.12 ± 515.81	865.92 ± 472.18	0.94	0.11	0.78	0.71
	Obese	978.53 ± 633.29	1037.26 ± 568.91	0.50			
Glucose							
	Normal weight	2.40 ± 2.20	1.88 ± 1.93	0.28	0.14	0.79	0.66
	Obese	2.76 ± 3.92	2.84 ± 4.08	0.74			
Insulin							
	Normal weight	80.63 ± 63.97	76.94 ± 58.09	0.78	**<0.01**	0.85	0.98
	Obese	146.41 ± 135.78	143.98 ± 110.16	0.86			
Data expressed as mean and standard deviation; *Difference in the postprandial response between FO and placebo response per group was analysed by paired t test;* *The main effects of fish oil and placebo were analysed using 2 way ANOVA (data fixed for age and sex)*							

Table 6.1b iAUC of plasma lipids, glucose and insulin during HFM challenge containing fish oil or placebo in normal weight (n=36) and obese subjects (n=40).

6.3.2 The acute effect of fish oil on the postprandial TLR2 and TLR4 response in normal weight and obese subjects

The postprandial TLR2 and TLR4 response in normal weight subjects after the HFM containing fish oil or placebo is described in **Figure 6.2** and **Table 6.2**. No significant differences in the subjects' response was detected after they consumed fish oil or placebo.

In obese subjects, the consumption of a HFM containing fish oil significantly increased TLR2 expression on monocytes at 6h when compared with a HFM containing placebo (**Figure 6.3**). Conversely, TLR4 expression was significantly lower at 6h after the HFM containing fish oil when compared with the HFM containing placebo. AUC and iAUC analysis revealed no significant differences between the fish oil or placebo treatment (**Table 6.3**).

In agreement with the results described in **Chapter 5.3**, the HFM (containing fish oil and placebo) resulted in significant differences between normal weight and obese subjects in the postprandial TLR4 and TLR2 expression. The postprandial % of $CD14^{+}TLR4^{+}$ monocytes was higher in obese compared with normal weight subjects (AUC and iAUC) (**Table 6.2a and Table 6.2b**). Conversely, the postprandial expression of $TLR2^{+}$ on monocytes was lower in obese subjects compared with normal weight (AUC) (**Table 6.2a**).

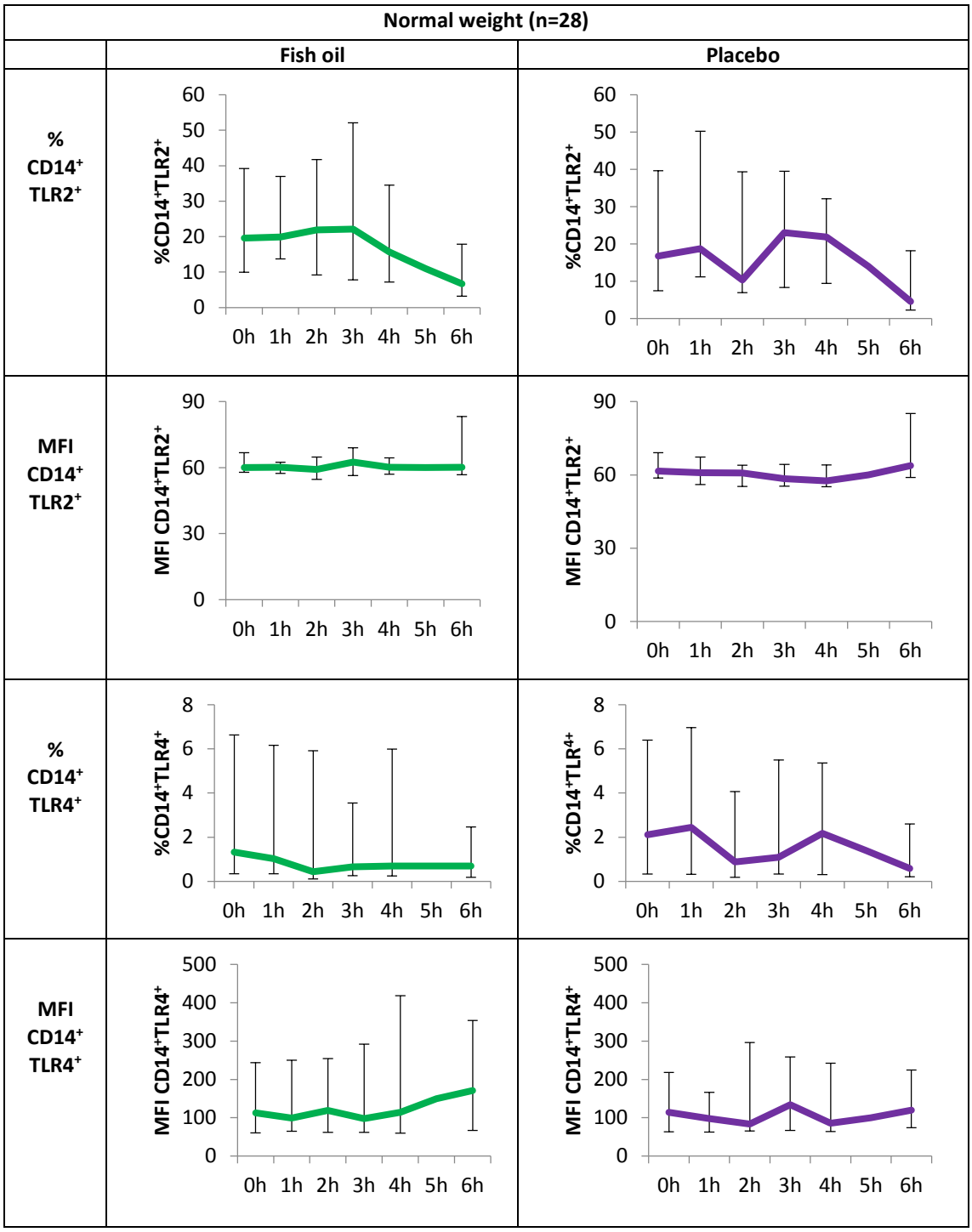


Figure 6.2a Postprandial changes in $CD14^+$ $TLR2^+$ and $CD14^+$ $TLR4^+$ monocytes after a HFM with placebo or HFM with fish oil in normal weight subjects. Changes in the % and MFI of $CD14^+TLR2^+$ and % and MFI of $CD14^+TLR4^+$ after fish oil (**-green-**) or placebo (**-purple-**). Time points expressed as median and error bars as 25^{TH} and 75^{TH} percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*$\rho<0.05$).

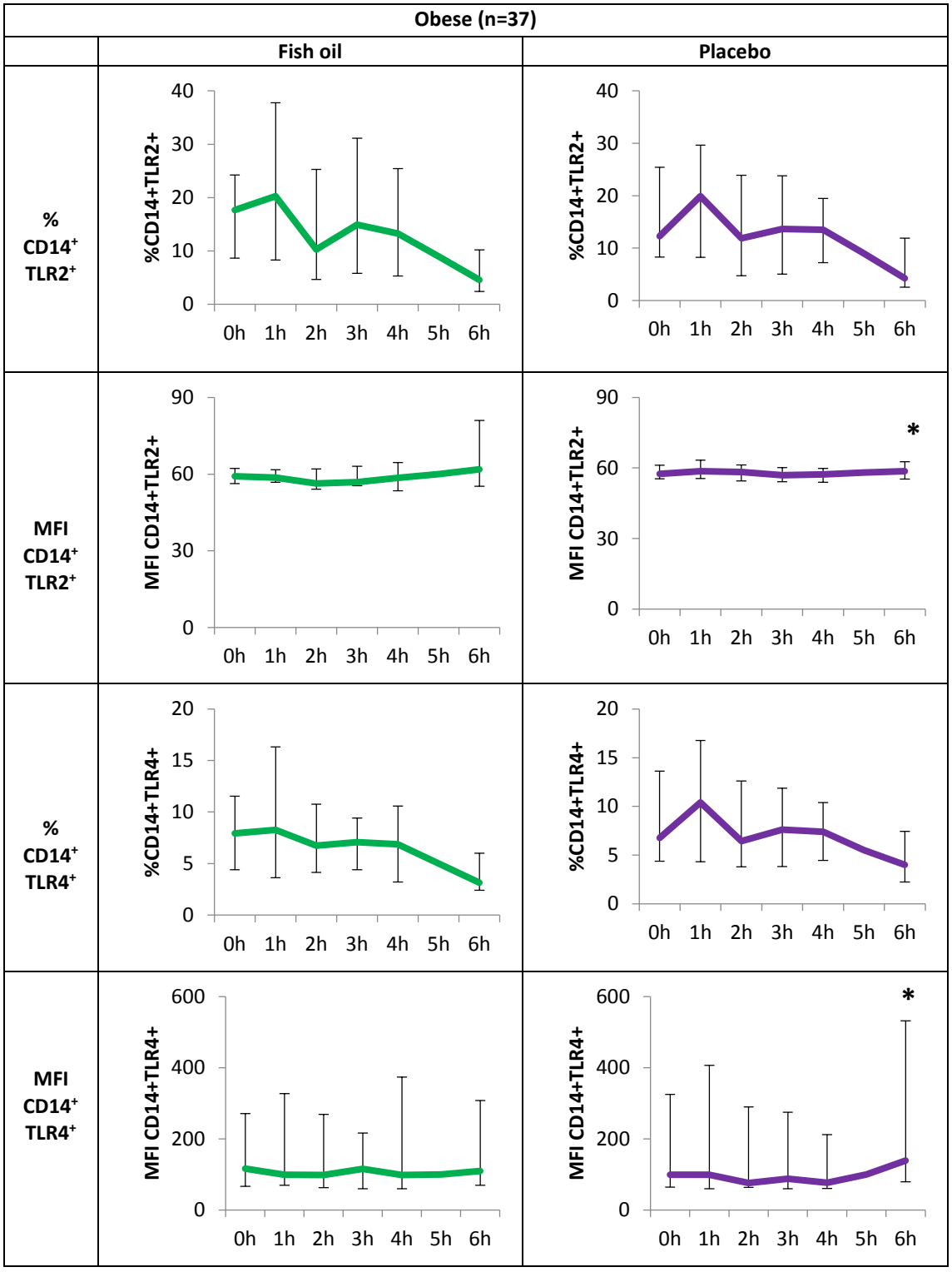


Figure 6.2b Postprandial changes in $CD14^+$ $TLR2^+$ and $CD14^+$ TLR4 monocytes after a HFM with placebo or HFM with fish oil in obese subjects. Changes in the % and MFI of $CD14^+TLR2^+$ and % and MFI of $CD14^+TLR4^+$ after fish oil (**-green-**) or placebo (**-purple-**). Time points expressed as median and error bars as 25^{TH} and 75^{TH} percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*$p<0.05$).

				Fish oil vs Placebo ρ-value	Main Effects ρ-value		Interaction ρ-value
		Fish oil	Placebo		*group*	*treatment*	*group*treatment*
% CD14⁺TLR2⁺							
	Normal weight	117.60 (49.75-189.50)	101.45 (50.16-217.92)	0.71	0.09	0.51	0.98
	Obese	80.40 (55.12-144.90)	75.16 (45.45-128.60)	0.70			
MFI CD14⁺TLR2⁺							
	Normal weight	362.00 (347.92-393.12)	383.20 (353.92-429.87)	0.65	**<0.01**	0.70	0.30
	Obese	357.60 (340.50-389.50)	352.60 (329.60-364.20)	0.57			
% CD14⁺TLR4⁺							
	Normal weight	6.94 (1.52-27.18)	17.80 (1.49-26.98)	0.37	**<0.01**	0.41	0.93
	Obese	38.04 (29.14-69.44)	42.18 (24.56-79.80)	0.78			
MFI CD14⁺TLR4⁺							
	Normal weight	1223.00 (381.30-2378.25)	529.80 (437.80 -2219.00)	0.63	0.63	0.82	0.94
	Obese	801.70 (447.40-3229.50)	856.65 (439.03-2032.25)	0.65			
Data expressed as median and percentiles 25 and 75; data was log10 transformed before statistical analysis. Difference in the postprandial response between FO and placebo response per group was analysed by paired t test; The main effects of fish oil and placebo were analysed using 2 way ANOVA (data fixed for age and sex)							

Table 6.2a. AUC of CD14⁺TLR2⁺ and CD14⁺TLR4⁺ cells after a HFM challenge containing fish oil or placebo in normal weight (n=28) and obese subjects (n=37).

				Fish oil vs Placebo ρ-value	Main Effects ρ-value		Interaction ρ-value
		Fish oil	Placebo		*group*	*treatment*	*group*treatment*
% CD14⁺TLR2⁺							
	Normal weight	5.25 (1.18-9.76)	9.88 (1.03-27.60)	0.37	0.37	0.77	0.25
	Obese	11.34 (2.81-29.42)	7.98 (1.73-32.53)	0.06			
MFI CD14⁺TLR2⁺							
	Normal weight	10.00 (1.39-15.07)	12.99 (0.60-40.81)	0.46	0.27	0.62	0.15
	Obese	7.34 (0.98-22.18)	9.75 (0.07-23.62)	0.76			
% CD14⁺TLR4⁺							
	Normal weight	0.32 (0.00-1.79)	0.38 (0.00-2.09)	0.30	**<0.01**	0.39	0.96
	Obese	1.28 (0.00-10.80)	6.06 (0.73-12.73)	0.53			
MFI CD14⁺TLR4⁺							
	Normal weight	66.88 (3.83-1968.75)	83.42 (0.00-840.23)	0.36	0.72	0.81	0.36
	Obese	49.02 (0.00-340.60)	57.97 (23.85-363.70)	0.32			

Data expressed as median and percentiles 25 and 75; data was log10 transformed before statistical analysis.
Difference in the postprandial response between FO and placebo response per group was analysed by paired t test;
The main effects of fish oil and placebo were analysed using 2 way ANOVA (data fixed for age and sex)

Table 6.2b. iAUC of CD14⁺TLR2⁺ and CD14⁺TLR4⁺ cells after a HFM challenge containing fish oil or placebo in in normal weight (n=28) and obese subjects (n=37).

6.3.3 The acute effect of fish oil on the postprandial cytokine response in normal weight and obese subjects

The overall postprandial cytokine response (AUC and iAUC) after a meal containing fish oil or placebo was not significantly different for both groups (normal weight or obese subjects) (**Figure 6.3a, Figure 6.3b, Figure 6.3ca, Figure 6.3d and Table 6.3a and b**).

Postprandial GM-CSF, TNF-α and IL-10 concentrations after a HFM (fish oil and placebo) were lower in obese than normal weight while postprandial VEGF plasma concentrations were higher obese than normal weight subjects ($\rho<0.05$).

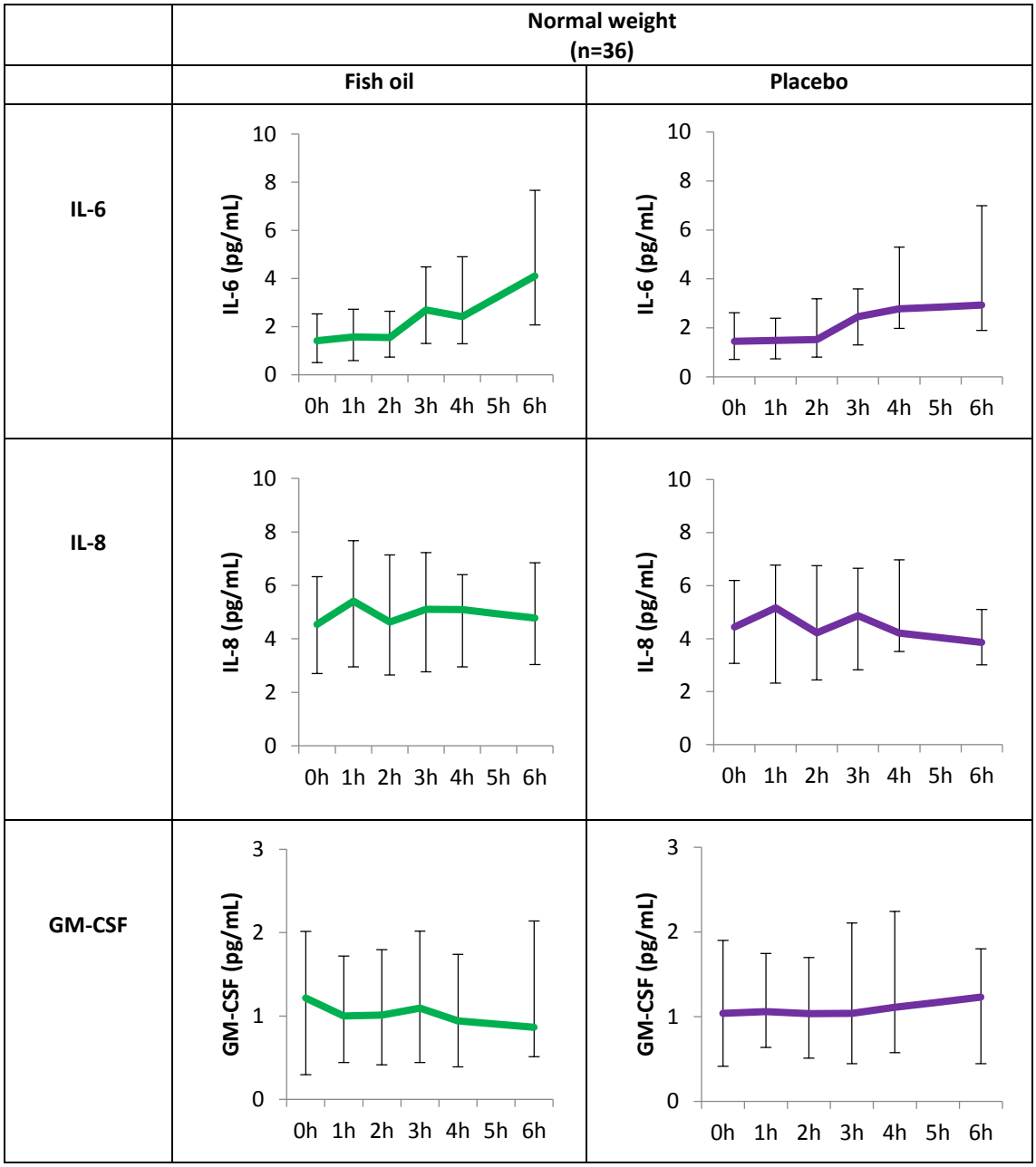


Figure 6.3a Postprandial changes in cytokines after a HFM with placebo or fish oil in normal weight subjects. Changes in cytokines after fish oil (**-green-**) or placebo (**-purple-**). Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*ρ<0.05).

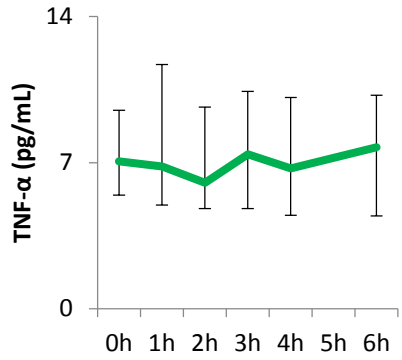
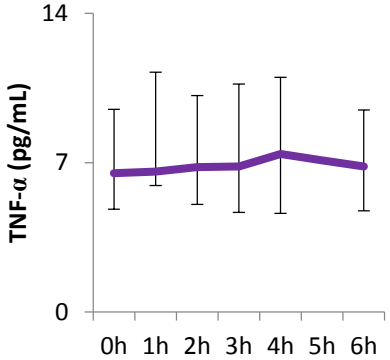
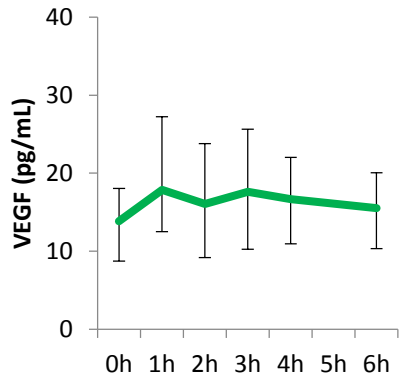
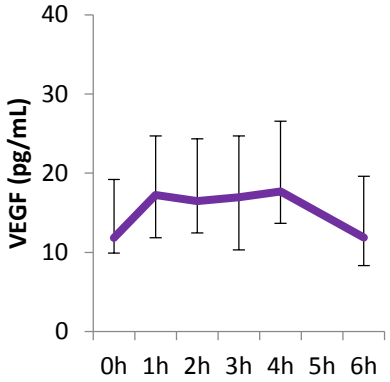
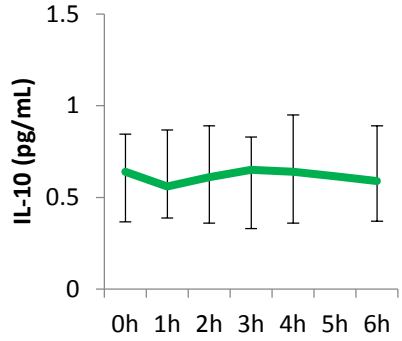
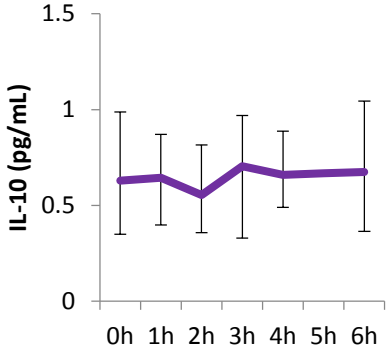
	Normal weight (n=36)	
	Fish oil	**Placebo**
TNF-α		
VEGF		
IL-10		

Figure 6.3b Postprandial changes in cytokines after a HFM with placebo or fish oil in normal weight subjects. Changes in cytokines after fish oil (**-green-**) or placebo (**-purple-**). Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*p<0.05).

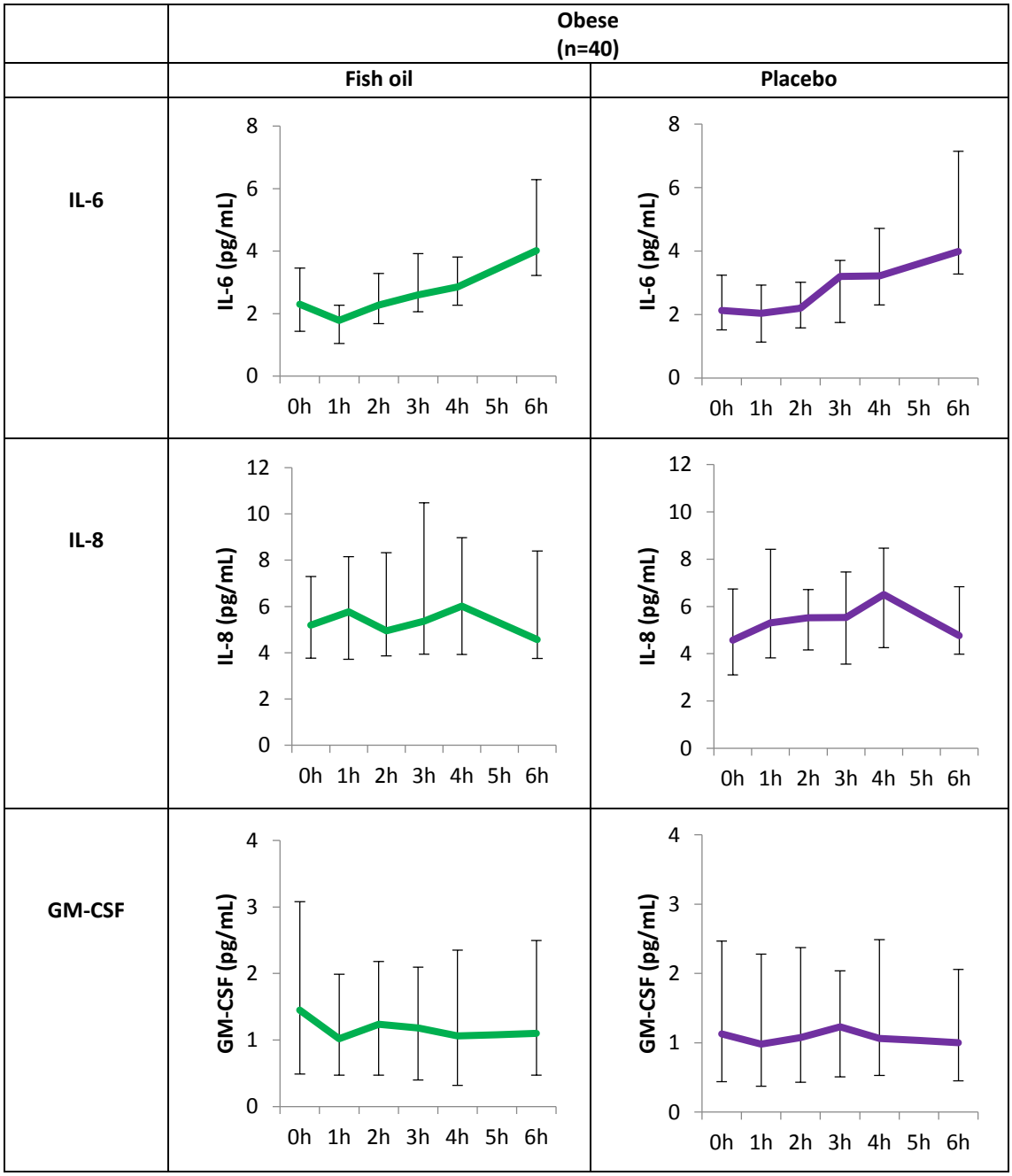


Figure 6.3c Postprandial changes in cytokines after a HFM with placebo or fish oil in obese subjects. Changes in cytokines after fish oil (**-green-)** or placebo (**-purple-**). Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*ρ<0.05).

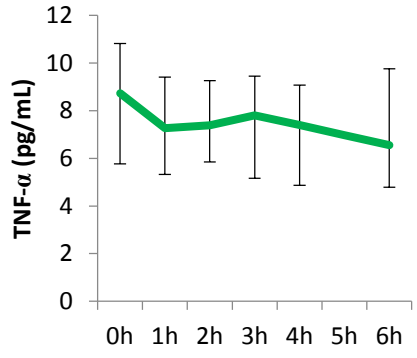
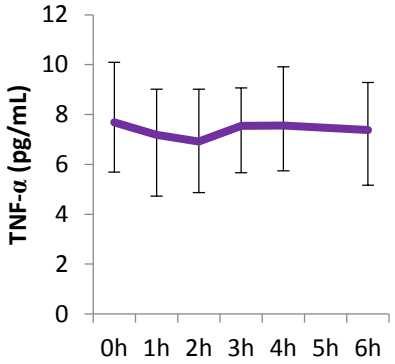
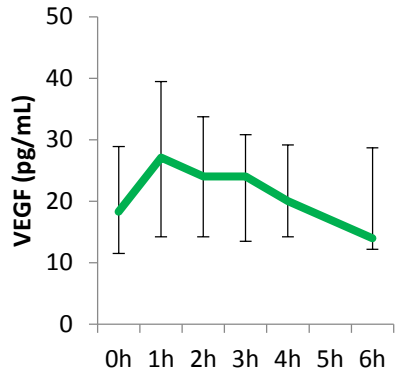
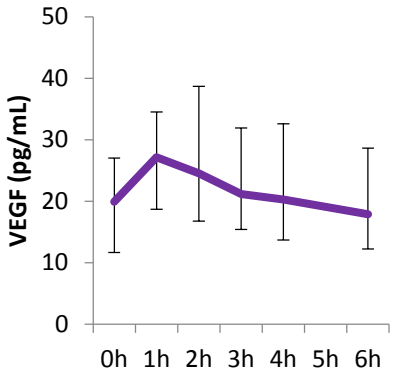
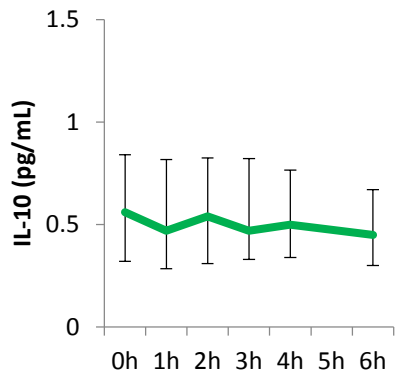
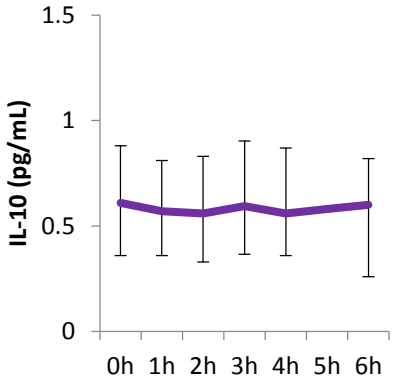
	Obese (n=40)	
	Fish oil	Placebo
TNF-α		
VEGF		
IL-10		

Figure 6.3d Postprandial changes in cytokines after a HFM with placebo or fish oil in obese subjects. Changes in cytokines after fish oil (-green-) or placebo (-purple-). Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*ρ<0.05).

				Fish oil vs Placebo *p-value*	Main Effects *p-value*		Interaction *p-value*
		Fish oil	**Placebo**		*group*	*treatment*	*group* treatment*
IL-6							
	Normal weight	14.61 (9.35-24.25)	15.90 (10.68-25.06)	0.98	0.11	0.92	0.90
	Obese	16.31 (13.57-23.90)	18.94 (13.78-24.62)	0.83			
IL-8							
	Normal weight	30.73 (19.25-43.56)	28.79 (20.36-36.99)	0.39	0.07	0.42	0.86
	Obese	35.73 (25.84-56.93)	35.45 (25.64-45.49)	0.18			
GM-CSF							
	Normal weight	5.73 (3.01-11.37)	7.17 (3.58-11.40)	0.80	0.47	0.98	0.91
	Obese	7.75 (3.12-12.56)	7.19 (2.57-13.43)	0.79			
TNF-α							
	Normal weight	43.44 (28.30-61.43)	45.88 (31.27-59.35)	0.37	0.92	0.78	0.76
	Obese	44.47 (32.93-57.76)	45.23 (33.27-56.06)	0.91			
VEGF							
	Normal weight	99.43 (65.21-138.8)	104.00 (71.36-130.32)	0.54	**<0.05**	0.64	0.92
	Obese	125.10 (80.67-197.80)	134.80 (102.70-190.00)	0.35			
IL-10							
	Normal weight	3.42 (2.47-4.85)	4.05 (2.64-5.30)	0.37	**<0.05**	0.39	0.79
	Obese	3.24 (1.86-4.67)	3.59 (2.19-5.13)	0.12			

Data expressed as median and percentiles 25 and 75; data were log10 transformed before statistical analysis
Difference in the postprandial response between FO and placebo response per group was analysed by paired t test;
The main effects of fish oil and placebo were analysed using 2 way ANOVA (data fixed for age and sex)

Table 6.3a. AUC of cytokines after a HFM challenge containing fish oil or placebo in normal weight (n=36) and obese subjects (n=40).

				Fish oil vs Placebo *p-value*	Main Effects *p-value*		Interaction *p-value*
		Fish oil	**Placebo**		*group*	*treatment*	*group* treatment*
IL-6							
	Normal weight	4.92 (1.45-10.84)	5.12 (1.13-14.12)	0.90	0.19	0.46	0.44
	Obese	4.87 (1.35-7.51)	4.62 (1.22-14.40)	0.56			
IL-8							
	Normal weight	2.94 (1.21-10.08)	2.42 (0.35-6.32)	0.31	0.33	0.55	0.94
	Obese	5.37 (2.11-14.82)	6.00 (1.45-11.22)	0.63			
GM-CSF							
	Normal weight	0.47 (0.00-1.50)	0.73 (0.00-2.36)	0.88	**<0.05**	0.81	0.19
	Obese	0.30 (0.00-0.80)	0.39 (0.00-1.00)	0.32			
TNF-α							
	Normal weight	1.58 (0.00-5.20)	1.72 (0.00-4.81)	0.31	**<0.01**	0.51	0.53
	Obese	0.50 (0.00-1.96)	0.80 (0.10-2.80)	0.98			
VEGF							
	Normal weight	15.96 (3.16-27.05)	12.41 (5.58-30.12)	0.61	0.80	0.71	0.99
	Obese	23.44 (11.88-43.49)	32.89 (11.95-57.39)	0.77			
IL-10							
	Normal weight	0.20 (0.00-0.97)	0.25 (0.01-0.84)	0.93	0.40	0.47	0.81
	Obese	0.12 (0.00-0.36)	0.13 (0.01-0.39)	0.72			

Data expressed as median and percentiles 25 and 75; data were log10 transformed before statistical analysis
Difference in the postprandial response between FO and placebo response per group was analysed by paired t test;
The main effects of fish oil and placebo were analysed using 2 way ANOVA (fixed for age and sex)

Table 6.3b. iAUC of cytokines after a HFM challenge containing fish oil or placebo in obese subjects.

6.3.4 The acute effect of fish oil on the postprandial adhesion molecules response in normal weight and obese subjects

The consumption of a HFM containing fish oil did not affect adhesion molecules concentrations compared with placebo as shown in **Figure 6.4** and **Figure 6.4.**

The P-selectin iAUC in obese subjects was significantly lower when subjects consumed the HFM with fish oil compared with placebo ($\rho<0.01$) (**Table 6.4**).

In agreement with the results presented in Chapter 6, postprandial VCAM-1 concentrations were lower after the HFM (fish oil and placebo) in obese compared with normal weight subjects ($\rho<0.01$) (**Table 6.4a and Table 6.4b**).

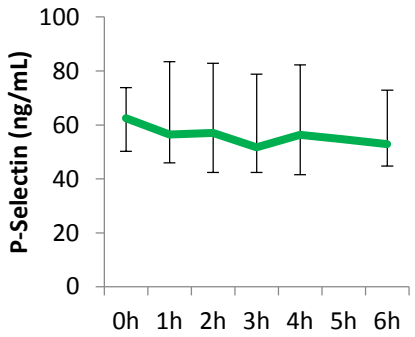
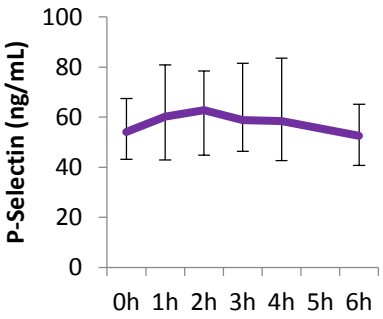
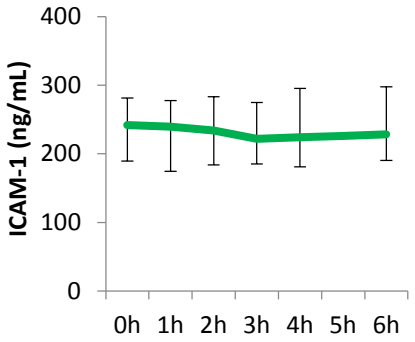
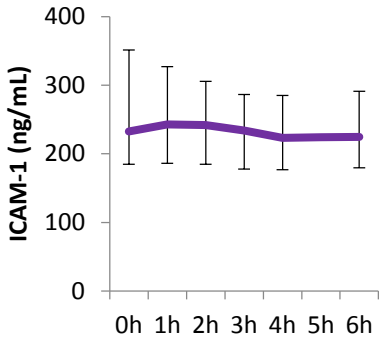
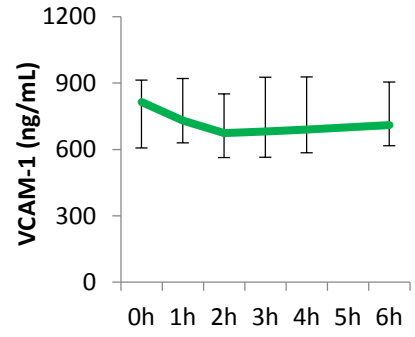
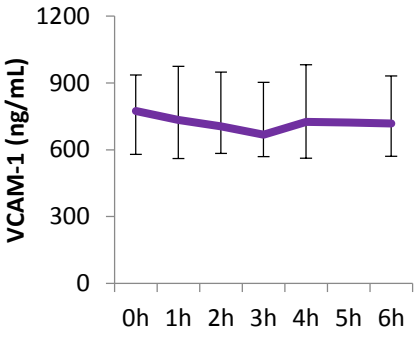
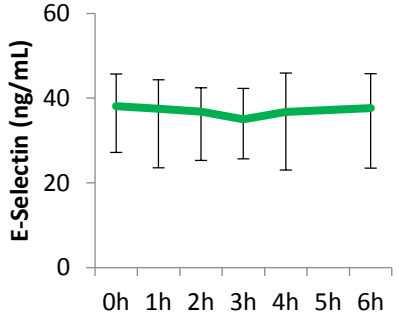
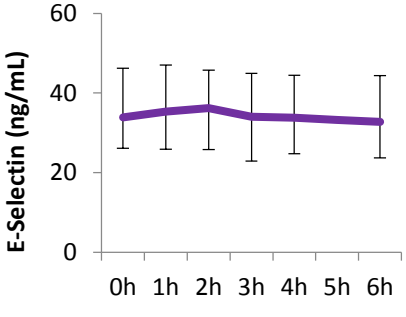
	Normal weight (n=36)	
	Fish oil	Placebo
P-Selectin		
ICAM-1		
VCAM-1		
E-Selectin		

Figure 6.4a Postprandial changes in adhesion molecules after a HFM with placebo or fish oil in normal weight subjects. Changes in adhesion molecules concentrations after fish oil (**-green-**) or placebo (**-purple-**). Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*ρ<0.05).

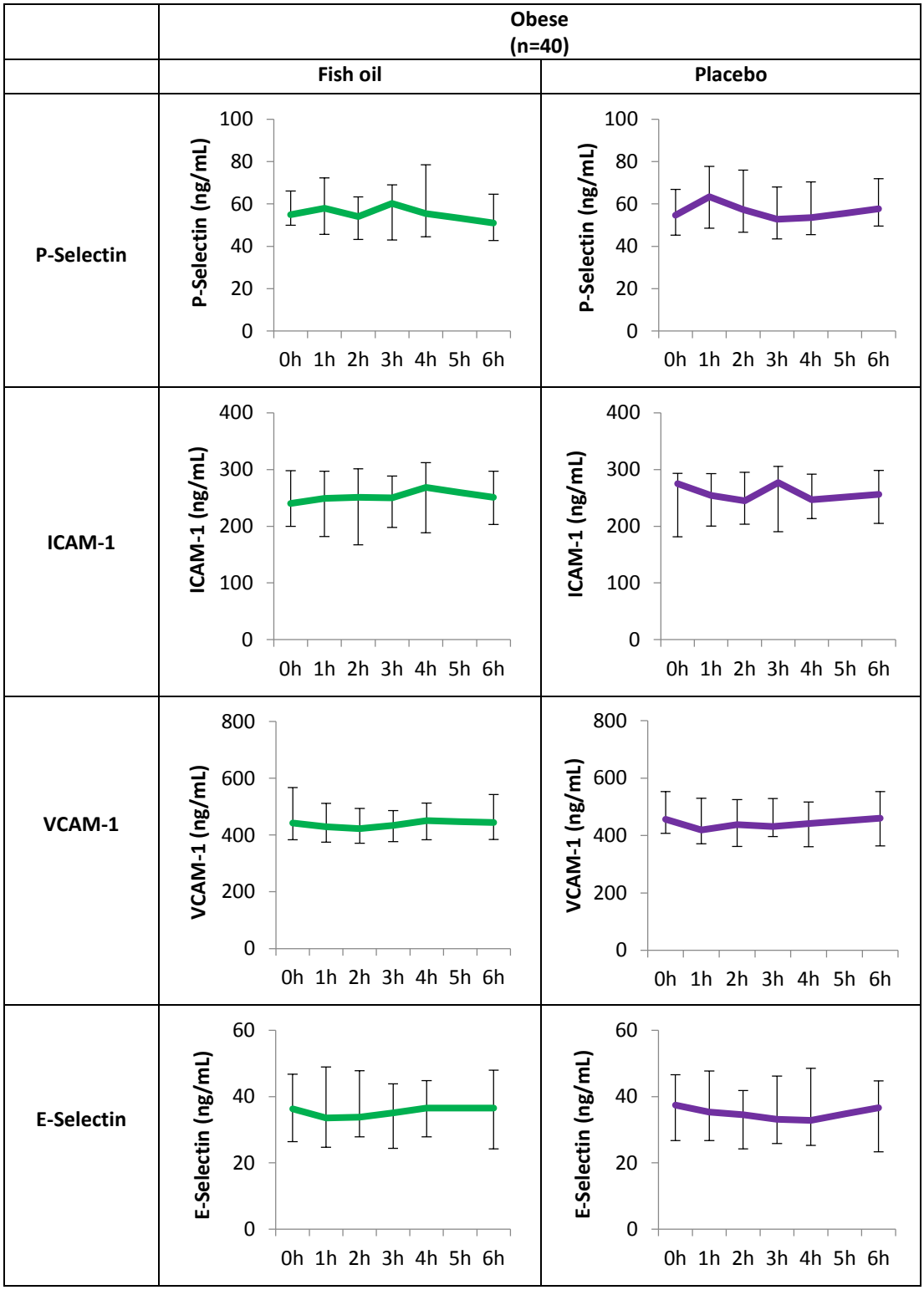


Figure 6.4b Postprandial changes in adhesion molecules after a HFM with placebo or fish oil in obese subjects. Changes in adhesion molecules concentrations after fish oil (**-green-**) or placebo (**-purple-**). Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*ρ<0.05).

				Fish oil vs Placebo ρ-value	Main Effects ρ-value		Interaction ρ-value
		Fish oil	Placebo		Group	Treatment	group* treatment
P- Selectin							
	Normal weight	33.70 (27.04-49.03)	35.58 (26.82-46.93)	0.15	0.51	0.57	0.85
	Obese	32.62 (28.79-43.37)	32.92 (30.05-42.89)	0.61			
ICAM-1							
	Normal weight	139.60 (111.50-168.10)	140.80 (109.25-183.70)	0.45	0.31	0.86	0.98
	Obese	150.10 (121.70-177.60)	148.65 (120.80-177.07)	0.60			
VCAM-1							
	Normal weight	412.40 (356.00-546.30)	449.90 (348.05-579.65)	0.91	**<0.01**	0.89	0.93
	Obese	258.70 (238.30-305.50)	264.15 (222.15-307.9)	0.67			
E-Selectin							
	Normal weight	22.89 (14.34-27.19)	20.36 (14.28-27.33)	0.36	0.30	0.85	0.87
	Obese	20.07 (16.76-26.25)	20.88 (15.01-27.39)	0.84			

Data expressed as median and percentiles 25 and 75; data were log10 transformed before statistical analysis
Difference in the postprandial response between FO and placebo response per group was analysed by paired t test;
The main effects of fish oil and placebo were analysed using 2 way ANOVA (fixed for age and sex)

Table 6.4a. AUC of adhesion molecules after a HFM challenge containing fish oil or placebo in normal weight (n=36) and obese subjects (n=40).

				Fish oil vs Placebo ρ-value	Main Effects ρ-value		Interaction ρ-value
		Fish oil	**Placebo**		Group	Treatment	group* treatment
P- Selectin							
	Normal weight	1.92 (0.74-7.44)	1.62 (0.32-5.29)	0.42	0.93	0.75	0.32
	Obese	1.04 (0.39-5.03)	2.11 (0.62-4.61)	**<0.01**			
ICAM-1							
	Normal weight	1.68 (0.00-5.85)	1.48 (0.11-7.89)	0.61	0.81	0.82	0.16
	Obese	1.46 (0.31-7.23)	2.62 (0.00-9.22)	0.44			
VCAM-1							
	Normal weight	4.92 (0.00-24.42)	1.81 (0.00-22.43)	0.93	**<0.01**	0.44	0.64
	Obese	3.31 (0.00-14.19)	0.46 (0.00-9.21)	0.39			
E-Selectin							
	Normal weight	0.27 (0.09-1.76)	0.12 (0.00-1.10)	0.87	0.06	0.91	0.65
	Obese	0.15 (0.00-1.21)	0.15 (0.00-0.97)	0.82			

Data expressed as median and percentiles 25 and 75; data were log10 transformed before statistical analysis
Difference in the postprandial response between FO and placebo response per group was analysed by paired t test;
The main effects of fish oil and placebo were analysed using 2 way ANOVA (fixed for age and sex)

Table 6.4b. iAUC of adhesion molecules after a HFM challenge containing fish oil or placebo in normal weight (n=36) and obese subjects (n=40)..

6.3.5 The acute effect of fish oil on the postprandial adipokines response in normal weight and obese subjects

Time point's analysis revealed no significant differences in the HFM containing FO or placebo (**Figure 6.5a** and **Figure 6.5b**). The MCP-1 AUC was higher when subjects received a HFM containing FO than placebo (**Table 6.5**).

The postprandial response to the HFM (fish oil/ placebo) was associated with a higher CRP, PAI-1 and leptin but lower adiponectin concentrations in obese compared with normal weight subjects ($\rho<0.01$).

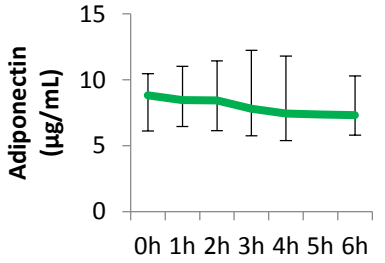
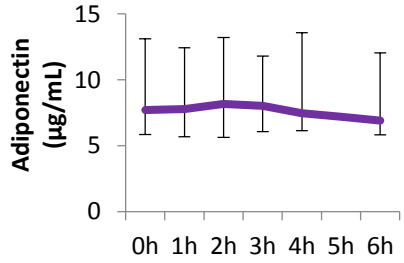
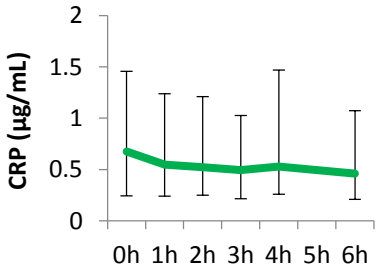
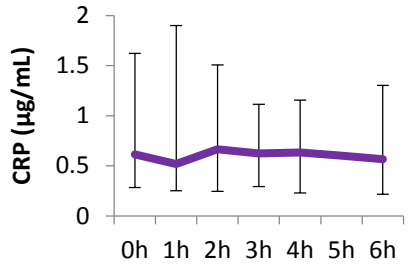
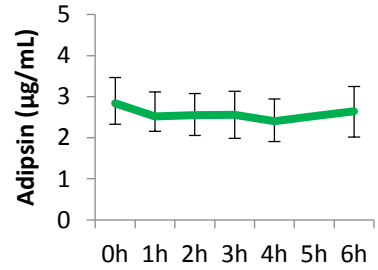
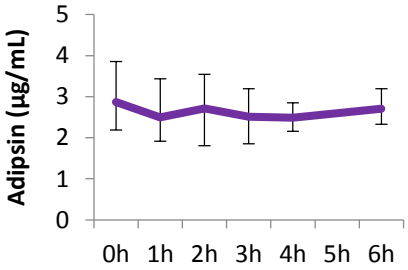
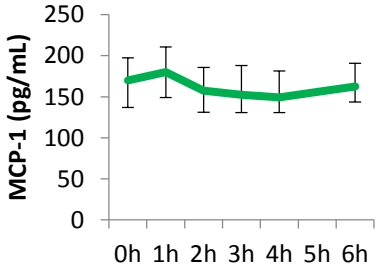
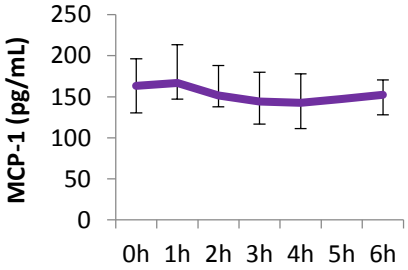
	Normal weight (n=36)	
	Fish oil	**Placebo**
Adiponectin		
CRP		
Adipsin		
MCP-1		

Figure 6.5a Postprandial changes in adipokines after a HFM with placebo or fish oil in normal weight subjects. Changes in adipokines concentrations after fish oil (**-green-**) or placebo (**-purple-**). Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*p<0.05).

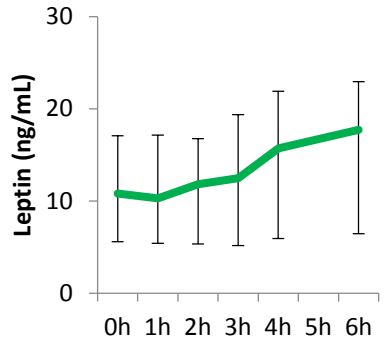
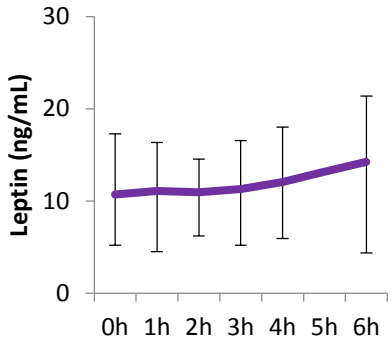
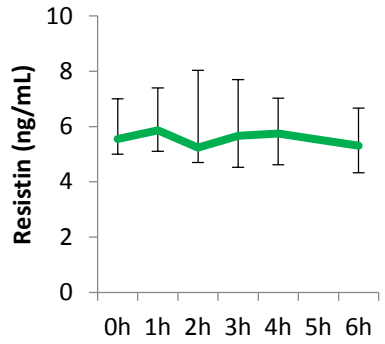
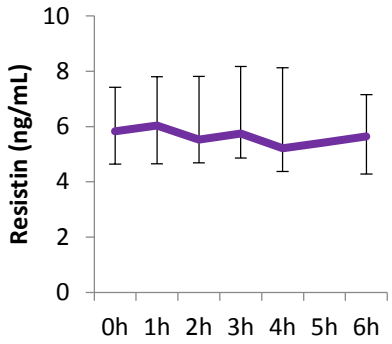
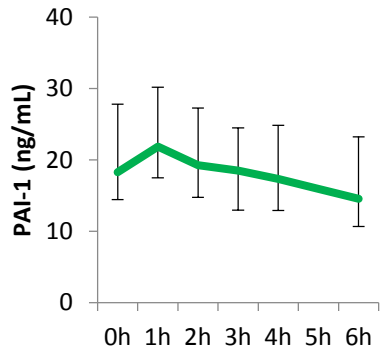
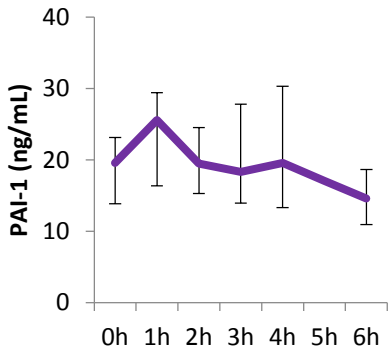
	Normal weight (n=36)	
	Fish oil	**Placebo**
Leptin		
Resistin		
PAI-1		

Figure 6.5b Postprandial changes in adipokines after a HFM with placebo or fish oil in normal weight subjects. Changes in adipokines concentrations after fish oil (**-green-**) or placebo (**-purple-**). Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*ρ<0.05).

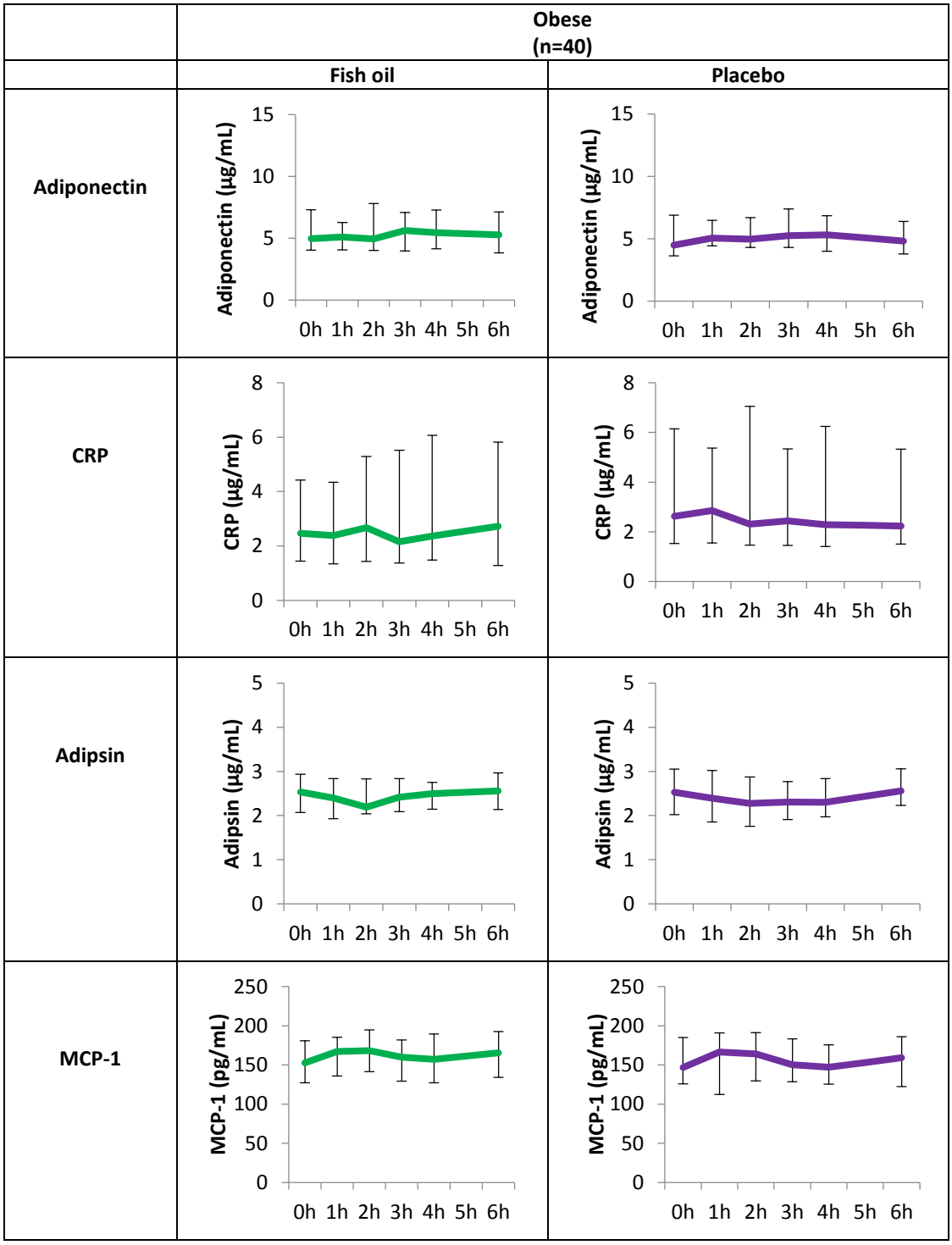


Figure 6.5c Postprandial changes in adipokines after a HFM with placebo or fish oil in obese subjects. Changes in adipokines after fish oil (-green-) or placebo (-purple-). Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*ρ<0.05).

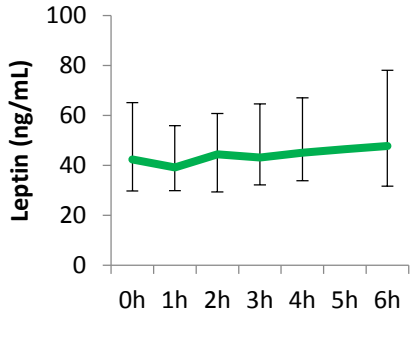
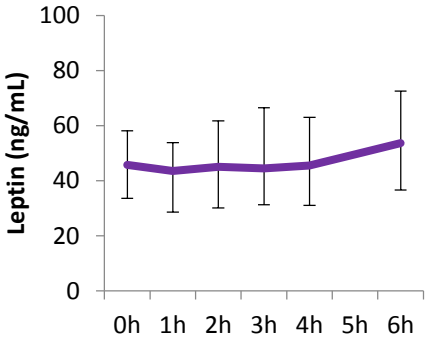
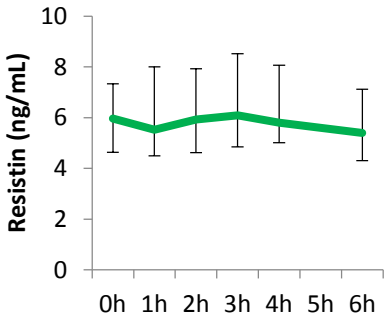
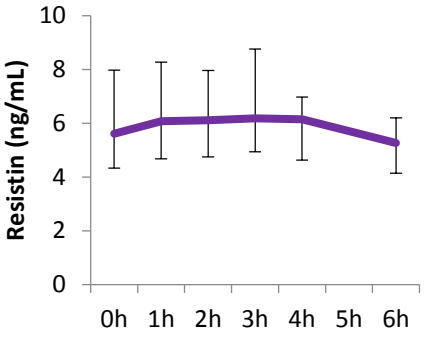
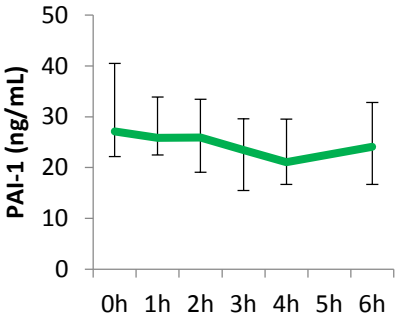
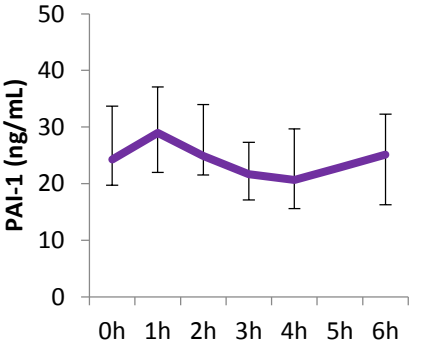
	Obese (n=40)	
	Fish oil	**Placebo**
Leptin		
Resistin		
PAI-1		

Figure 6.5d Postprandial changes in adipokines after a HFM with placebo or fish oil in obese subjects. Changes in adipokines after fish oil (-green-) or placebo (-purple-). Time points expressed as median and error bars as 25TH and 75TH percentiles. Data were log10 transformed and the time points differences between groups were evaluated using a paired t-test (*ρ<0.05).

				Fish oil vs Placebo ρ-value	Main Effects ρ-value		Interaction ρ-value
		Fish oil	Placebo		Group	Treatment	
Adiponectin							
	Normal weight	4.87 (3.48-6.56)	4.55 (3.72-8.02)	0.47	**<0.01**	0.92	0.47
	Obese	3.11 (2.66-4.19)	3.02 (2.55-3.95)	0.06			
CRP							
	Normal weight	0.35 (0.14-0.77)	0.38 (0.15-0.91)	0.82	**<0.01**	0.72	0.94
	Obese	1.48 (0.85-3.30)	1.45 (0.88-3.61)	0.34			
Adipsin							
	Normal weight	1.56 (1.28-1.95)	1.63 (1.27-2.00)	0.38	0.29	0.99	0.55
	Obese	1.49 (1.25-1.670	1.47 (1.24-1.71)	0.09			
MCP-1							
	Normal weight	96.77 (85.22-112.30)	89.45 (79.14-107.45)	0.60	0.25	0.46	0.70
	Obese	98.95 (79.55-110.30)	90.85 (78.28-111.00)	**<0.05**			
Leptin							
	Normal weight	78.40 (34.85-119.24)	77.93 (43.33-108.24)	0.61	**<0.01**	0.86	0.99
	Obese	272.84 (19.38-435.02)	272.71 (187.94-385.97)	0.49			
Resistin							
	Normal weight	32.66 (28.21-45.52)	34.19 (27.63-47.18)	0.85	0.76	0.89	0.82
	Obese	35.18 (28.28-46.44)	36.02 (28.08-45.84)	0.55			
PAI-1							
	Normal weight	12.08 (8.68-14.79)	12.26 (8.84-15.63)	0.63	**<0.05**	0.92	0.79
	Obese	15.78 (11.72-19.95)	15.34 (11.58-20.79)	0.85			
Data expressed as median and percentiles 25 and 75; data were log10 transformed before statistical analysis *Difference in the postprandial response between FO and placebo response per group was analysed by paired t test;* *The main effects of fish oil and placebo were analysed using 2 way ANOVA (fixed for age and sex)*							

Table 6.5a. AUC of adhesion molecules after a HFM challenge containing fish oil or placebo in obese subjects.

				Fish oil vs Placebo ρ-value	Main Effects ρ-value		Interaction ρ-value
		Fish oil	Placebo		Group	Treatment	
Adiponectin							
	Normal weight	0.10 (0.00-0.49)	0.21 (0.00-0.56)	0.77	0.92	0.84	0.85
	Obese	0.23 (0.02-0.47)	0.27 (0.00-0.57)	0.99			
CRP							
	Normal weight	0.00 (0.00-0.05)	0.00 (0.00-0.04)	0.82	**<0.01**	0.81	0.94
	Obese	1.11 (0.11-2.84)	0.12 (0.00-0.39)	0.89			
Adipsin							
	Normal weight	0.00 (0.00-0.05)	0.00 (0.00-0.15)	0.59	0.27	0.68	0.70
	Obese	0.03 (0.00-0.13)	0.03 (0.00-0.08)	0.98			
MCP-1							
	Normal weight	16.11 (0.00-102.80)	11.00 (0.00-74.20)	0.43	0.06	0.24	0.88
	Obese	87.32 (1.54-143.80)	26.38 (2.72-77.11)	0.33			
Leptin							
	Normal weight	6.11 (0.83-14.91)	6.54 (0.51-13.31)	0.38	0.21	0.71	0.70
	Obese	20.75 (5.93-55.74)	19.08 (1.63-31.06)	0.99			
Resistin							
	Normal weight	1.33 (0.36-3.98)	0.77 (0.25-3.51)	0.28	0.59	0.48	0.41
	Obese	1.87 (0.19-5.02)	2.09 (0.29-5.95)	0.94			
PAI-1							
	Normal weight	1.15 (0.00-4.08)	1.11 (0.21-3.07)	0.46	0.64	0.68	0.55
	Obese	1.30 (0.00-3.41)	0.63 (0.00-3.29)	0.89			
Data expressed as median and percentiles 25 and 75; data were log10 transformed before statistical analysis *Difference in the postprandial response between FO and placebo response per group was analysed by paired t test;* *The main effects of fish oil and placebo were analysed using 2 way ANOVA (fixed for age and sex)*							

Table 6.5b. iAUC of adhesion molecules after a HFM challenge containing fish oil or placebo in obese subjects.

6.4 Discussion

The acute effect of fish oil on a number of metabolic and inflammatory molecules following the consumption of a HFM was described in this chapter. As illustrated in **Table 6.6** some markers (mainly involved in cardiovascular health) were significantly reduced after a HFM containing fish oil in both normal weight and obese subjects.

The differences in the postprandial response of normal weight and obese subjects after a HFM (including fish oil or placebo) are summarized in **Table 6**.7. Some of these differences are in agreement with the results presented in **Chapter 5** (HFM + placebo). These include the group differences observed in some molecules such as, triglycerides, TLR4, VEGF, VCAM-1 adiponectin and CRP.

Normal weight subjects		**Obese subjects**		**Both groups**
Reduced after FO	**Increased after FO**	**Reduced after FO**	**Increased after FO**	**No changes**
Metabolism				
AUC: Triglycerides **iAUC**: Triglycerides	-	-	-	**AUC**: Insulin, glucose and NEFA **iAUC**: Insulin, glucose and NEFA
TLR expression on monocytes				
-	-	**Time points:** MFI $CD14^+TLR4^+$ (6 h)	**Time points:** MFI $CD14^+TLR2^+$ (6 h)	All AUC and iAUC
Plasma				
Cytokines				
-	-	-	-	All AUC and iAUC
Adhesion Molecules				
-	-	**iAUC**: P-Selectin	-	**AUC**: all **iAUC**: all except P-Selectin
Adipokines				
-	-	-	**AUC**: MCP-1	**AUC**: all except MCP-1 **iAUC**: all

Table 6.6 Summary of the main differences in the postprandial response to a HFM containing fish oil or placebo in normal weight and obese subjects.

Higher in normal weight subjects	Higher in obese subjects	Not different between groups
Metabolism		
-	**AUC:** Triglycerides, NEFA, Glucose & insulin **iAUC:** Triglycerides & insulin **% NEFA suppression**: -	**iAUC**: - Glucose **% NEFA suppression**:
TLR expression on monocytes		
AUC: MFI $CD14^+TLR2^+$	**AUC**: $\%CD14^+TLR4^+$ **iAUC:** $-\%CD14^+TLR4^+$	**AUC:** $\%CD14^+TLR2^+$, MFI $CD14^+TLR4^+$ **iAUC:** - $\%CD14^+TLR2^+$, MFI $CD14^+TLR2^+$, MFI $CD14^+TLR4^+$
Plasma		
Cytokines		
AUC: IL-10 **iAUC**: GM-CSF & TNF-α	**AUC**: VEGF	**AUC**: IL-6, IL-8, TNF-α, & GM-CSF **iAUC**: IL-6, IL-8, TNF-α, VEGF & IL-10
Adhesion molecules		
AUC: VCAM-1 **iAUC**: VCAM-1 & E-Selectin	-	**AUC**: P-Selectin, E-Selectin & ICAM-1 **iAUC**: P-Selectin & ICAM-1
Adipokines		
AUC: Adiponectin,	**AUC**: CRP, Leptin and PAI-1 **iAUC**: CRP	**AUC**: MCP-1, Resistin, & Adipsin **iAUC**: Adiponectin, Leptin and PAI-1, MCP-1, Resistin, & Adipsin

Table 6.7 Summary of the main group differences in the postprandial response to a HFM (containing fish oil and placebo)

In this section, some cytokines and adhesion molecules responses, including TNF-α, GM-CSF, E-selectin and IL-10, were found to be significantly lower in obese then normal weight subjects. These findings are consistent with those presented in **Chapter 4**, where it was observed that monocytes from obese subjects were less responsive to an inflammatory challenge than those from normal weight subjects. Importantly, this is the first time the postprandial differences between obese and normal weight subjects in

plasma inflammatory markers, such as IL-10, VEGF and GM-CSF, are reported. Some studies have documented no differences in the postprandial plasma IL-10 concentrations in healthy normal weight (Fogarty *et al.*2014). Postprandial concentrations of VEGF has been studied in healthy normal weight subjects after a HFM (Brandauer *et al.* 2013); plasma VEGF and GM-CSF concentrations have been documented in healthy obese subjects after a meal containing protein from different sources (e.g. cod, whey, casein) (Holmer-Jensen *et al.* 2011). These studies observed a postprandial increment of plasma VEGF and GM-CSF concentrations, although they do not provide information about the link between obesity, metabolism and inflammatory mediators. Thus, the findings reported in this section contribute to the understanding of the role of obesity in the changes of the metabolic, hormonal and inflammatory signals during the postprandial response.

In this study, the addition of fish oil to a HFM resulted in a significant reduction of postprandial triglyceride concentrations in normal weight subjects. Some studies have reported an alteration of lipoprotein metabolism after the ingestion of a HFM containing fish oil (Heath *et al*. 2003; Burdge *et al.* 2009), while others documented a reduction on postprandial triglycerides in response to acute exposure to n-3 PUFA (Williams *et al.* 1997). The results described in this study are in agreement with this prior work, and suggest a beneficial effect of fish oil in lipid metabolism.

P-selectin is found at higher plasma concentrations in patients with cardiovascular disease and is thought to mediate the attachment and rolling of leukocytes at the early stages of atherosclerosis (Blann *et al*. 2003). Studies in P-selectin knockout rodents observed a reduction of atherosclerotic lesions in absence of P-selectin (Collins *et al.* 2000; Dong *et al.* 2000). In this study, postprandial P-selectin was significantly reduced by fish oil in obese subjects. Moreover, this effect was only noticed in obese subjects. This difference may be related to other events occurring during the postprandial response, including the prolonged exposure to exogenous fatty acids or other factors inferring on P-selectin synthesis (e.g. thrombin).

Fatty acids have been shown to be important factors determining endothelium activation and homeostasis. Keogh *et al*, (2005) observed a dysregulation of endothelial

function measured by flow mediated dilatation (FMD), and an increment of P-selectin after a high saturated fatty acid diet. Similarly Newens *et al,* (2001) reported a detrimental effect of SFA in FMD but this effect was prevented by acute consumption of fish oil. In support of these findings, Esser *et al*. (2013) showed lower postprandial P-selectin concentrations in healthy subjects after an n-3 PUFA rich milkshake compared with a high SFA shake.

In agreement with human studies, *in vitro* experiments in primary endothelial cells reported that cytokine and oxLDL induced production of adhesion molecules, including ICAM-1 and P-selectin, was prevented by n-3 PUFA treatment after 2 -5 h of treatment (De Caterina *et al*. 1994; Chen *et al.* 2003; Wang *et al.* 2003). Thus, the lower postprandial P-selectin response observed after the HFM including fish oil compared with HFM with placebo is in agreement with previous studies. In addition, in comparison with the reports described before, this study also demonstrated that fish oil is able to prevent the detrimental increment of P-selectin induced by saturated fatty acids in obese subjects. The mechanisms involved in n-3 PUFA prevention of adhesion molecules production by endothelial cells have been linked to its effect on protein kinase B, a downstream effector of NF-κB (Chen *et al.* 2003). These findings suggest that acute exposure to fish oil has immediate beneficial effects in obese patients.

In addition to the reduction of P-selectin, some differences in TLRs expression were observed 6 h after the HFM challenge in obese subjects. Saturated fatty acids have been shown to be potent activators of the NF-κB pathway via TLRs in *in vitro* macrophage models (Hwang *et al*. 2001; Lee *et al.* 2003). In contrast, n-3 PUFA had an antagonistic effect on NF-κB activation in the same model (Hwang *et al.* 2001; Lee *et al*. 2003). During the postprandial response, n-3 PUFA reaches the bloodstream rapidly and incorporates in endogenous pools 3-6 h after a meal (Burdge *et al*. 2003; Heath *et al.* 2003). Thereby, the reduction on TLR-4 expression at 6 h may be a result of a dysregulation of NF-κB pathway by n-3 PUFA, and this effect may also be related to the reduction of plasma P-selectin concentrations previously described.

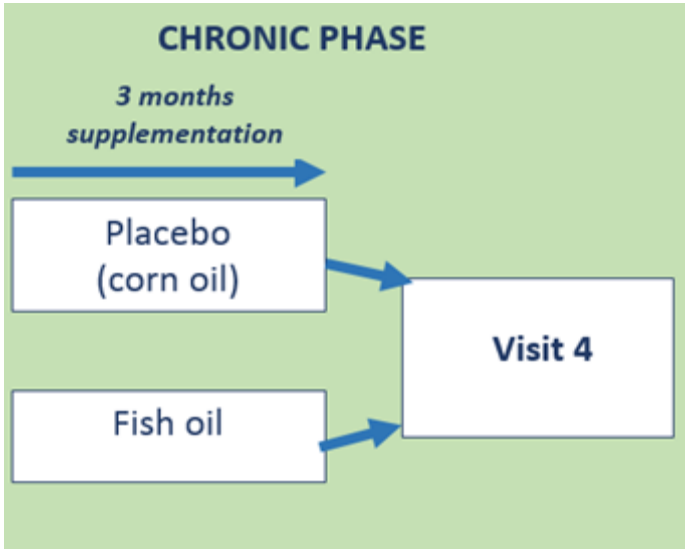
In contrast with these results, MCP-1, a chemokine involved in monocyte migration and inflammation, was upregulated after a HFM containing FO when compared with

placebo. Some studies have suggested a role of insulin in MCP-1 modulation. In these studies, serum MCP-1 levels were reduced after 6 h of a euglycemic hyperinsulinemic clamp in insulin sensitive subjects, while these changes were not observed in obese insulin resistant subjects (Dandona, *et al.* 2001; Westerbacka *et al.* 2008).

To date, the acute effect of fish oil on postprandial MCP-1 plasma concentrations have not been reported. A study by van Djik *et al.* (2012) documented an increase in MCP-1 mRNA levels after a meal containing fish oil and MUFA compared with a SFA. Thus, this study is consistent with the literature, although the high MCP-1 concentrations observed in normal weight subjects and the induction of MCP-1 by fatty acids (n-3 PUFA and MUFA) considered to be protective in cardiovascular health raise some questions about the role of this chemokine in metabolism.

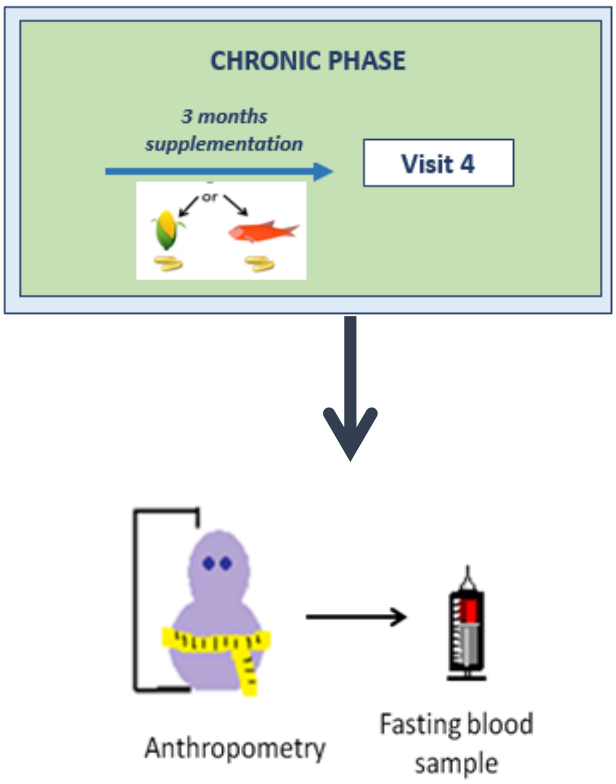
6.5 Conclusions

This study demonstrates that fish oil has an acute effect on postprandial lipid metabolism in healthy normal weight subjects. In addition, it mitigated inflammation in obese subjects by reducing postprandial P-selectin and TLR4 expression, although it also increases plasma of MCP-1. Thus, this study provides novel and detailed information about the acute effect of n-3 PUFA on the postprandial metabolic and inflammatory responses.


CHRONIC PHASE
3 months
supplementation
Placebo
(corn oil)
Fish oil
Visit 4

CHAPTER 7

The effect of chronic fish oil supplementation on fasting metabolic and inflammatory markers in normal weight and obese subjects



CHAPTER 7

The effect of chronic fish oil supplementation on fasting metabolic and inflammatory markers in normal weight and obese subjects

7.1 Introduction, objectives, hypothesis and aims

7.1.1 Introduction

Epidemiological studies have reported an association of long-term consumption of n-3 PUFA with the lowered risk of cardiovascular events and mortality (Kromhout *et al.* 1985; Shekelle *et al.* 1985; Dolecek *et al.* 1991). Furthermore intervention studies in patients with cardiovascular disease (CVD) have shown lower mortality with n-3 PUFA (GISSI, 1999; Yokoyama *et al.* 2007). Other trials have consistently reported the ability of n-3 PUFA to lower fasting plasma triglyceride concentrations in healthy normal weight people (Brown and Roberts, 1991; Egert *et al.* 2009), and in severely obese (Itariu *et al.* 2012), hypertriglyceridemic (Tinker *et al.* 1999; Krysiak *et al.* 2010; Maki *et al*. 2011; Koh *et al.* 2011; Derosa *et al.* 2011; Bosch *et al*. 2012; Derosa *et al.* 2012) and diabetic patients (Kabir *et al.* 2007; Rizza *et al.* 2009). Similarly, n-3 PUFA supplementation caused changes in fasting lipoprotein concentrations, including a reduction of VLDL (Tinker *et al.* 1999; Westphal *et al.* 2000) and chylomicrons (Brown and Roberts, 1991) and an increase in HDL (Egert *et al.* 2009; Clark *et al.* 2012; Bragt and Mensink, 2012; Derosa *et al.* 2012). Other studies have explored the effect of n-3 PUFA on glucose metabolism, although these studies report contradictory results (Flachs, 2014). Thus, the ability of n-3 PUFA to reduce plasma lipids is one mechanism underlying their beneficial effects on cardiovascular health.

The association of metabolic diseases with inflammation reported in epidemiological studies led to a number of clinical studies exploring the role of n-3 PUFA in this context (Pradhan *et al.* 2001; Cushman *et al.* 2005; Matthijs *et al.* 2006). In healthy overweight subjects (Albert *et al.* 2014) and in patients with cardiovascular risk (e.g. hypertriglyceridemic) (Derosa *et al.* 2006; Browing *et al.* 2006; Kelley *et al.* 2009) chronic

supplementation (3-4 months) with n-3 PUFA significantly reduced fasting plasma concentrations of CRP. In addition, these and other reports observed reductions in plasma and serum IL-6, TNF-α and GM-CSF in healthy overweight subjects (Zhang *et al.* 2012) and in hypertriglyceridemic (Kelley *et al.* 2009; Derosa *et al.* 2012) and heart failure patients (Moert *et al.* 2011). In contrast, studies in healthy normal weight and obese subjects (Chang, 2002) and hyperlipidemic patients (Koh *et al.* 2011; Chiang *et al.* 2012) reported no changes in plasma concentrations of these inflammatory markers. Clinical studies have also reported no changes in plasma concentrations of other cytokines including IL-10 and IL-8 after n-3 PUFA supplementation (Schubert *et al.* 2007; Pot *et al*. 2009; Roost *et al*. 2013). Some of the factors influencing the outcome of n-3 PUFA effects may be related to the supplementation duration, n-3 PUFA dose, subjects' genetic background and baseline inflammation levels (Calder, 2006).

The mechanisms involved in the anti-inflammatory effects of n-3 PUFA have been attributed to the ability to influence mononuclear cell proinflammatory cytokine production. Studies in healthy middle aged normal weight subjects observed that supplementation with fish oil (from 2-18 g/day) for 4-6 weeks resulted in a significant reduction in the ex-vivo production of TNF-α, IL-6 and IL-1β by PBMCs in basal conditions and in response to endotoxin (Endres *et al.* 1989; Caughey *et al.* 1996; Trebble *et al.* 2003; Wallace *et al.* 2003). The changes in the cell inflammatory profile were associated with the cells n-3 PUFA content (Caughey *et al.* 1996). Ex-vivo incubation of PBMCs with 100 μM of DHA and EPA increased IL-10 production and induced a switch in the lipid mediator production (↓ TXB_2 & LTB_4 while ↑ TXB_3 & LTB_5) (Jandszus *et al*. 2013). Thus, the incorporation of n-3 PUFA into the cell membrane modifies cell responses; this effect may be associated with a switch in lipid mediators production (Calder, 2014) and modulation of inflammatory signalling pathways (NF-κB, PPAR-Y, TLRs) (Shaikh, 2010; Calder, 2006).

Adhesion to and trans endothelial migration through the vascular wall, facilitated by adhesion molecules, is a key event in the development of atherosclerosis. *In vitro* studies in endothelial cells have reported a reduction in VCAM-1 and E-selectin expression in response to TNF-α and IL-1β when cells are pre treated with DHA (5-10 μM) (DeCaterina *et al.* 1995; Yates *et al.* 2011). Both, EPA and DHA (10 μM), induced suppression of

platelet activating factor (PAF) synthesis, monocyte rolling and adherence *in vitro* (Mayer *et al.* 2002). Human studies in elderly and middle age subjects have shown a reduction in systemic E-selectin and VCAM-1 after 12 weeks intervention with fish oil (1-1.2 g/day of EPA + DHA) (Miles *et al.* 2001; Thies *et al.* 2001; Derosa *et al.* 2012), while others have reported no changes in plasma VCAM-1, P-selectin and ICAM-1 after fish oil intervention (Damsgaard *et al.* 2008; Din *et al.* 2008; Pot *et al.* 2009; Bragt and Mensink, 2012). Moreover, authors have observed a consistent improvement in endothelial function (measured by FMD) (Koh *et al.* 2011; Moertl *et al.* 2011) and reduction in platelet monocyte and collagen aggregates (Woodman *et al.* 2003; Din *et al.* 2008). Thus, both *in vitro* and human studies suggest a cardioprotective effect of n-3 PUFA on plasma adhesion molecule concentrations and other markers associated with atherosclerosis.

Emerging evidence has revealed the role of n-3 PUFA in the regulation of adipose tissue derived molecules. Studies in normal weight and obese subjects, and patients with stable coronary artery disease (CAD) and triglyceridemia have reported an increase in circulating adiponectin after chronic supplementation with fish oil (1- 3 g fish oil / day for 1- 6 months) (Itoh *et al.* 2007; Kondo *et al.* 2010; Derosa *et al.* 2011; Mostowik *et al.* 2013;) while others observed a modest effect (Rizza *et al*. 2009) or no changes (Patel *et al.* 2007; Kratz *et al.* 2008; Damsgaard *et al.* 2008). Kondo e*t al.* (2010) reported an increase in plasma adiponectin concentrations only in women supplemented with fish oil, similarly other studies which included both male and female volunteers found an increment in adiponectin (Itoh *et al.* 2007; Rizza *et al.* 2009; Derosa *et al.* 2011) while conversely studies conducted only in men (healthy and myocardial infraction patients) reported no n-3 PUFA effect on plasma adiponectin concentrations (Patel *et al.* 2007; Damsgaard *et al.* 2008).

In CAD patients, fish oil supplementation induced a reduction of plasma leptin and resistin while increasing adiponectin concentrations and the adiponectin/leptin ratio (Mostowick *et al.* 2013). Derosa *et al* (2011) observed a similar pattern for these adipokines in hypertriglyceridemic patients. Nevertheless, the effect of n-3 PUFA on these molecules in healthy normal weight and obese subjects has not been studied in detail. MCP-1 concentrations in plasma and expression in adipose tissue were reduced

after fish oil supplementation in severely obese and diabetic patients (Spencer *et al.* 2013; Itariu *et al.* 2012). This effect was not observed in elderly normal weight and moderately obese subjects (Pot *et al.* 2009; Plat *et al.* 2009).

Overall, the role of fish oil in inflammation and metabolism has been explored by several authors. These studies agreed that chronic fish oil supplementation is followed by a significant reduction of plasma lipids (TGs), that may be explained by changes in LPL activity and lipoprotein metabolism. Some studies have reported a reduction of plasma inflammatory cytokines and adhesion molecules while reducing their *ex vivo* production. The effect of n-3 PUFA on plasma adipokines concentrations present conflicting results and requires further investigation. The differences in n-3 PUFA dose, patient characteristics, supplementation duration, age and gender are some factors that may impact the outcome of these n-3 PUFA on human health.

7.1.2 Aim

The aim of this research is to explore the effect of chronic n-3 PUFA supplementation on metabolic and inflammatory markers (including cytokines, adipokines and adhesion molecules) in healthy normal weight and obese subjects.

7.1.3 Hypothesis

Chronic supplementation with n-3 PUFAs will lower blood lipids and related analytes (glucose, insulin) and markers of inflammation in both normal weight and obese subjects.

7.1.4 Objectives

To evaluate the effect of a 12 week period of n-3 PUFA supplementation on the following markers in normal weight and obese subjects:

- fasting plasma triglycerides, cholesterol, non-esterified fatty acids, glucose and insulin;
- fasting plasma concentrations of molecules related to inflammation including cytokines, adipokines, adhesion molecules and growth factors;
- fasting TLR-2 and -4 expression on monocytes;
- *ex vivo* intracellular production of IL-6, TNF-α and IL-1β by monocytes

- *ex vivo* production of inflammatory cytokines in whole blood cultures.

7.2 Methods

Subject characteristics, treatment (capsules composition), the blood collection protocol, and the methods employed for the analysis of the metabolic profile, inflammatory molecules, adipokines and TLR expression are described in **Chapters 3.2 and 4.2**. The results reported in this chapter describe the changes in fasting metabolic and inflammatory markers in normal weight and obese subjects after 12 weeks supplementation with n-3 PUFA.

Subjects compliance was calculated based on the capsules returned by the volunteers after 12 weeks supplementation with either treatment as shown in the following formula:

(Number of capsules expected to be consumed)/(number of capsules dispensed – number of capsules returned)*100

Red blood cells fatty acid composition data were provided by Annette West and experimental work and data analysis for fatty acid analysis was performed essentially as described in **Chapter 2**.

The chronic effect of fish oil or placebo on fasting metabolic and inflammatory markers was evaluated using a paired t-test. The main effects of fish oil and placebo on the changes in fasting metabolic inflammatory markers were evaluated using 2-way ANOVA (fixed for age and sex). Metabolic data are expressed as mean and standard deviation. Due to the skewed distribution of the TLR data and other inflammatory variables, these data were log10 transformed before statistical analysis and data are expressed as median and percentiles.

7.3 Results

7.3.1 Subjects compliance after 12 weeks supplementation with n-3 PUFA

The average duration of the supplementation was 90 days and 75 % of the volunteers recruited finished the study. Compliance was evaluated based on the number of capsules returned and the n-3 PUFA incorporation into erythrocytes. Compliance was estimated to be 94% based on the number of capsules returned. The fatty acid profile analysis revealed a 4% increase of n-3 PUFA (before = 8.09%; after = 12.33% of total fatty acids) in erythrocytes at the expense of n-6 PUFA (before = 14.55%; after = 12.25% of total fatty acids) (A. West, unpublished results). A increase of n-3 PUFA on adipose tissue was also observed (A. West, unpublished results).

7.3.2 Changes in fasting metabolic markers after 12 weeks supplementation with n-3 PUFA

Changes in fasting plasma lipids were evaluated before and after each treatment. Fasting HDL cholesterol and HOMA %S were increased after fish oil supplementation in the normal weight group ($\rho<0.05$) (**Table 7.1a**). As HDL particles transfer cholesterol from tissue (including artery walls) to the liver this is a protective effect. Moreover, the increment of HOMA %S also represents a beneficial effect as it suggest a better insulin sensitivity. As shown in **Table 7.1b**, the significant changes observed in normal weight subjects were not extended to the obese group.

The changes after fish oil and placebo in metabolic markers are summarized in **Table 7.2**. 2 way ANOVA analysis revealed a significant treatment*group interaction for HDL and HOMA%S ($\rho<0.05$),

	Normal weight (n=35)					
	Fish oil			Placebo		
	Before	After	ρ-value	Before	After	ρ-value
Total Cholesterol	4.32 ± 1.08	4.36 ± 0.98	0.76	4.29 ± 0.71	4.40 ± 0.84	0.28
HDL Cholesterol	1.55 ± 0.48	1.67 ± 0.49	**<0.05**	1.56 ± 0.29	1.52 ± 0.25	0.45
LDL Cholesterol	2.60 ± 0.89	2.54 ± 0.81	0.49	2.58 ± 0.70	2.72 ± 0.76	0.12
Triglycerides	0.84 ± 0.37	0.72 ± 0.30	0.24	0.75 ± 0.26	0.76 ± 0.25	0.87
NEFA	449 ± 233	468 ± 267	0.79	500 ± 199	562 ± 247	0.39
Glucose	4.88 ± 0.54	4.84 ± 0.44	0.39	4.71 ± 0.41	4.87 ± 0.45	0.19
Insulin	5.49 ± 2.49	4.56 ± 2.53	0.25	6.99 ± 5.21	6.44 ± 2.56	0.74
HOMA %B	78.90 ± 31.02	69.05 ± 24.61	0.23	95.53 ± 49.42	88.69 ± 25.20	0.63
HOMA %S	168.29 ± 65.23	230.22 ± 138.38	**<0.05**	156.04 ± 77.44	139.16 ± 54.50	0.15
HOMA-IR	0.70 ± 0.33	0.59 ± 0.33	0.24	0.87 ± 0.66	0.83 ± 0.33	0.79
Data expressed as mean ± SD; all units are mmol/L, except NEFA (µmol/L) and insulin (µIU/mL); HOMA=homeostasis model assessment; HOMA IR = insulin resistance; HOMA %B= beta cell function; HOMA %S: insulin sensitivity. Differences in the fasting metabolic markers before and after fish oil or placebo supplementation were assessed using paired t test.						

Table 7.1a Fasting metabolic markers before and after 12 weeks supplementation with fish oil or placebo in normal weight subjects

Obese (n=40)						
Obese	**Fish oil**			**Placebo**		
	Before	**After**	**ρ-value**	**Before**	**After**	**ρ-value**
Total Cholesterol	5.56 ± 1.02	5.29 ± 1.11	0.28	4.92 ± 0.61	5.02 ± 0.72	0.26
HDL Cholesterol	1.54 ± 0.37	1.54 ± 0.40	0.63	1.39 ± 0.31	1.40 ± 0.27	0.76
LDL Cholesterol	3.73 ± 0.91	3.48 ± 0.85	0.30	3.28 ± 0.54	3.23 ± 0.76	0.45
Triglycerides	1.41 ± 0.78	1.34 ± 0.99	0.73	1.22 ± 0.52	1.39 ± 0.71	0.18
NEFA	584 ± 161	522 ± 172	0.10	621 ± 331	577 ± 191	0.55
Glucose	5.55 ± 1.12	5.76 ± 1.00	0.32	5.75 ± 1.31	5.66 ± 1.37	0.43
Insulin	11.25 ± 5.89	13.02 ± 6.23	0.11	16.39 ± 8.26	14.01 ± 7.67	0.12
HOMA %B	102.40 ± 45.81	102.80 ± 34.95	0.95	124.14 ± 44.84	115.59 ± 48.09	0.36
HOMA %S	91.83 ± 68.79	76.91 ± 50.26	0.13	57.76 ± 25.71	73.85 ± 45.87	0.06
HOMA-IR	1.48 ± 0.77	1.73 ± 0.83	0.09	2.16 ± 1.09	1.85 ± 1.01	0.11
Data expressed as mean ± SD; all units are mmol/L, except NEFA (µmol/L) and insulin (µIU/mL); HOMA=homeostasis model assessment; HOMA IR = insulin resistance; HOMA %B= beta cell function; HOMA %S: insulin sensitivity. Differences in the fasting metabolic markers before and after fish oil or placebo supplementation were assessed using paired t test.						

Table 7.1b Fasting metabolic markers before and after 12 weeks supplementation with fish oil or placebo in obese subjects

	Normal weight (n=35)		Obese (n=40)		Treatment ρ-value	Obesity ρ-value	Interaction ρ-value
	Δ Fish oil	**Δ Placebo**	**Δ Fish oil**	**Δ Placebo**			
Total Cholesterol	0.09 (-0.19-0.35)	0.14 (-0.08-0.32)	-0.08 (-0.52-0.23)	-0.02 (-0.15-0.31)	0.13	0.35	0.32
HDL Cholesterol	0.17 (-0.05-0.25)	-0.07 (-0.13-0.03)	-0.02 (-0.13-0.11)	0.00 (-0.05-0.13)	0.22	0.23	**<0.05**
LDL Cholesterol	-0.04 (-0.22-0.18)	0.15 (-0.02-0.32)	-0.07 (-0.38-0.15)	0.07 (-0.19-0.21)	0.46	0.44	0.22
Triglycerides	-0.03 (-0.24-0.01)	0.01 (-0.09-0.14)	-0.03 (-0.26-0.11)	0.18 (-0.08-0.46)	0.10	0.25	0.22
NEFA	13.74 (-53.72-74.25)	4.72 (-74.54-160.73)	-94.20 (-206.26-100.25)	-41.21 (-163.93-89.96)	0.48	0.10	0.39
%NEFA suppression	-2.00 (-16.00-10.66)	5.00 (-10.00-19.50)	-1.82 (-12.00-3.00)	-6.00 (-18.75-9.50)	0.48	0.59	0.86
Glucose	-0.04 (-0.46-0.21)	0.00 (-0.15-0.42)	-0.01 (-0.24-0.46)	-0.11 (-0.40-0.22)	0.88	0.97	0.26
Insulin	-0.74 (-3.26-0.81)	0.69 (-0.52-1.45)	1.34 (-0.90-4.39)	-1.65 (-3.40-0.77)	0.09	0.68	0.08
HOMA %B	-7.80 (-20.15-11.15)	7.20 (-8.90-17.75)	2.15 (-20.57-19.62)	-1.85 (-22.32-18.85)	0.72	0.62	0.86
HOMA %S	34.80 (-42.60-126.90)	-12.80 (-46.70-8.40)	-4.15 (-36.00-2.80)	4.25 (-3.00-20.90)	0.30	0.15	**<0.05**
HOMA-IR	-0.08 (-0.43-0.09)	0.07 (-0.07-0.19)	0.19 (-0.11-0.59)	-0.25 (-0.45-0.11)	0.18	0.67	0.09
Data expressed as median and percentile 25 and 75; main effects of treatment, obesity and their interaction were assessed by 2 way ANOVA							

Table 7.2 Changes in metabolic markers after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

7.3.3 Changes in fasting TLR expression on CD14+ cells after 12 weeks supplementation with n-3 PUFA in normal weight and obese subjects

After 12 weeks supplementation with fish oil or placebo the subjects fasting % of TLR positive cells and expression were evaluated. **Table 7.3a, 7.3b** and **7.4** show the TLR2 and TLR4 expression after fish oil supplementation and placebo; no significant changes in the % or the expression of TLR2 and TLR4 were detected.

	Normal weight					
	Fish oil (n=16)			Placebo (n=10)		
	Before	After	ρ-value	Before	After	ρ-value
% $CD14^+TLR2^+$	17.96 (8.37-39.83)	20.18 (7.23-32.56)	0.72	24.85 (11.65-38.34)	19.18 (8.56-55.52)	0.38
MFI $CD14^+TLR2^+$	61.29 (58.44-68.72)	62.52 (54.96-65.20)	0.66	60.85 (58.75-84.78)	57.06 (53.73-69.12)	0.06
% $CD14^+TLR4^+$	1.42 (0.39-6.41)	4.19 (2.64-8.13)	0.09	1.33 (0.31-7.11)	3.83 (2.93-7.76)	0.13
MFI $CD14^+TLR4^+$	152.29 (62.08-297.84)	66.50 (58.06-102.79)	**<0.05**	104.01 (83.81-248.55)	74.77 (69.48-148.65)	0.71

Table 7.3a TLR4 and TLR2 expression on $CD14^+$ monocytes before and after 12 weeks supplementation with fish oil or placebo in normal weight subjects

	Obese					
	Fish oil (n=16)			Placebo (n=20)		
	Before	After	p-value	Before	After	p-value
% $CD14^+TLR2^+$	16.52 (9.63-25.40)	9.12 (7.43-14.95)	0.08	15.58 (7.19-30.18)	12.24 (10.02-24.35)	0.97
MFI $CD14^+TLR2^+$	58.21 (57.16-63.12)	58.43 (53.70-68.45)	0.73	59.55 (57.17-60.82)	56.35 (55.48-60.36)	0.68
% $CD14^+TLR4^+$	7.03 (5.48-14.26)	9.14 (5.34-15.59)	0.60	8.51 (5.38-13.53)	7.13 (4.44-13.92)	0.74
MFI $CD14^+TLR4^+$	10.34 (65.51-282.11)	69.66 (60.79-189.83)	0.22	166.43 (91.69-383.12)	80.56 (67.01-301.21)	0.15
Data are expressed as median and percentile 25 and 75; data were log10 transformed and differences in the fasting TLRs before and after fish oil or placebo supplementation were assessed using paired t test.						

Table 7.3b TLR4 and TLR2 expression on $CD14^+$ monocytes before and after 12 weeks supplementation with fish oil or placebo in obese subjects

	Normal weight		Obese		Treatment ρ-value	Obesity ρ-value	Interaction ρ-value
	Δ Fish oil	**Δ Placebo**	**Δ Fish oil**	**Δ Placebo**			
% $CD14^+TLR2^+$	-0.38 (-14.45-19.69)	-6.16 (-10.90-0.15)	-3.63 (-13.78-1.89)	2.42 (-3.71-6.42)	0.78	0.69	0.62
MFI $CD14^+TLR2^+$	-1.71 (-4.77-6.82)	-3.61 (-27.66-(-0.92))	-0.06 (-4.35-3.66)	-0.69 (-3.98-1.99)	0.11	0.44	0.12
% $CD14^+TLR4^+$	0.93 (-1.49-4.69)	3.04 (-4.44-7.57)	0.65 (-3.92-4.85)	-1.1 (-4.05-5.37)	0.89	0.49	0.90
MFI $CD14^+TLR4^+$	-44.14 (-212.06-6.24)	-15.45 (-58.78-10.47)	-9.05 (-94.67-2.12)	-26.50 (-163.59-26.65)	0.45	0.77	0.89

Data are expressed as median and percentile 25 and 75; main effects of treatment, obesity and their interaction were assessed by 2 way ANOVA

Table 7.4 Changes in TLR4 and TLR2 expression on $CD14^+$ monocytes after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

7.3.4 Changes in fasting cytokine, adhesion molecule and obesity-related molecule concentrations in plasma in normal weight and obese subjects plasma cytokines after 12 weeks supplementation with n-3 PUFA

Chronic supplementation with fish oil was followed by changes in the plasma concentrations of some cytokines, adhesion molecules and adipokines in the obese and normal weight group. As shown in **Table 7.5a** and **7.5b**, plasma IL-10 concentrations were significantly lower following chronic fish oil consumption in obese subjects ($\rho<0.05$), while no changes in other cytokine concentrations were observed in either the normal weight or obese group.

Fasting plasma P-selectin and ICAM-1 concentrations were significantly reduced by fish oil supplementation in the normal weight group ($\rho<0.01$) (**Table 7.6a and 7.6b**). Similarly plasma adiponectin, CRP and adipsin concentrations were lower after fish oil supplementation in normal weight subjects ($\rho<0.05$) (**Table 7.7a** and **7.7b**).

Subsequent analysis revealed modest differences in treatment effect on inflammatory molecules (**Table 7.8**). When compared with placebo, fish oil induced a greater reduction in TNF-α and PAI-1 concentrations while inducing a lower suppression of plasma IL-10 concentrations ($\rho<0.01$).

	Normal weight					
	Fish oil (n=19)			Placebo (n=15)		
	Before	After	ρ-value	Before	After	ρ-value
IL-6 *(pg/mL)*	1.98 (0.85-2.53)	1.25 (0.52-2.37)	0.12	1.44 (0.78-1.57)	1.17 (0.60-2.00)	0.49
IL-8 *(pg/mL)*	4.84 (3.43-6.69)	3.82 (2.28-6.02)	0.17	3.77 (2.58-6.18)	3.95 (3.46-5.19)	0.42
GM-CSF *(pg/mL)*	1.11 (0.43-1.84)	0.67 (0.24-1.48)	0.28	1.34 (0.59-2.41)	1.20 (0.76-1.95)	0.49
TNF-α *(pg/mL)*	8.29 (5.03-9.17)	6.91 (4.13-9.01)	0.25	6.78 (5.34-9.64)	7.52 (4.80-12.21)	0.67
VEGF *(pg/mL)*	13.13 (9.27-15.18)	12.35 (7.52-18.31)	0.53	15.65 (14.01-25.72)	17.78 (12.11-26.42)	0.60
IL-10 *(pg/mL)*	0.66 (0.43-1.04)	0.65 (0.44-1.23)	0.95	0.50 (0.40-0.80)	0.76 (0.43-1.01)	0.65
	Data are expressed as median and percentile 25 and 75; data were log10 transformed and differences in the fasting cytokines before and after fish oil or placebo supplementation were assessed using paired t test					

Table 7.5a Fasting plasma cytokine concentrations before and after 12 weeks supplementation with fish oil or placebo in normal weight subjects

	Obese					
	Fish oil (n=20)			Placebo (n=20)		
	Before	**After**	**ρ-value**	**Before**	**After**	**ρ-value**
IL-6 *(pg/mL)*	2.24 (1.38-3.21)	2.06 (1.33-2.73)	0.61	2.28 (1.77-3.07)	2.82 (2.51-3.23)	0.71
IL-8 *(pg/mL)*	4.81 (3.16-5.92)	4.36 (2.61-5.69)	0.30	5.34 (4.28-6.52)	5.62 (3.45-6.27)	0.09
GM-CSF *(pg/mL)*	1.37 (0.52-2.85)	1.09 (0.49-2.43)	0.45	1.35 (0.51-2.86)	0.78 (0.49-1.36)	0.16
TNF-α *(pg/mL)*	8.49 (7.44-9.69)	8.02 (5.96-9.67)	0.35	7.10 (4.50-10.87)	6.19 (4.78-10.05)	0.59
VEGF *(pg/mL)*	16.06 (10.41-29.15)	16.04 (8.65-24.85)	0.32	19.26 (14.03-27.38)	19.60 (15.03-26.31)	0.82
IL-10 *(pg/mL)*	0.82 (0.42-0.91)	0.69 (0.26-0.85)	**<.0.05**	0.49 (0.33-0.78)	0.39 (0.29-0.69)	0.21
	Data are expressed as median and percentile 25 and 75; data were log10 transformed and differences in the fasting cytokines before and after fish oil or placebo supplementation were assessed using paired t test					

Table 7.5b Fasting plasma cytokine concentrations before and after 12 weeks supplementation with fish oil or placebo in obese subjects

	Normal weight					
	Fish oil (n=19)			Placebo (n=15)		
	Before	**After**	**ρ-value**	**Before**	**After**	**ρ-value**
P-Selectin *(ng/mL)*	56.42 (45.80-68.63)	44.97 (35.20-69.98)	**<0.01**	59.86 (48.87-73.18)	51.70 (47.15-67.40)	0.14
ICAM-1 *(ng/mL)*	242.03 (182.34-311.34)	187.32 (173.20-291.25)	**<0.01**	232.23 (198.30-334.31)	234.29 (177.75-248.21)	0.06
VCAM-1 *(ng/mL)*	802.44 (625.88-869.28)	725.86 (592.39-845.14)	0.10	874.91 (584.33-1097.17)	660.26 (577.25-953.17)	0.07
E-Selectin *(ng/mL)*	35.88 (28.49-46.27)	31.54 (21.57-44.26)	0.19	34.79 (27.80-41.65)	34.17 (23.83-45.94)	0.11
Data are expressed as median and percentile 25 and 75; data were log10 transformed and differences in the fasting adhesion molecules before and after fish oil or placebo supplementation were assessed using paired t test						

Table 7.6a Fasting plasma adhesion molecule concentrations before and after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

	Obese					
	Fish oil (n=20)			Placebo (n=20)		
	Before	**After**	**ρ-value**	**Before**	**After**	**ρ-value**
P-Selectin *(ng/mL)*	58.45 (50.96-68.89)	56.50 (47.70-75.45)	0.57	51.25 (46.17-62.54)	61.05 (53.07-70.85)	0.08
ICAM-1 *(ng/mL)*	253.31 (232.44-305.34)	259.92 (215.06-299.82)	0.98	239.56 (195.96-282.58)	234.42 (199.77-270.86)	0.97
VCAM-1 *(ng/mL)*	469.51 (421.27-513.65)	464.07 (384.79-553.19)	0.92	426.57 (389.70-553.24)	430.24 (397.34-542.51)	0.31
E-Selectin *(ng/mL)*	39.47 (30.09-46.26)	41.10 (30.05-46.98)	0.56	33.13 (25.30-41.16)	36.51 (26.39-49.08)	0.15
	Data are expressed as median and percentile 25 and 75; data were log10 transformed and differences in the fasting adhesion molecules before and after fish oil or placebo supplementation were assessed using paired t test					

Table 7.6b Fasting plasma adhesion molecule concentrations before and after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

	Normal weight					
	Fish oil (n=19)			Placebo (n=15)		
	Before	**After**	**ρ-value**	**Before**	**After**	**ρ-value**
Adiponectin *(µg/mL)*	80.49 (58.48-94.43)	62.36 (58.46-77.98)	**<0.01**	88.75 (63.72-148.06)	111.49 (59.60-146.20)	0.71
CRP *(µg/mL)*	10.27 (3.48-24.53)	5.38 (2.66-7.29)	**<0.05**	7.29 (4.08-14.58)	8.29 (3.50-15.88)	0.82
Adipsin *(µg/mL)*	29.67 (25.32-34.31)	26.03 (18.51-28.36)	**<0.01**	29.55 (22.05-41.21)	24.62 (18.69-36.40)	0.14
MCP-1 *(pg/mL)*	168.95 (147.80-198.62)	168.40 (129.75-180.70)	0.13	153.73 (128.29-185.97)	138.63 (120.70-162.12)	0.07
Leptin *(ng/mL)*	8.63 (2.52-19.45)	7.66 (2.30-19.72)	0.84	12.67 (8.44-16.07)	14.33 (9.31-17.86)	092
Resistin *(ng/mL)*	5.33 (4.93-7.03)	5.72 (4.76-7.19)	0.42	6.56 (4.98-7.29)	6.72 (4.45-7.81)	0.24
PAI-1 *(ng/mL)*	19.86 (15.90-24.69)	18.02 (12.45-26.69)	0.62	18.63 (14.61-23.77)	20.91 (18.67-25.85)	0.58

Data are expressed as median and percentile 25 and 75; data were log10 transformed and differences in the fasting adipokines before and after fish oil or placebo supplementation were assessed using paired t test

Table 7.7a Fasting plasma adipokine concentrations before and after 12 weeks supplementation with fish oil or placebo in normal weight subjects

	Obese					
	Fish oil (n=20)			Placebo (n=20)		
	Before	After	ρ-value	Before	After	ρ-value
Adiponectin *(µg/mL)*	54.70 (40.68-82.79)	58.92 (39.68-81.29)	0.88	46.59 (39.78-61.48)	52.47 (40.44-62.44)	0.25
CRP *(µg/mL)*	25.21 (20.56-46.46)	22.23 (10.63-50.68)	0.11	23.88 (14.07-54.71)	21.79 (14.97-42.22)	0.93
Adipsin *(µg/mL)*	25.83 (22.95-31.78)	27.20 (21.08-28.54)	0.26	23.16 (20.35-29.19)	24.48 (20.65-28.56)	0.44
MCP-1 *(pg/mL)*	144.86 (120.45-185.90)	163.39 (124.30-181.59)	0.70	150.36 (134.79-176.68)	162.69 (129.21-178.34)	0.32
Leptin *(ng/mL)*	49.20 (27.19-57.91)	37.78 (27.69-59.28)	0.23	44.68 (35.59-73.20)	48.56 (38.40-67.35)	0.33
Resistin *(ng/mL)*	5.35 (3.65-6.69)	4.88 (3.82-6.61)	0.45	6.49 (5.28-9.16)	6.98 (5.15-8.81)	0.44
PAI-1 *(ng/mL)*	25.91 (22.94-61.87)	26.66 (17.88-45.50)	0.49	28.61 (22.34-35.39)	29.42 (21.58-45.90)	0.13

Data are expressed as median and percentile 25 and 75; data were log10 transformed and differences in the fasting adipokines before and after fish oil or placebo supplementation were assessed using paired t test

Table 7.7b Fasting plasma adipokine concentrations before and after 12 weeks supplementation with fish oil or placebo in obese subjects

		Normal weight (n=35)		Obese (n=40)		Treatment *p-value*	Obesity *p-value*	Interaction *p-value*
		Δ Fish oil	**Δ Placebo**	**Δ Fish oil**	**Δ Placebo**			
Cytokines								
	IL-6	0.14 (-0.95-0.31)	0.78 (-0.14-0.23)	0.18 (-0.29-0.52)	-0.09 (-0.94-0.57)	0.72	0.73	0.87
	IL-8	0.51 (-1.79-0.65)	-0.44 (-1.18-0.25)	0.21 (-1.28-0.15)	-0.40 (-1.07-0.06)	0.39	0.21	0.71
	GM-CSF	-0.09 (-0.44-0.16)	-0.06 (-0.47-0.55)	-0.13 (-0.40-0.01)	-0.11 (-0.59-0.13)	0.97	0.24	0.13
	TNF-α	0.30 (-1.94-0.72)	0.45 (-0.78-1.64)	0.70 (-1.79-0.44)	-0.93 (-1.92-1.34)	**<0.01**	0.61	0.07
	VEGF	1.72 (-4.23-3.08)	-1.59 (-5.68-4.26)	2.25 (-6.71-1.46)	-0.06 (-4.18-5.18)	0.48	0.25	0.56
	IL-10	0.04 (-0.12-0.21)	0.11 (-0.24-0.22)	0.16 (-0.24-0.03)	-0.12 (-0.19-[-2.77])	**<0.01**	**<0.01**	**<0.01**
Adhesion Molecules								
	P-Selectin	-11.19 (-17.52-0.55)	-4.53 (-11.35-5.68)	5.46 (-7.27-7.61)	5.72 (-4.41-14.88)	0.78	**<0.01**	0.49
	ICAM-1	-22.22 (-47.34-[-3.12])	-18.92 (-77.59-6.86)	0.69 (-33.02-25.87)	-9.27 (-28.39-16.57)	0.40	**<0.05**	0.98
	VCAM-1	-32.94 (-128.11-1.11)	-49.00 (-262.01-9.92)	25.93 (-102.58-58.80)	-6.93 (-79.63-49.25)	0.76	0.42	0.13
	E-Selectin	1.17 (-6.43-2.71)	-4.23 (-7.77-3.13)	0.10 (-2.84-2.26)	0.95 (-1.34-5.70)	0.99	0.33	0.85
Adipokines								
	Adiponectin	1.93 (-2.35-0.17)	0.65 (-1.54-1.14)	-0.07 (-0.67-0.78)	0.11 (-0.39-1.15)	0.20	0.15	0.29
	CRP	0.17 (-0.92-0.07)	-0.08 (-0.29-0.48)	-0.41 (-0.85-1.21)	0.06 (-1.01-1.24)	0.08	0.22	0.89
	Adipsin	0.68 (-1.05-[-0.03])	-0.21 (-0.52-0.03)	-0.07 (-0.45-0.26)	0.06 (-0.15-0.23)	0.40	0.18	0.94
	MCP-1	-22.51 (-37.26-18.46)	-18.35 (-51.17-7.06)	1.34 (-16.86-21.55)	7.12 (-19.05-27.63)	0.82	0.04	0.44
	Leptin	0.22 (-1.28-2.37)	0.83 (-2.63-5.82)	1.85 (-9.71-6.22)	5.92 (-3.84-16.32)	0.94	0.67	0.33
	Resistin	0.17 (-0.55-0.84)	-0.31 (-1.66-1.31)	-0.27 (-0.62-0.17)	0.06 (-0.79-1.44)	0.88	0.81	0.26
	PAI-1	0.10 (-4.93-4.91)	2.77 (-5.17-6.51)	0.58 (-5.97-4.30)	3.77 (-0.18-8.93)	**<0.01**	**<0.01**	**<0.01**

Data are expressed as median and percentile 25 and 75; main effects of treatment, obesity and their interaction were assessed by 2 way ANOVA

Table 7.8 Changes in fasting plasma cytokines, adhesion molecules and adipokines concentrations after 12 weeks supplementation with fish oil and placebo in normal weight and obese subjects

7.3.5 Changes in ex-vivo production of TNF-α, IL-1β and IL-6 by CD14+ monocytes from obese and normal weight subjects after 12 weeks supplementation with n-3 PUFA

The % and expression of IL-1β, TNF-α and IL-6 in CD14+ monocytes before and after chronic fish oil supplementation are summarized in **Table 7.9** and **Table 7.10**. Fish oil supplementation resulted in a significant reduction of the %$CD14^{+}IL\text{-}1^{+}$ cells in the basal state in obese subjects ($\rho<0.01$) (**Table 7.10a**). Moreover, placebo reduced the expression of TNF in CD14+ cells in the basal state in the obese group and increased the expression of TNF in CD14+ monocytes in response to LPS ($\rho<0.05$) (**Table 7.10a** and **7.10b**).

The changes in % and MFI OF CD14+IL-1β+ monocytes after in the basal state after supplementation were different between treatments. Fish oil supplementation induced a significant reduction of the basal % and MFI of CD14+IL-1β+ monocytes compared with placebo supplementation (**Table 7.11**).

		Normal weight					
		Basal					
		Fish oil (n=5)			Placebo (n=6)		
		Before	After	ρ-value	Before	After	ρ-value
CD14⁺ IL-1β⁺	**%**	1.59 (1.13-2.14)	2.06 (1.01-4.41)	0.87	2.74 (1.89-3.15)	3.46 (2.01-17.03)	0.27
	MFI	221.15 (218.16-228.78)	205.80 (202.25-314.21)	0.53	202.27 (187.94-205.28)	207.69 (198.54-217.49)	0.37
CD14⁺ IL-6⁺	**%**	2.04 (1.77-3.20)	0.93 (0.88-2.56)	0.08	1.06 (1.03-2.61)	1.17 (0.72-4.82)	0.89
	MFI	43.59 (33.94-49.30)	44.03 (41.19-100.71)	0.25	33.90 (32.39-38.61)	53.89 (43.54-78.15)	0.38
CD14⁺ TNF-α⁺	**%**	3.94 (3.92-4.70)	2.26 (1.94-4.55)	0.44	5.12 (3.96-7.44)	5.13 (3.12-7.44)	0.75
	MFI	72.69 (63.77-143.99)	118.60 (72.35-249.94)	0.42	94.37 (76.43-114.22)	106.87 (82.34-115.71)	0.63

Data are expressed as median and percentile 25 and 75; data were log10 transformed and differences before and after fish oil or placebo supplementation were assessed using paired t test

Table 7.9a Intracellular cytokine production by $CD14^+$ monocytes from whole blood cultured for 6 h with or without LPS before and after 12 weeks supplementation with fish oil or placebo (normal weight subjects).

		Normal weight					
		LPS					
		Fish oil (n=5)			Placebo (n=6)		
		Before	After	ρ-value	Before	After	ρ-value
CD14$^+$ IL-1β$^+$	**%**	82.07 (75.45-84.67)	88.98 (87.60-92.05)	0.72	94.67 (76.98-95.73)	96.98 (96.63-97.48)	0.37
	MFI	495.88 (457.45-535.30)	609.19 (560.83-739.03)	0.11	581.96 (497.22-589.51)	520.93 (450.09-634.52)	0.93
CD14$^+$ IL-6$^+$	**%**	64.47 (59.26-79.38)	86.33 (85.27-88.85)	0.81	83.47 (68.29-89.05)	94.05 (87.61-94.20)	0.45
	MFI	81.16 (76.12-96.87)	84.76 (74.15-97.02)	0.82	75.55 (64.08-132.26)	66.56 (60.63-89.35)	0.68
CD14$^+$ TNF-α$^+$	**%**	76.83 (76.19-83.56)	89.84 (78.80-91.47)	0.65	87.55 (79.19-96.67)	96.54 (83.81-97.50)	0.35
	MFI	668.16 (488.23-794.83)	569.05 (464.43-832.00)	0.65	770.66 (746.01-942.770	748.00 (486.39-1024.92)	0.46
		Data are expressed as median and percentile 25 and 75; data were log10 transformed and differences before and after fish oil or placebo supplementation were assessed using paired t test					

Table 7.9b Intracellular cytokine production by CD14$^+$ monocytes from whole blood cultured for 6 h with or without LPS before and after 12 weeks supplementation with fish oil or placebo (normal weight subjects).

		Obese					
		Basal					
		Fish oil (n=14)			Placebo (n=10)		
		Before	After	ρ-value	Before	After	ρ-value
CD14+ IL-1β+	**%**	6.37 (2.54-7.08)	2.02 (1.67-4.31)	**<0.01**	2.59 (1.64-4.33)	2.75 (1.06-6.61)	0.15
	MFI	208.76 (171.05-231.10)	193.86 (183.74-219.51)	0.61	229.61 (204.22-242.28)	214.51 (170.98-227.42)	0.44
CD14+ IL-6+	**%**	2.69 (1.93-3.77)	1.01 (0.60-1.99)	0.14	2.63 (1.29-3.58)	1.37 (0.65-2.52)	0.14
	MFI	58.45 (36.15-78.82)	42.40 (35.90-92.44)	0.78	60.98 (41.05-89.68)	48.54 (35.39-61.36)	0.79
CD14+ TNF-α+	**%**	7.32 (5.82-10.69)	4.77 (3.33-7.42)	0.11	6.32 (3.25-7.68)	4.27 (3.09-5.86)	0.17
	MFI	83.52 (51.99-146.18)	65.29 (52.36-88.96)	0.87	118.01 (81.62-350.35)	79.32 (49.23-114.83)	**<0.04**

Data are expressed as median and percentile 25 and 75; data were log10 transformed and differences before and after fish oil or placebo supplementation were assessed using paired t test

Table 7.10a Intracellular cytokine production by $CD14^+$ monocytes from whole blood cultured for 6 h with or without LPS before and after 12 weeks supplementation with fish oil or placebo (obese subjects).

		Obese					
		LPS					
		Fish oil (n=14)			Placebo (n=10)		
		Before	After	ρ-value	Before	After	ρ-value
CD14+ IL-1β+	**%**	79.04 (37.15-87.15)	84.98 (69.11-91.01)	0.52	71.09 (55.02-86.12)	83.79 (72.35-88.81)	0.12
	MFI	680.17 (474.67-757.77)	618.12 (467.69-723.46)	0.63	673.55 (608.11-852.61)	664.00 (501.06-725.98)	0.16
CD14+ IL-6+	**%**	70.19 (36.52-83.42)	71.04 (50.48-84.79)	0.23	62.55 (47.48-73.56)	73.52 (68.24-82.730	0.23
	MFI	90.47 (70.10-96.90)	53.68 (44.65-100.76)	0.12	115.09 (94.54-126.89)	75.99 (70.83-92.74)	0.12
CD14+ TNF-α+	**%**	54.22 (34.62-90.18)	87.43 (67.76-92.13)	0.27	68.31 (56.46-80.99)	85.33 (80.19-93.28)	**<0.01**
	MFI	509.11 (353.11-623.37)	343.85 (256.44-569.91)	0.31	636.56 (423.30-806.59)	578.46 (415.79-664.53)	0.36
		Data are expressed as median and percentile 25 and 75; data were log10 transformed and differences before and after fish oil or placebo supplementation were assessed using paired t test					

Table 7.10b Intracellular cytokine production by $CD14^{+}$ monocytes from whole blood cultured for 6 h with or without LPS before and after 12 weeks supplementation with fish oil or placebo (obese subjects).

		Basal						
		Normal weight		Obese		Treatment ρ-value	Obesity ρ-value	Interaction ρ-value
		Δ Fish oil	Δ Placebo	Δ Fish oil	Δ Placebo			
$CD14^+IL\text{-}1\beta^+$	**%**	-0.08 (-0.12-1.81)	13.88 (-0.20-42.03)	-2.67 (-4.35-0.15)	-0.41 (-1.21-0.22)	**<0.01**	**0.01**	**0.02**
	MFI	-22.98 (-35.67-127.35)	29.56 (14.04-128.24)	-14.95 (-32.39-3.80)	-16.18 (-42.91-11.18)	**<0.02**	0.57	0.28
$CD14^+IL\text{-}6^+$	**%**	-0.89 (-1.11-(-0.43))	-0.80 (-1.90-3.34)	-1.23 (-2.76-0.22)	-1.10 (-1.56-0.06)	0.65	0.42	0.29
	MFI	7.25 (-5.27-47.78)	19.99 (4.93-45.77)	-3.32 (-19.70-3.43)	-0.44 (-21.52-10.10)	0.24	0.58	0.49
$CD14^+TNF\text{-}\alpha^+$	**%**	-1.68 (-2.45-0.96)	-2.61 (-4.57-8.58)	-2.28 (-5.37-(-1.92))	-2.84 (-3.59-0.66)	0.25	0.48	0.67
	MFI	45.91 (-22.90-209.40)	-3.25 (-6.47-16.50)	0.14 (-25.76-9.12)	-37.33 (-181.99-(-17.91))	0.92	0.31	0.48
Data are expressed as median and percentile 25 and 75; main effects of treatment, obesity and their interaction was assessed by 2 way ANOVA								

Table 7.11a Changes in intracellular cytokine production by $CD14^+$ monocytes from whole blood cultured for 6 h in the basal state after 12 weeks supplementation with fish oil or placebo (normal weight and obese subjects).

		LPS						
		Normal weight		Obese		Treatment ρ-value	Obesity ρ-value	Interaction ρ-value
		Δ Fish oil	Δ Placebo	Δ Fish oil	Δ Placebo			
$CD14^+IL\text{-}1\beta^+$	**%**	5.49 (2.93-6.91)	1.16 (-0.13-11.41)	1.51 (-6.27-14.81)	17.13 (-0.16-25.53)	0.61	0.61	0.77
	MFI	111.55 (19.98-203.73)	-16.62 (-40.18-37.76)	-5.18 (-62.05-71.32)	-125.59 (-236.91-66.77)	0.13	0.36	0.81
$CD14^+IL\text{-}6^+$	**%**	9.91 (4.98-27.07)	-0.28 (-1.29-20.34)	2.86 (-10.74-25.45)	24.18 (7.68-30.64)	0.65	0.58	0.65
	MFI	-2.25 (-11.48-11.16)	2.08 (-60.17-19.34)	-12.97 (-44.77-5.52)	-27.16 (-44.62-(-10.88))	0.58	0.29	0.68
$CD14^+TNF\text{-}\alpha^+$	**%**	7.91 (-11.56-13.65)	0.56 (0.52-20.02)	4.68 (-6.04-30.14)	16.42 (11.43-29.36)	0.15	0.56	0.72
	MFI	58.16 (-99.11-543.77)	-82.63 (-358.02-121.97)	-161.77 (-208.36-133.65)	-46.03 (-134.13-33.32)	0.31	0.36	0.35
Data are expressed as median and percentile 25 and 75; main effects of treatment, obesity and their interaction were assessed by 2 way ANOVA								

Table 7.11b Changes in intracellular cytokine production by $CD14^+$ monocytes from whole blood cultured for 6 h with LPS after 12 weeks supplementation with fish oil or placebo (normal weight and obese subjects).

7.3.6 Changes in ex-vivo production of cytokines from fasting whole blood samples after 12 weeks supplementation with n-3 PUFA

Table 7.12 and **7.13** show the changes induced by 12 weeks supplementation with fish oil or placebo in the *ex vivo* production of cytokines from fasting whole blood treated with or without LPS. In the basal state, IL-6 and IL-8 *ex vivo* production from fasting whole blood was lower in the obese group treated with fish oil for 12 weeks ($\rho<0.01$ and $\rho<0.01$, respectively) (**Table 7.13a**). In contrast, chronic supplementation with the placebo significantly increased the production of IL-1β and TNF-α in response to LPS, in normal weight and obese subjects, respectively.

Normal weight						
Basal						
pg/mL	Fish oil (n=11)		ρ-value	Placebo (n=9)		ρ-value
	Before	After		Before	After	
IL-2	0.00 (0.00-134.54)	0.00 (0.00-131.08)	0.96	87.07 (0.00-120.33)	97.49 (0.00-162.98)	0.73
IL-10	0.00 (0.000.00)	0.00 (0.00-0.00)	0.65	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.31
IL-8	0.00 (0.00-3.21)	0.00 (0.00-36.80)	0.75	0.00 (0.00-0.00)	0.00 (0.00-69.87)	0.68
IL-6	0.00 (0.00-2.82)	0.00 (0.00-3.99)	0.89	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.31
IL-4	0.00 (0.00-35.65)	0.00 (0.00-12.97)	0.73	32.68 (0.00-66.23)	44.17 (41.11-49.28)	0.32
IL-1β	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.31	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.99
TNF-α	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.18	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.31
Data are expressed as median and percentile 25 and 75; units=pg/mL; data were log10 transformed and differences before and after fish oil or placebo supplementation were assessed using paired t test						

Figure 7.12a Ex-vivo production of cytokines from fasting whole blood without LPS (basal) stimulation for 24 h from normal weight subjects before and after fish oil or placebo supplementation

Normal weight						
LPS						
pg/mL	Fish oil (n=11)		ρ-value	Placebo (n=9)		ρ-value
	Before	**After**		**Before**	**After**	
IL-2	0.00 (0.00-195.49)	46.35 (0.00-175.13)	0.99	0.00 (0.00-172.79)	0.00 (0.00-199.11)	0.71
IL-10	215.33 (192.22-407.18)	304.79 (234.75-490.75)	0.09	210.64 (195.89-515.25)	441.17 (391.09-486.43)	0.13
IL-8	6040.95 (5654.02-6990.61)	5206.48 (4932.51-6471.91)	0.72	8131.18 (5717.34-8828.52)	6873.56 (4959.06-7002.50)	0.59
IL-6	6579.70 (5497.40-7387.590	5641.51 (4904.92-7016.64)	0.24	5631.52 (4721.02-7977.32)	6951.28 (5881.53-7262.87)	0.26
IL-4	0.00 (0.00-43.18)	0.00 (0.00-53.48)	0.34	0.00 (0.00-68.26)	18.76 (0.00-59.01)	0.91
IL-1β	2376.12 (2183.14-2751.71)	2140.79 (1954.83-2884.59)	0.59	2255.04 (2059.25-2329.68)	2658.71 (2368.36-2767.59)	**<0.05**
TNF-α	2734.16 (1383.57-5021.82)	5642.73 (3097.99-9502.27)	0.11	2615.79 (2270.57-7040.54)	7324.71 (5284.19-10808.90)	0.11
Data are expressed as median and percentile 25 and 75; units=pg/mL; data were log10 transformed and differences before and after fish oil or placebo supplementation were assessed using paired t test						

Table 7.12b *Ex-vivo* production of cytokines from fasting whole blood with LPS stimulation for 24 h from normal weight subjects before and after fish oil or placebo supplementation

Obese						
Basal						
	Fish oil (n=19)			**Placebo (n=19)**		
pg/mL	**Before**	**After**	**ρ-value**	**Before**	**After**	**ρ-value**
IL-2	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.89	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.65
IL-10	0.00 (0.00-0.72)	0.00 (0.0-2.27)	0.31	0.00 (0.00-2.69)	0.00 (0.00-0.00)	0.17
IL-8	26.98 (0.00-180.50)	0.00 (0.00-67.16)	**<0.05**	25.65 (0.00-157.5)	0.00 (0.00-22.54)	0.06
IL-6	6.78 (0.00-16.03)	0.00 (0.00-5.53)	**<0.01**	0.00 (0.00-6.62)	0.00 (0.00-0.00)	0.09
IL-4	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.28	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.99
IL-1β	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.08	0.00 (0.00-2.93)	0.00 (0.00-0.00)	0.24
TNF-α	0.00 (0.00-0.00)	0.00 (0.00-0.59)	0.22	0.00 (0.00-10.88)	0.00 (0.00-0.00)	0.17
Data are expressed as median and percentile 25 and 75; units= pg/mL; data were log10 transformed and differences before and after fish oil or placebo supplementation were assessed using paired t test						

Table 7.13a Ex-vivo production of cytokines from fasting whole blood without LPS (basal) stimulation for 24 h from obese subjects before and after fish oil or placebo supplementation

Obese						
LPS						
	Fish oil (n=19)			Placebo (n=19)		
pg/mL	**Before**	**After**	**ρ-value**	**Before**	**After**	**ρ-value**
IL-2	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.27	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.31
IL-10	421.82 (225.91-708.90)	599.69 (297.67-901.34)	0.22	538.32 (228.15-1084.45)	572.78 (330.42-843.40)	0.61
IL-8	5460.17 (4197.20-5912.49)	4736.71 (3819.55-5200.04)	0.26	4875.16 (4615.03-5606.44)	4846.60 (4069.52-5045.18)	0.47
IL-6	6517.00 (5492.12-7748.66)	6955.14 (5719.09-7323.43)	0.61	6481.35 (4975.28-7777.06)	6537.26 (5558.65-7366.17)	0.90
IL-4	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.32	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.99
IL-1β	2155.83 (1981.95-2586.22)	2065.16 (1849.89-2344.76)	0.13	1922.00 (1644.85-2105.69)	2056.11 (1511.83-2198.76)	0.65
TNF-α	4061.25 (2470.74-4964.35)	4210.24 (3315.39-7080.42)	0.58	2217.23 (1845.85-3102.48)	2988.26 (2007.71-4195.38)	**<0.05**
Data are expressed as median and percentile 25 and 75; units= pg/mL; data were log10 transformed and differences before and after fish oil or placebo supplementation were assessed using paired t test						

Table 7.13b *Ex-vivo* production of cytokines from fasting whole blood with stimulation for 24 h from obese subjects before and after fish oil or placebo supplementation

	Basal						
					Treatment ρ-value	Obesity ρ-value	Interaction ρ-value
	Normal weight		Obese				
	Δ Fish oil	Δ Placebo	Δ Fish oil	Δ Placebo			
IL-2	0.00 (-134.54-131.08)	0.00 (-14.12-82.64)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.92	0.43	0.37
IL-10	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (-0.38-0.00)	0.00 (-1.17-0.00)	0.23	0.62	0.49
IL-8	0.00 (-3.22-5.26)	0.00 (0.00-57.34)	0.00 (-138.25-0.00)	0.00 (-118.70-0.00)	0.97	0.62	0.24
IL-6	0.00 (-0.45-0.00)	0.00 (0.00-0.00)	-3.23 (-14.49-0.00)	0.00 (-2.91-0.00)	0.14	0.43	0.74
IL-4	0.00 (-35.62-12.97)	5.96 (0.00-41.11)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.94	0.48	0.27
IL-1β	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.14	0.8	0.78
TNF-α	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (-6.25-0.00)	0.17	0.51	0.68
Data are expressed as median and percentile 25 and 75; main effects of treatment, obesity and their interaction was assessed by 2 way ANOVA							

Table 7.14a Changes in *ex-vivo* production of cytokines from fasting whole blood without LPS (basal) stimulation for 24 h from normal weight and obese subjects before and after fish oil or placebo supplementation

	LPS						
					Treatment ρ-value	Obesity ρ-value	Interaction ρ-value
	Normal weight		Obese				
	Δ Fish oil	Δ Placebo	Δ Fish oil	Δ Placebo			
IL-2	0.00 (-31.71-23.17)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.75	0.88	0.78
IL-10	114.70 (8.27-295.46)	121.93 (36.50-245.28)	47.57 (-146.31-249.15)	-19.01 (-185.06-115.82)	0.32	0.28	0.53
IL-8	273.85 (-1537.54-470.96)	-758.28 (-1330.30-824.99)	-380.90 (-1256.75-412.90)	0.00 (-1473.74-561.64)	0.55	0.51	0.53
IL-6	-551.54 (-1863.10-705.35)	552.14 (339.79-1202.58)	-117.11 (-1944.24-931.98)	-217.71 (-1111.33-1300.90)	0.66	0.22	0.62
IL-4	0.00 (0.00-10.30)	0.00 (-9.25-18.76)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.76	0.52	0.85
IL-1β	-250.97 (-330.14-300.03)	329.03 (208.72-529.65)	-128.79 (-395.41-40.76)	49.16 (-249.66-300.31)	0.19	**<0.05**	0.63
TNF-α	2258.19 (-676.65-5829.99)	2691.45 (140.34-6670.58)	-791.63 (-1943.41-2638.94)	656.52 (-65.06-2517.12)	**<0.05**	0.68	0.74

Data are expressed as median and percentile 25 and 75; main effects of treatment, obesity and their interaction was assessed by 2 way ANOVA

7.14b Changes in *ex-vivo* production of cytokines from fasting whole blood with LPS stimulation for 24 h from normal weight and obese subjects before and after fish oil or placebo supplementation

7.4 Discussion

Chronic consumption of n-3 PUFA has been associated with beneficial effects for human health. Early studies demonstrated that chronic consumption of these fatty acids was associated with an improvement in metabolic markers in patients with metabolic conditions (Brown and Roberts, 1991; Egert *et al.* 2009). N-3 PUFA has been also associated with changes in the synthesis of inflammatory mediators *ex vivo* by mononuclear cells (Endres *et al.* 1989; Caughey *et al.* 1996; Trebble *et al.* 2003; Wallace *et al*. 2003). Even though there is strong evidence in the literature about the link of fish oil with human health, only a few studies have explored the changes in metabolic and inflammatory markers in response to chronic supplementation with fish oil in normal weight and obese subjects. The results presented in this chapter provided robust information about the modulation of metabolism and inflammation, as well as recently characterised molecules involved in the regulation of human health by fish oil. Chronic supplementation with fish oil resulted in different effects on the normal weight and obese subjects metabolic and inflammatory markers, as summarized in **Table 7.15**.

The findings reported in this chapter regarding the effect of chronic fish oil supplementation on metabolic markers are in agreement with previous reports. In normal weight subjects, chronic fish oil consumption significantly increased the plasma HDL cholesterol and HOMA %S.

The increment on HDL cholesterol is consistent with previous studies exploring the effect of fish oil on metabolism in healthy normolipemic adults (Egert *et al*. 2009) and in subjects with features of metabolic syndrome (Silvkoff-Clark *et al.* 2012; Derosa *et al.* 2012; Bragt and Mensink, 2012). The modulation of lipoprotein metabolism by fish oil, as described in section 7.1, may explain the changes in plasma HDL cholesterol concentrations induced by fish oil supplementation.

Clinical studies exploring the role of fish oil in insulin sensitivity have reported inconsistent results (Kabir *et al.* 2007; Tsitouras *et al*.2008; Ramel *et al.* 2008). In this study a significant increase in HOMA %S was detected in normal weight subjects after fish oil supplementation. Moreover, an unexpected reduction in plasma adiponectin concentration was detected while others have reported an increase of adiponectin in plasma after fish oil supplementation. In addition, adipsin concentrations were reduced

after fish oil supplementation. To date the role of this adipokine in beta cell function has not been elucidated in humans. However, based on this series of observations the precise role of fish oil in glucose homeostasis is unclear

Normal weight subjects		Obese subjects		Both groups
Reduced after FO	**Increased after FO**	**Reduced after FO**	**Increased after FO**	**No changes**
Metabolism				
-	HDL cholesterol HOMA % S	-	-	Insulin, glucose and NEFA, LDL cholesterol, total cholesterol , HOMA-IR & HOMA %B
TLR expression on monocytes				
MFI $CD14^+TLR4^+$	-	-	-	All except MFI $CD14^+TLR4^+$
Plasma				
Cytokines				
-	-	IL-10		All except IL-10
Adhesion Molecules				
P-selectin ICAM-1	-	-	-	E-selectin & VCAM-1
Adipokines				
Adiponectin CRP Adipsin	-	-	-	Resistin, PAI-1, MCP-1 & leptin
Intracellular cytokines				
	-	Basal: IL-1β	-	Basal: all except IL-1 β LPS: all
Cytokine production from whole blood (24 h)				
-	-	Basal: IL-8, IL-6	-	Basal: IL-10, IL-2, IL-4, TNF-α LPS: all

Table 7.15 Summary of the main effects of fish oil supplementation in metabolism and inflammation

Inflammation has been postulated as an important element influencing metabolic homeostasis. In addition to the changes in metabolic markers, n-3 PUFA supplementation resulted in a significant reduction in plasma P-selectin, ICAM-1 and CRP concentrations. In terms of adhesion molecules, Derosa *et al* (2012) reported similar results than those described in this study, although others documented no effect of fish oil in these markers (Din *et al.* 2008; Damsgaard *et al*. 2008; Pot *et al.* 2009; Bragt

and Mensink, 2012). The supplementation period used in this study and by Derosa *et al* (2012) (3-6 months) were longer than studies reporting no effects after fish oil supplementation (1-2 months). In addition, some authors have also found changes in E-selectin and VCAM-1 after 3 months of fish oil treatment (Miles *et al.* 2001; Thies *et al.* 2001). This evidence together suggests that fish oil can affect circulating concentrations of these markers when it is consumed for at least 3 months.

CRP was significantly reduced by fish oil supplementation in normal weight subjects and this result is consistent with the literature (Derosa *et al.* 2006; Browning *et al.* 2007; Kelley *et al.* 2009; Albert *et al.* 2014). CRP is a well-documented marker of inflammation and a risk factor for cardiovascular disease (Cushman *et al.* 2005; Matthijs *et al.* 2006). CRP is produced rapidly (approximately 2 h) in response to IL-6 by macrophages or adipose tissue in response to an infection (bacterial, viral and fungal), injury or necrosis. Elevations of plasma CRP concentrations have been also described in the presence of chronic inflammatory diseases (e.g. atherosclerosis, inflammatory bowel syndrome, renal failure). The reductions of plama CRP reflects somehow a reduction of inflammatory signals. Therefore, the changes of plasma adhesion molecule and CRP concentrations observed here represent a relevant positive effect of fish oil and may be one of the underlying mechanisms explaining the reduction of cardiovascular risk in populations with high n-3 PUFA consumption.

Fish oil supplementation decreased the basal production of IL-1β by monocytes and of IL-8 and IL-6 from whole blood in obese subjects but not in normal weight subjects. Previous studies have reported significant reductions of *ex vivo* synthesis of cytokines by mononuclear cells after a period of fish oil consumption cells in normal weight subjects (Caughey *et al.* 1996; Trebble *et al.* 2003; Wallace *et al*. 2003). This discrepancy between our study and previous reports may be explained by the small sample size in the normal weight group (n=16-28 vs n=5) (Caughey *et al.* 1996; Trebble *et al.* 2003). This effect may be linked to the ability of n-3 PUFA to modulate cell signalling (TLRs and NF-κB) and lipid mediator's profile (Calder, 2006; Calder, 2014). These changes may also explain the reduction in plasma IL-10 concentrations. Similarly to inflammatory cytokines, IL-10 is produced following TLR ligation and activation of NF-κB. Moreover, the production of IL-10 after TLR activation is regulated by the extracellular signal-

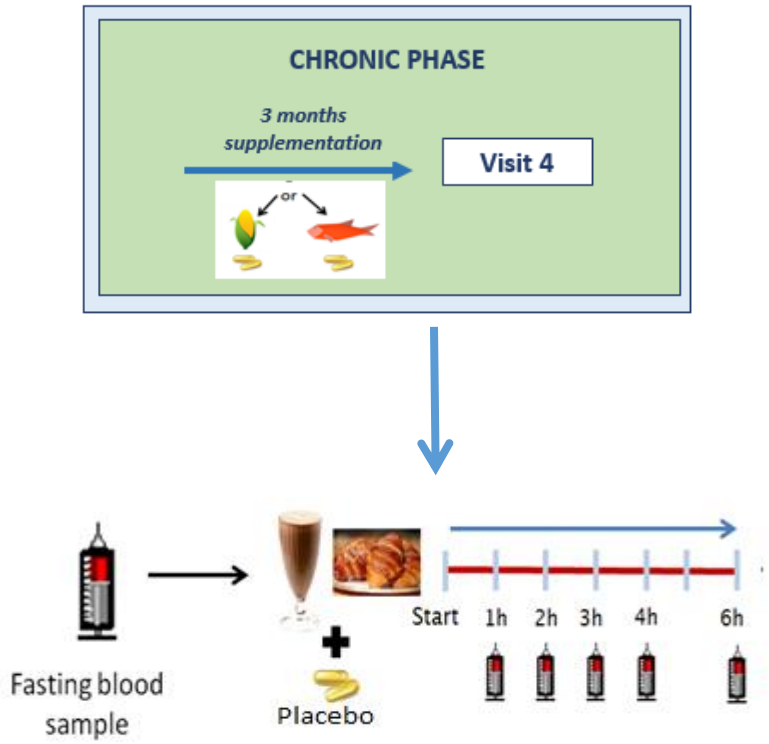
regulating kinases (ERK). ERK and NF-κB pathway are affected by lipid mediators (lipoxins & resolvins) (Serhan, 2008; Saraiva *et al.* 2010). Overall, these results suggest that n-3 PUFA induces changes in inflammatory mediators that culminate with the reduction of cytokines related to the NF-κB pathway.

7.5 Conclusion

Fish oil had a positive effect on some metabolic and inflammatory markers in normal weight subjects although these effects were not observed in the obese group. Nevertheless, fish oil supplementation reduced the production of inflammatory cytokines in whole blood and in monocytes in obese subjects.

CHAPTER 8

The effect of chronic fish oil supplementation on the postprandial metabolic and inflammatory responses in normal weight and obese subjects



CHAPTER 8

The effect of chronic fish oil supplementation on the postprandial metabolic and inflammatory responses in normal weight and obese subjects

8.1 Introduction, objective, hypothesis and aims

8.1.1 Introduction

A diet rich in n-3 PUFA has been associated with an improvement in fasting lipemia in healthy and metabolically compromised subjects. Some of these changes, previously described, include the lowered concentrations of triglycerides and VLDL. Chronic fish oil consumption reduces postprandial concentrations of chylomicrons (Harris *et al.* 1988; Brown and Roberts 1991; Westpal *et al.* 2000), triglycerides (Williams *et al.* 1997; Tinker *et al***.** 1999; Rivellese *et al.* 2002; Shirmer *et al.* 2012; Jackson *et al.* 2012) and apoB48 and apo100 (Harris *et al.* 1988). These effects have been described not only in subjects at risk of CVD but also in healthy normal weight individuals. The mechanisms of action involved in these effects have been attributed to the ability of fish oil to modulate fatty acid and triglyceride synthesis and metabolism of triglyceride rich lipoproteins partly through increased activity of lipoprotein lipase (Harris, 1998; Murphy, 1999).

Fish oil reduces the hepatic rates of triglyceride and VLDL production *in vitro* and this is reflected in human studies where long term fish oil feeding resulted in lower fasting triglyceride and VLDL concentrations (Harris *et al.* 1988; Brown and Roberts, 1991). Another hypothesis explaining the beneficial effects of fish oil on postprandial lipemia has been related to effects on LPL activity. Some studies suggest that incorporation of fish oil into cell membranes has the ability to enhance LPL activity and thus accelerate lipid clearance from circulation after a fatty meal (Harris *et al.* 1988; Murphy *et al*. 1999; Khan *et al.* 2002). The evaluation was performed measuring LPL expression on adipose tissue, lipoprotein lipase and hepatic lipase activity *in vitro*.

In addition to the improvement in the lipemic response, a greater dietary intake of fish oil may also improve postprandial inflammation. The literature and results described in **Chapter 6** and **7** suggest a beneficial acute effect of fish oil on some markers related to

cardiovascular disease, although the long term exposure to n-3 PUFA in relation to postprandial inflammation has not been studied in detail.

Studies in patients with hypertriglyceridemia (men and women) observed a reduction of plasma adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and inflammatory cytokines (IL-6, CRP and TNF-α) and changes in adipokine concentrations (↓ resistin, ↑ adiponectin) after a combined intervention including 6 months of fish oil treatment (3 g/day), diet (American Heart Association recommendations) and exercise (Derosa *et al*. 2011; Derosa *et al.* 2012). In contrast, studies exploring the independent effect of fish oil and a weight loss diet on postprandial inflammation reported a significant reduction in postprandial MCP-1 and a trend for reduction in IL-6 after fish oil intervention (6 weeks) in healthy obese subjects (Jellerma *et al.* 2004). Moreover, a weight loss diet reduced significantly the postprandial MCP-1, ICAM-1 and CRP response in this population. In healthy normal weight men, no effect of fish oil (3.1 g per day n-3 PUFA) on the postprandial CRP, IL-6, P-selectin and adiponectin response were detected after 8 weeks of supplementation (Damsgaard *et al.* 2008).

These studies suggest a modest or no effect of fish oil for some markers of postprandial inflammation. Although the doses used in these studies are comparable to those reporting a beneficial effect of fish oil on inflammatory makers measured in the fasting state, the supplementation period is shorter. Additionally, these studies made no comparisons between healthy subjects and those at risk of metabolic complications. Thus the literature regarding to the effect of long-term fish oil supplementation on postprandial inflammation is limited and some molecules involved in metabolism and regulation of inflammation have not been explored in this context (including TLR).

8.1.2 Aim

To evaluate the effect of fish oil supplementation on the postprandial metabolic and inflammatory response in normal weight and obese subjects

8.1.3 Hypothesis

- Chronic supplementation with n-3 PUFAs will lower the postprandial metabolic and inflammatory responses in normal weight and obese subjects after a HFM.

8.1.4 Objectives

To evaluate the effect of a 12 week period of n-3 PUFA supplementation on the following in normal weight and obese subjects following a HFM:

- metabolic response;
- TLR expression on monocytes;
- molecules related to inflammation (cytokines, adhesion molecules and adipokines).

8.2 Methods

The results reported in this chapter describe the changes in postprandial metabolic and inflammatory markers in normal weight and obese subjects after 12 weeks supplementation with n-3 PUFA. Subject characteristics, treatment (capsules composition), the blood collection protocol, and the methods employed for the analysis of the metabolic profile, inflammatory molecules, adipokines and TLR expression are described in **Chapter 3.2 and 4.2**. AUC and iAUC were calculated as described in **Chapter 5.2.**

Comparisons of intra-individual responses to the HFM before and after fish oil supplementation were made using a paired t-test. Metabolic data are expressed as mean and standard deviation. Due to the skewed distribution of the TLR data and other inflammatory variables, these data were log10 transformed before statistical analysis and data are expressed as median and percentiles. The time point differences before and after fish oil supplementation were tested using paired t-test per time point, if the data were normally distributed, or using a Wilcoxon test if data were not normally distributed or negative.

Comparisons of the postprandial metabolic and inflammatory responses to the different treatments (fish oil or placebo) and the group differences were evaluated using 2-way-ANOVA (fixed for age and sex).

8.3 Results

8.3.1 The effect of chronic fish oil supplementation on the postprandial metabolic response in normal weight and obese subjects

Postprandial plasma lipids and other metabolic markers responses before and after fish oil and placebo intervention are displayed in **Figure 8.1a and 8.1b**. Plasma concentrations of triglycerides in response to a HFM were significantly lower at 4 h and 6 h while glucose was significantly higher at 4 h after 12 weeks fish oil supplementation in normal weight subjects ($\rho<0.05$).

AUC and iAUC from 0 to 6 h for all metabolic markers are shown in **Table 8.1a** and **8.1b**. Fish oil supplementation significantly lowered the postprandial plasma triglyceride iAUC in normal weight subjects ($\rho<0.05$). Moreover, fish oil supplementation increased the AUC and iAUC for insulin in obese subjects ($\rho<0.05$).

Supplementation with fish oil resulted in a significantly greater reduction in postprandial NEFA concentrations when compared with placebo ($\rho<0.05$) (**Table 8.2**).

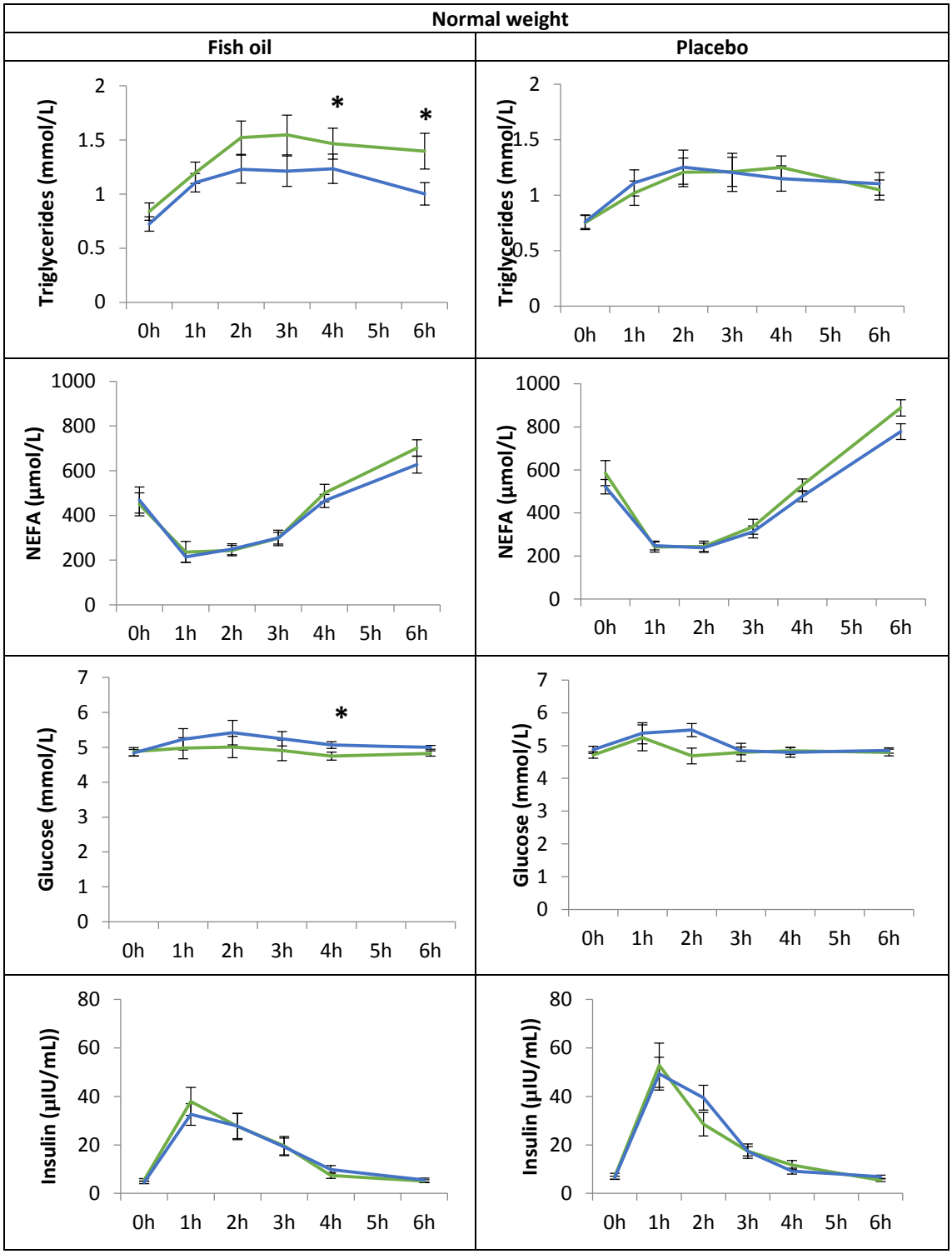


Figure 8.1a Metabolic response to a HFM challenge in normal weight subjects after 12 weeks fish oil supplementation. **Before** (-green-) or **after** (-blue-) fish oil or placebo. Data are expressed as mean concentration and SE bars; *p-value <0.05, comparison between before and after supplementation time points.

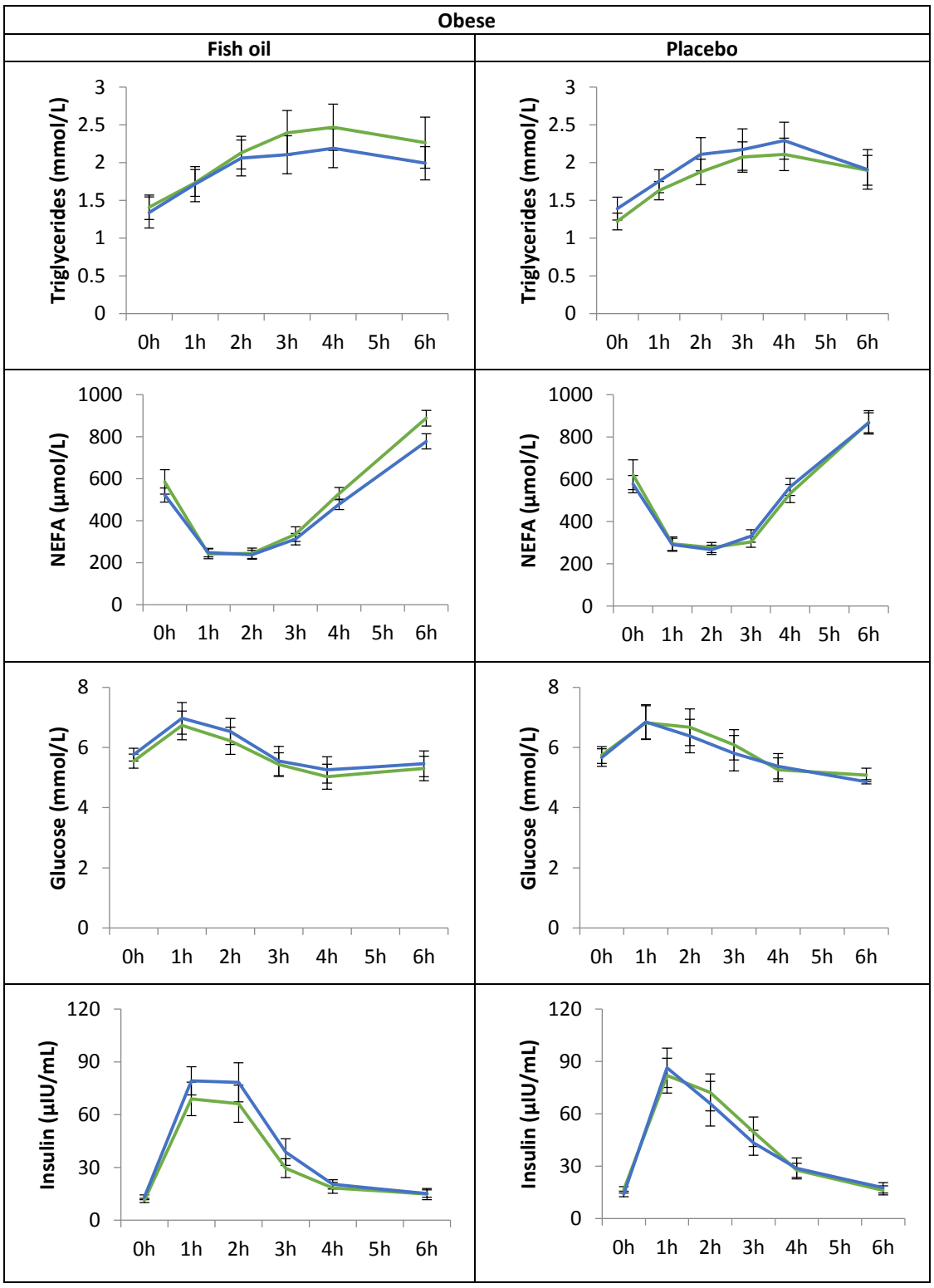


Figure 8.1b Metabolic response to a HFM challenge in obese subjects after 12 weeks fish oil supplementation. Before (-green-) or after (-blue-) fish oil or placebo. Data are expressed as mean concentration and SE bars; *p-value <0.05, comparison between before and after supplementation time points.

AUC		Fish oil			Placebo		
		Before	**After**	**ρ-value**	**Before**	**After**	**ρ-value**
Triglycerides	**Lean**	8.28 ± 3.49	6.68 ± 2.97	0.08	6.74 ± 2.23	6.78 ± 2.69	0.95
	Obese	12.94 ± 7.33	11.83 ± 6.60	0.29	11.25 ± 4.82	12.08 ± 5.80	0.37
NEFA	**Lean**	1871.00 ± 381.29	1682.93 ± 411.68	0.21	1900.47 ± 523.82	1869.20 ± 267.37	0.84
	Obese	2137.77 ± 328.56	1925.95 ± 449.57	0.06	2109.56 ± 572.21	2177.60 ± 482.40	0.5
% NEFA suppression	**Lean**	-34.57 ± 38.96	-29.59 ± 45.30	0.68	-41.04 ± 43.58	-52.55 ± 31.34	0.23
	Obese	-54.24 ± 24.44	-51.27 ± 21.43	0.54	-46.11 ± 30.62	-47.09 ± 28.77	0.35
Glucose	**Lean**	1.73 ± 1.91	2.77 ± 3.01	0.16	2.40 ± 2.03	1.94 ± 1.34	0.24
	Obese	3.22 ± 5.10	3.32 ± 5.76	0.78	2.94 ± 3.25	2.81 ± 3.00	0.81
Insulin	**Lean**	74.10 ± 52.60	75.88 ± 55.48	0.81	87.17 ± 68.55	92.58 ± 50.19	0.66
	Obese	137.40 ± 95.54	173.02 ± 107.99	**<0.05**	166.22 ± 98.31	182.32 ± 140.98	0.52

Data expressed as mean and standard deviation;
Difference in the postprandial response before and after treatment was analysed by paired t test

Table 8.1a AUC of plasma lipids, glucose and insulin following a HFM challenge after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects.

iAUC		Fish oil			Placebo		
		Before	**After**	**ρ-value**	**Before**	**After**	**ρ-value**
Triglycerides	**Lean**	3.31 ± 2.03	2.35 ± 1.68	**<0.05**	2.23 ± 1.12	2.32 ± 1.54	0.83
	Obese	4.39 ± 2.92	3.83 ± 2.35	0.44	3.99 ± 2.40	3.85 ± 2.18	0.71
NEFA	**Lean**	922.35 ± 423.01	762.19 ± 360.74	0.16	969.82 ± 427.26	1014.84 ± 306.61	0.75
	Obese	1160.61 ± 351.16	981.54 ± 448.15	0.13	1052.29 ± 662.89	1160.37 ± 554.80	0.41
Glucose	**Lean**	29.27 ± 3.72	30.39 ± 4.39	0.34	29.04 ± 3.51	30.17 ± 2.45	0.12
	Obese	34.01 ± 10.72	35.27 ± 11.30	0.08	35.44 ± 11.21	34.80 ± 10.66	0.48
Insulin	**Lean**	103.38 ± 56.69	102.56 ± 59.60	0.74	125.24 ± 65.50	130.06 ± 54.58	0.54
	Obese	208.41 ± 116.62	248.57 ± 132.90	**0.01**	266.46 ± 135.51	263.28 ± 159.32	0.90

Data expressed as mean and standard deviation;
Difference in the postprandial response before and after treatment was analysed by paired t test

Table 8.1b iAUC of plasma lipids, glucose and insulin following a HFM challenge after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects.

	Normal weight		Obese				
	Δ Fish oil	**Δ Placebo**	**Δ Fish oil**	**Δ Placebo**	**treatment**	**group**	**interaction**
iAUC							
Triglycerides	-1.53 (-3.63-0.15)	0.06 (-0.93-1.38)	-0.31 (-2.64-1.40)	0.71 (-0.11-2.49)	0.11	0.26	0.79
NEFA	-8.00 (-551.00-268.08)	170.22 (-180.00-312.00)	-273.50 (-548.00-246.00)	128.00 (-238.50-400.45)	**<0.05**	0.52	0.94
Glucose	-0.07 (-2.19-3.76)	0.97 (-0.31-2.31)	0.26 (-0.23-2.22)	0.07 (-3.41-2.03)	0.87	0.76	0.14
Insulin	0.10 (-30.13-22.09)	6.20 (-8.80-31.25)	43.20 (1.30-68.81)	-24.00 (-80.05-35.55)	0.88	0.22	0.24
iAUC							
Triglycerides	-1.17 (-1.88-0.17)	0.15 (-0.91-0.98)	-0.05 (-1.19-1.10)	-0.11 (-0.71-0.80)	0.12	0.98	0.66
NEFA	-107.00 (-457.35-136.85)	239.70 (-225.15-341.65)	-187.50 (-563.55-189.83)	118.00 (-287.75-551.55)	0.11	0.71	0.56
Glucose	0.30 (-0.75-2.85)	-0.52 (-1.43-0.87)	-0.24 (-1.12-0.66)	-0.59 (-1.35-0.52)	0.18	0.2	0.14
Insulin	-3.90 (-18.77-29.33)	6.58 (-21.66-27.42)	34.70 (-8.87-58.56)	0.20 (-42.34-54.23)	0.75	0.15	0.69

Data expressed as median and percentile 25th and 75th; the effect of treatment, group and interaction was evaluated using 2 way ANOVA

Table 8.2 Changes in AUC and iAUC for metabolic markers following a HFM challenge after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

8.3.2 The effect of chronic fish oil supplementation on the postprandial TLRs response in normal weight and obese subjects

In normal weight subjects, fish oil supplementation altered the TLR response to a HFM. The % of TLR 2 positive monocytes was lower at 4 h compared with baseline ($\rho<0.05$) (**Figure 8.2a**). The percentage of TLR4 positive monocytes and level of TLR 4 expression were significantly lower at 0 h, 1 h, 2 h, 3 h and 4 h as illustrated in **Figure 8.2a**.

After placebo supplementation, TLR2 positive monocytes were reduced at 4 h in response to the HFM in normal weight subjects($\rho<0.05$) (**Figure 8.2b**). In parallel, the percentage of TLR4 positive monocytes were significantly higher at this time point while TLR4 expression was lowered by placebo intervention at 3 h ($\rho<0.05$) (**Figure 8.2b**).

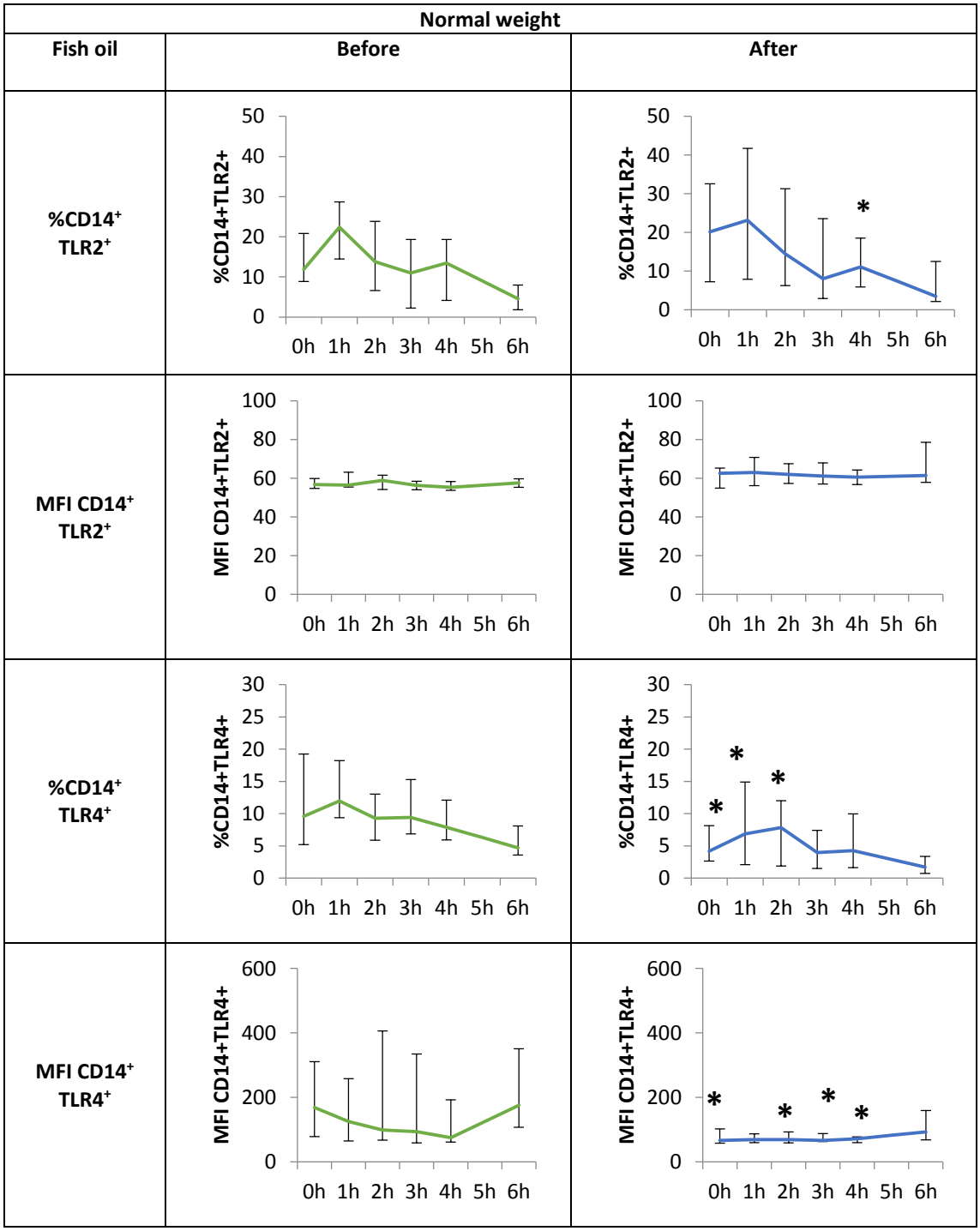


Figure 8.2a TLR response to a HFM challenge in normal weight subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points.

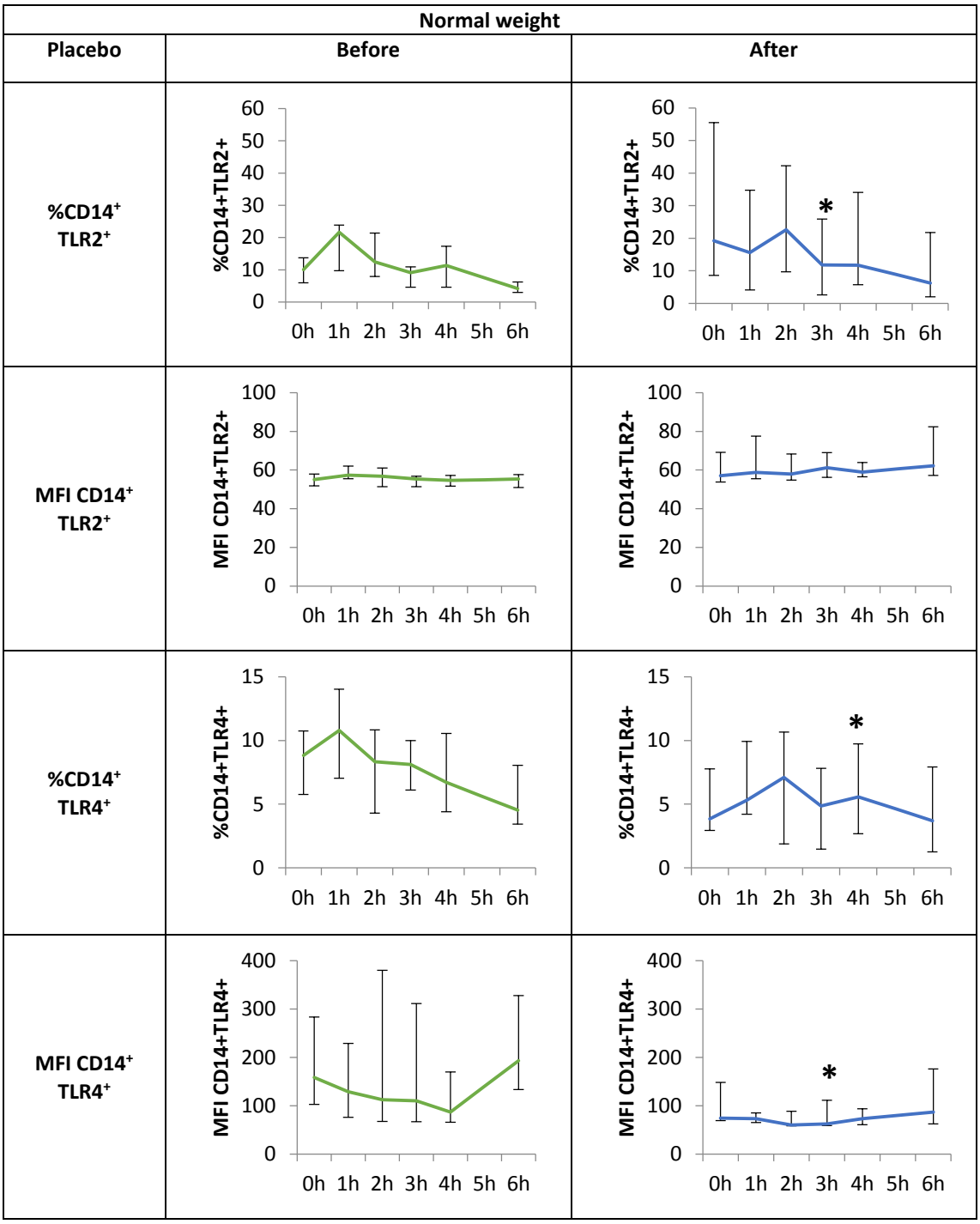


Figure 8.2b TLR response to a HFM challenge in normal weight subjects after 12 weeks placebo supplementation. Before (-green-) or after (-blue-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points.

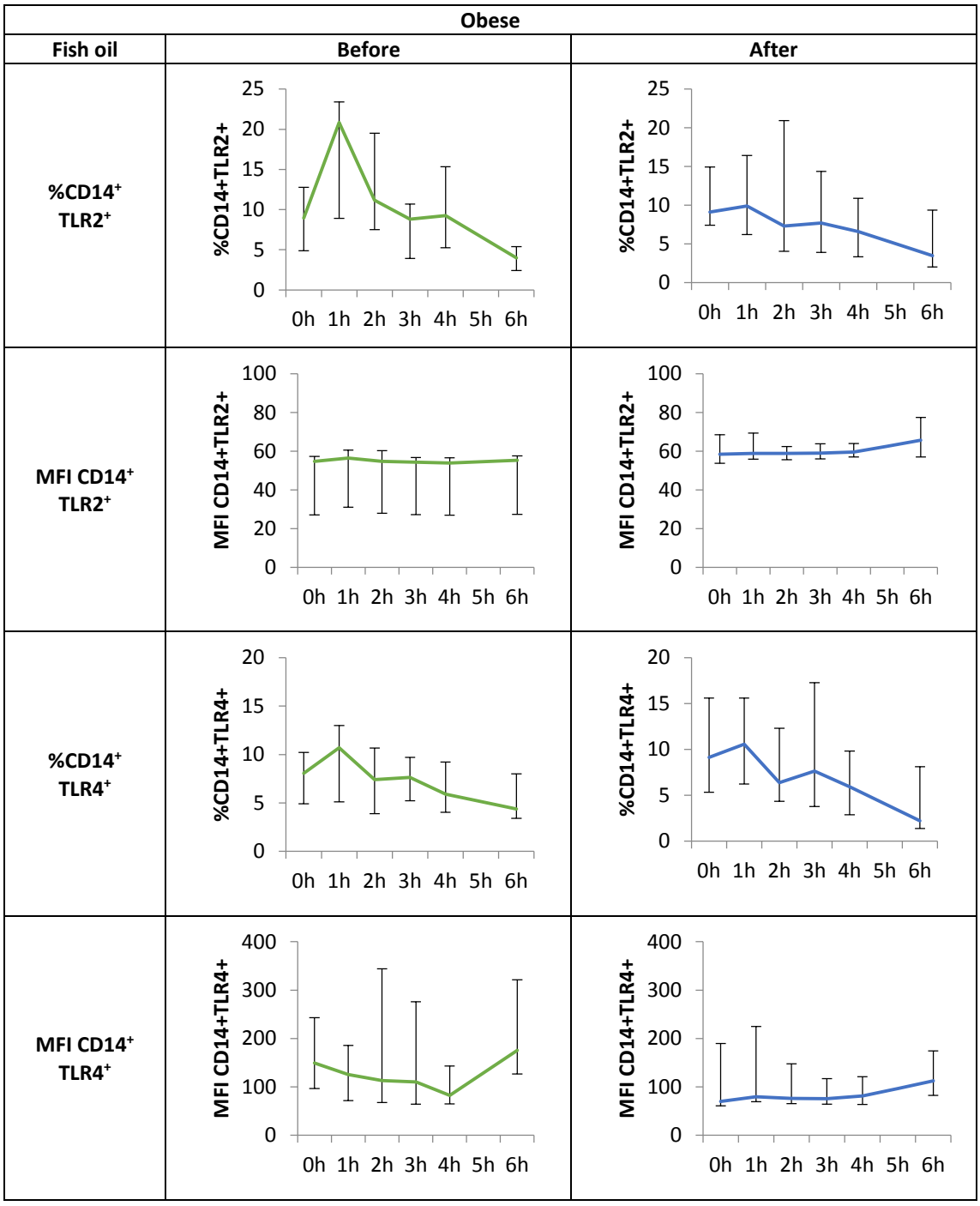


Figure 8.3a TLR response to a HFM challenge in obese subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points.

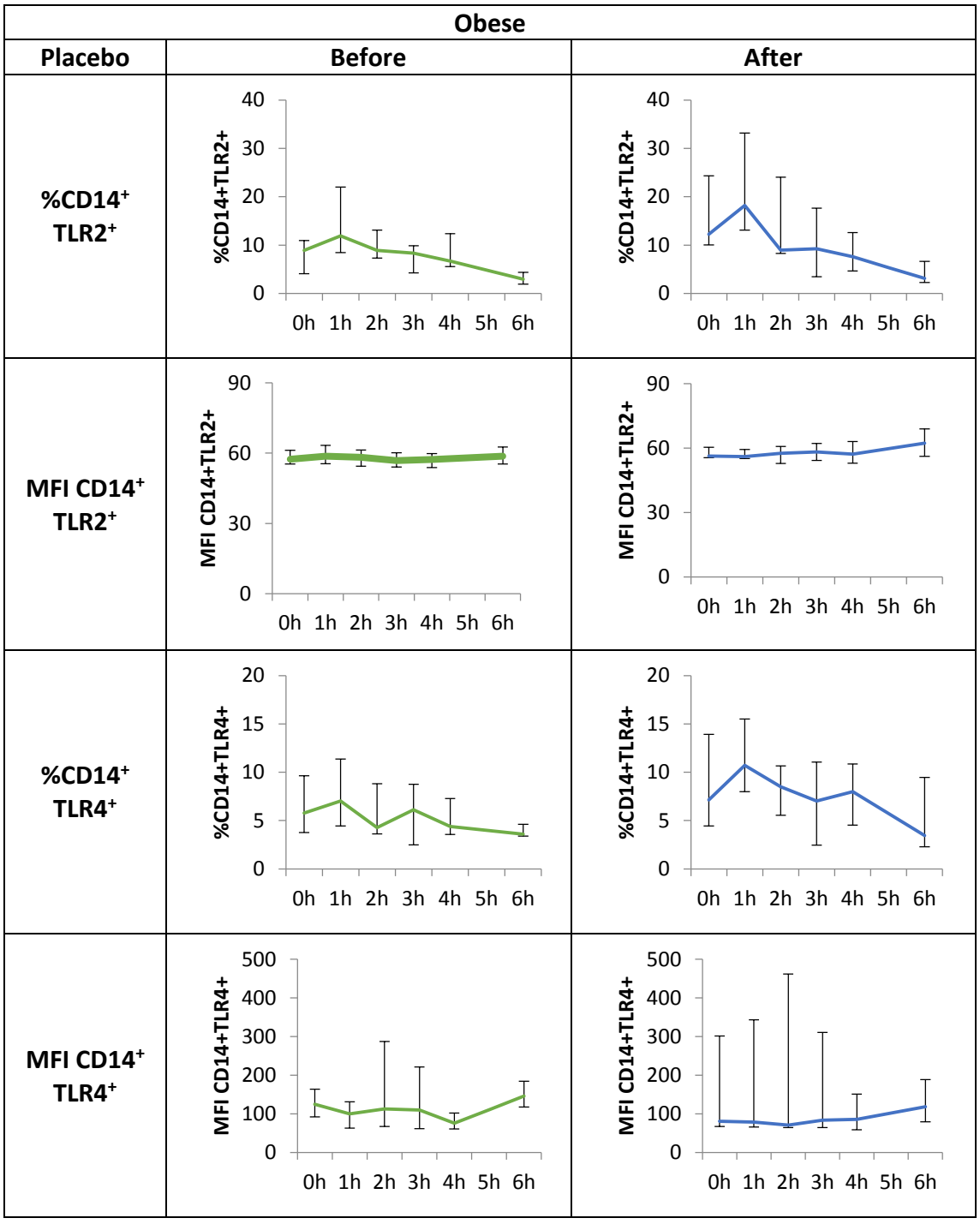


Figure 8.3b TLR response to a HFM challenge in obese subjects after 12 weeks placebo supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points.

AUC		Fish oil			Placebo		
		Before	**After**	**p-value**	**Before**	**After**	**p-value**
% $CD14^+TLR2^+$	**Lean**	73.62 (46.72-187.50)	107.50 (36.35-154.35)	0.33	122.05 (48.83-219.22)	90.56 (32.03-245.05)	0.26
	Obese	83.59 (64.02-121.35)	41.34 (29.38-76.25)	**<0.05**	64.41 (30.46-141.10)	59.57 (42.65-94.29)	0.91
MFI $CD14^+TLR2^+$	**Lean**	387.10 (358.40-421.05)	372.20 (354.90-408.65)	0.38	367.65 (345.13-439.47)	375.90 (345.10-428.17)	0.31
	Obese	352.60 (332.05-362.50)	355.20 (339.50-408.65)	0.39	355.00 (333.5-364.20)	353.35 (338.47-379.92)	0.92
% $CD14^+TLR4^+$	**Lean**	18.05 (2.23-29.97)	36.65 (12.32-58.67)	0.06	8.42 (1.27-22.54)	38.32 (20.86-41.76)	**<0.05**
	Obese	34.67 (24.07-53.99)	39.11 (26.88-57.11)	0.61	53.32 (26.03-81.67)	49.31 (31.21-67.74)	0.25
MFI $CD14^+TLR4^+$	**Lean**	484.90 (413.85-1831.00)	465.40 (417.70-630.25)	0.06	502.70 (446.45-1432.00)	491.60 (431.80-893.45)	0.14
	Obese	476.85 (416.45-1528.00)	569.25 (411.37-1264.25)	0.59	1097.00 (477.40-2453.00)	504.00 (430.62-1808.50)	0.99

Data expressed as median and percentile 25^{th} and 75^{th}; differences before and after treatment were evaluated using paired t-test; all data were log transformed before analysis.

Table 8.3a AUC of TLR2 and TLR4 following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects.

iAUC	Fish oil				Placebo		
		Before	After	p-value	Before	After	p-value
% $CD14^+TLR2^+$	Lean	10.70 (1.08-37.48)	6.35 (0.57-10.12)	0.22	4.49 (0.31-14.35)	2.04 (0.00-10.73)	0.23
	Obese	4.95 (0.02-21.69)	1.64 (0.00-12.96)	0.33	8.94 (3.66-35.38)	5.04 (0.00-11.04)	0.28
MFI $CD14^+TLR2^+$	Lean	16.85 (5.41-43.72)	7.89 (5.98-31.47)	0.35	1.57 (0.00-14.95)	12.43 (2.47-19.02)	0.45
	Obese	2.35 (0.06-30.27)	13.91 (0.64-35.36)	0.19	10.68 (0.20-15.42)	6.12 (0.07-22.06)	0.28
% $CD14^+TLR4^+$	Lean	0.73 (0.11-2.51)	3.79 (0.00-19.78)	0.13	0.00 (0.00-0.47)	8.47 (2.65-18.43)	**<0.05**
	Obese	7.24 (0.38-16.40)	1.32 (0.00-7.55)	0.33	5.76 (0.67-12.24)	4.85 (0.11-10.45)	0.92
MFI $CD14^+TLR4^+$	Lean	9.84 (0.00-111.85)	68.32 (12.42-126.37)	0.73	119.95 (77.59-304.07)	23.70 (0.00-42.32)	**0.03**
	Obese	61.19 (24.75-132.70)	162.90 (27.40-262.95)	0.17	54.28 (8.05-487.65)	53.92 (6.48-641.75)	0.76

Data expressed as median and percentile 25^{th} and 75^{th}; differences before and after treatment were evaluated using paired t-test; all data were log transformed before analysis.

Table 8.3b iAUC of TLR2 and TLR4 following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects.

	Normal weight		Obese		treatment	group	interaction
	Δ Fish oil	Δ Placebo	Δ Fish oil	Δ Placebo	**p-value**	**p-value**	**p-value**
% $CD14^+TLR2^+$	9.71 (-45.33-78.81)	-15.79 (-32.78-35.09)	-29.85 (-76.31-2.38)	-9.79 (-63.59-8.94)	0.62	0.48	0.88
MFI $CD14^+TLR2^+$	3.10 (-31.88-37.80)	-7.80 (-42.45-22.00)	0.60 (-15.85-53.53)	11.85 (-18.85-31.73)	0.48	0.93	0.83
% $CD14^+TLR4^+$	9.47 (0.00-27.06)	25.60 (-4.63-39.980	6.18 (-11.12-25.71)	4.89 (-52.91-31.00)	0.55	0.13	0.15
MFI $CD14^+TLR4^+$	0.00 (-633.00-103.60)	-39.15 (-434.18-0.00)	26.75 (-8.85-397.38)	-244.20 (-678.85-343.58)	0.89	0.48	0.72

Data expressed as median and percentile 25^{th} and 75^{th}; the effect of treatment, group and interaction was evaluated using 2 way ANOVA; all data were adjusted for age and sex and were log transformed before analysis

Table 8.4 Changes in AUC for TLR2 and TLR4 following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

	Normal weight		Obese		treatment	group	interaction
	Δ Fish oil	Δ Placebo	Δ Fish oil	Δ Placebo	**p-value**	**p-value**	**p-value**
% $CD14^+TLR2^+$	-2.98 (-32.65-2.64)	1.00 (-9.89-2.63)	-0.87 (-8.85-7.10)	-7.94 (-16.43-1.85)	0.81	0.39	0.55
MFI $CD14^+TLR2^+$	-1.51 (-14.62-5.04)	5.30 (-2.34-18.71)	6.05 (-4.20-21.95)	0.12 (-11.67-18.15)	**<0.01**	0.13	0.21
% $CD14^+TLR4^+$	0.79 (-0.59-18.44)	8.27 (0.67-18.43)	-0.02 (-8.37-1.94)	-2.46 (-8.84-4.84)	0.89	0.22	0.65
MFI $CD14^+TLR4^+$	2.75 (-10.20-60.66)	-40.89 (-116.67-0.00)	18.50 (-13.06-127.00)	2.03 (-37.87-270.08)	0.55	**<0.05**	0.14

Data expressed as median and percentile 25th and 75th; the effect of treatment, group and interaction was evaluated using 2 way ANOVA; all data were adjusted for age and sex and were log transformed before analysis

Table 8.5 Changes in iAUC for TLR2 and TLR4 following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

8.3.3 The effect of chronic fish oil supplementation on the postprandial cytokine response in normal weight and obese subjects

The postprandial cytokine responses after 12 weeks supplementation with fish oil or placebo are shown in **Figures 8.4**, **8.5**, **8.6** and **8.7**.

Fish oil supplementation induced significant reductions in the postprandial IL-10 and IL-6 responses as described **in Figure 8.6a** and **8.6b**. Postprandial IL-6 concentrations were significantly lowered at 4 h and 6 h after a HFM in obese subjects ($\rho<0.05$). Similarly a reduction in IL-10 concentrations were observed at 0 h, 1 h, 3 h, 4 h and 6 h ($\rho<0.05$).

In agreement with these observations, IL-6 and IL-10 AUC was lower in obese subjects after the fish oil intervention ($\rho<0.05$) (**Table 8.6a**).

Placebo treatment resulted in a significant reduction of VEGF AUC in obese subjects and an increment of IL-6 iAUC in normal weight subjects (**Table 8.6a**).

IL-10 changes after the fish oil and placebo supplementation were significantly different in groups and treatments ($\rho<0.05$) (**Table 8.7a** and **Table 8.7b**).

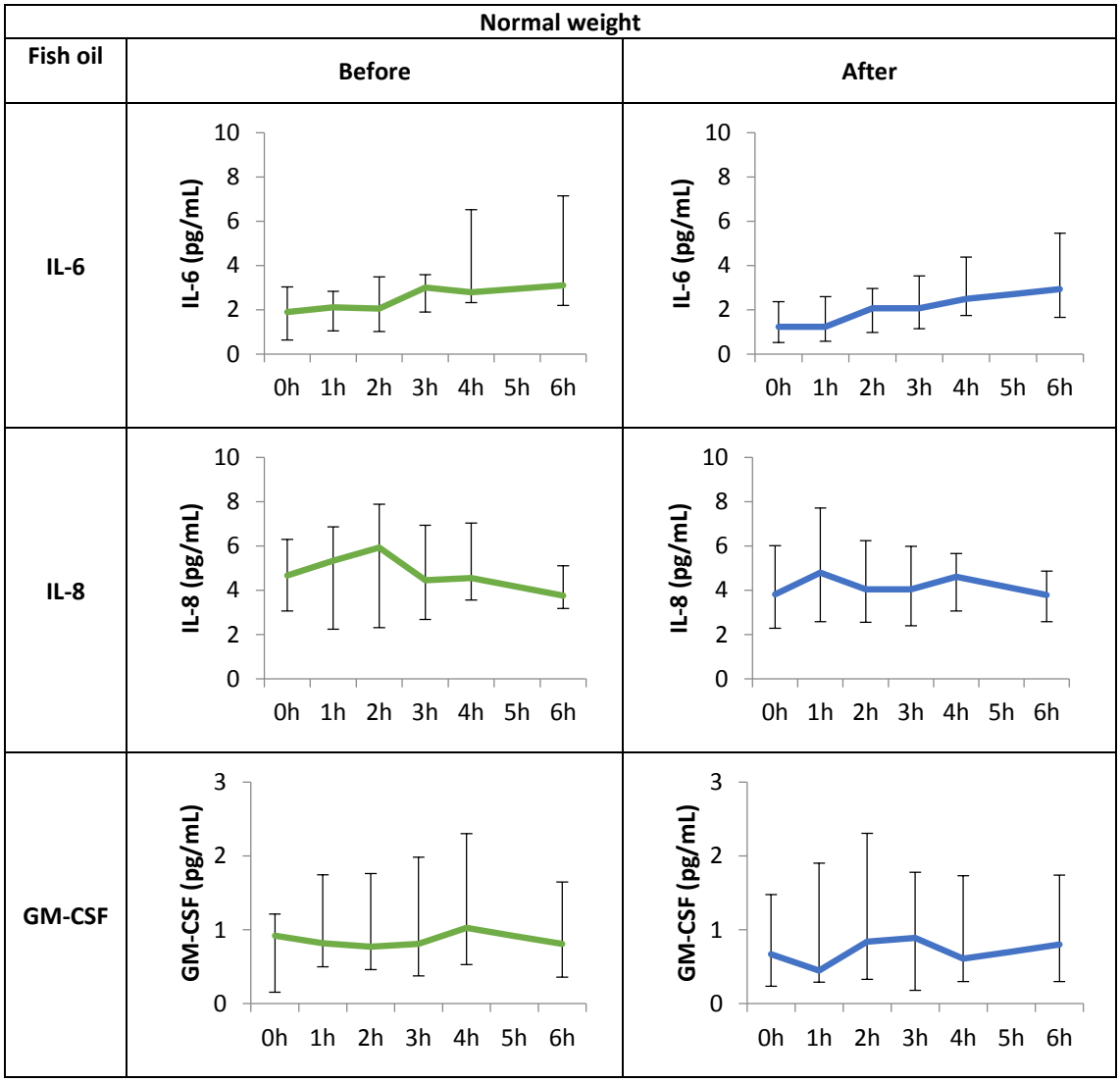


Figure 8.4a Cytokines response to a HFM challenge in normal weight subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points.

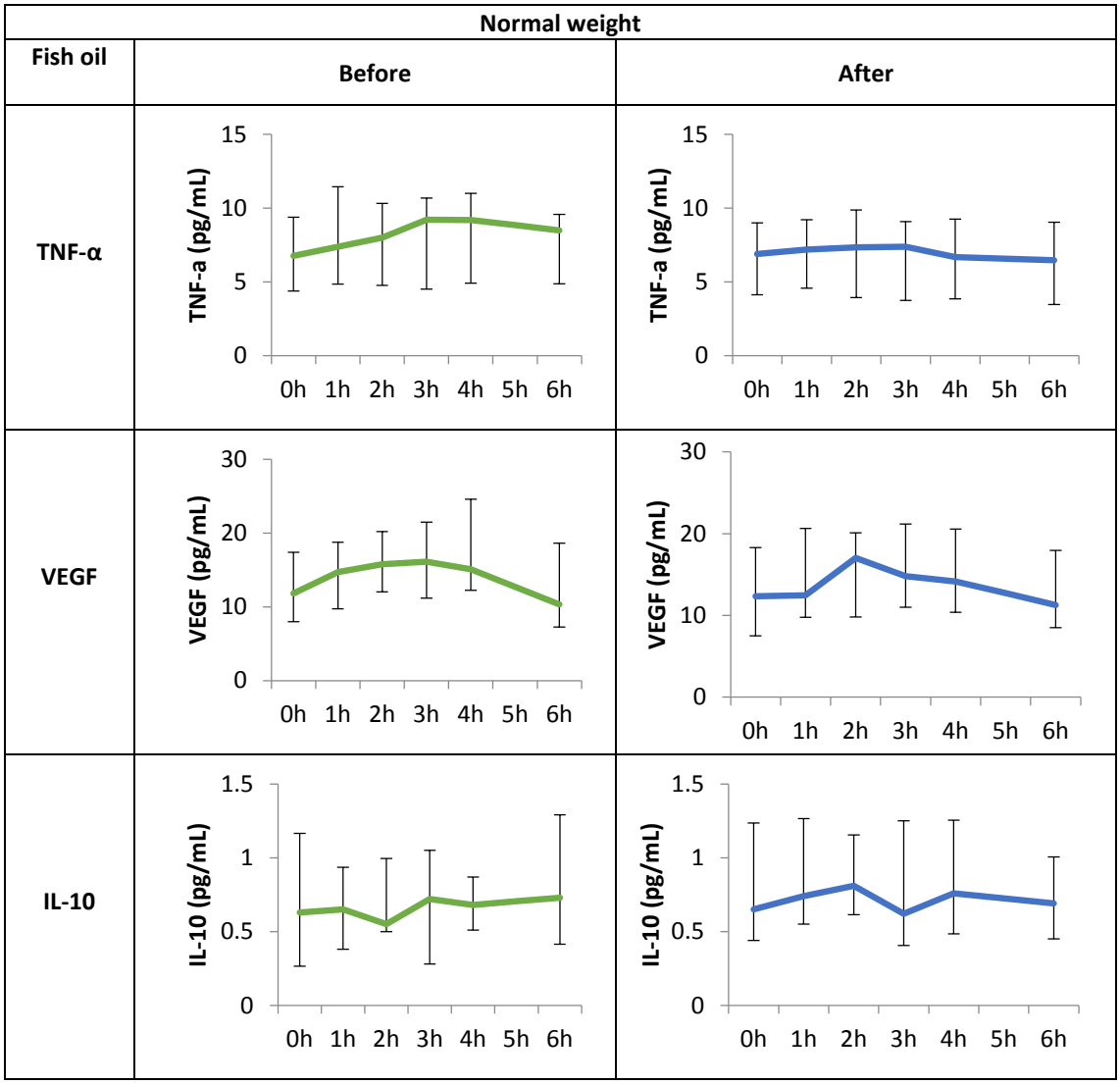


Figure 8.4b Cytokines response to a HFM challenge in normal weight subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation.Data are expressed as median concentration with 25th and 75th centile bars; *ρ-value <0.05, comparison between before and after supplementation time points.

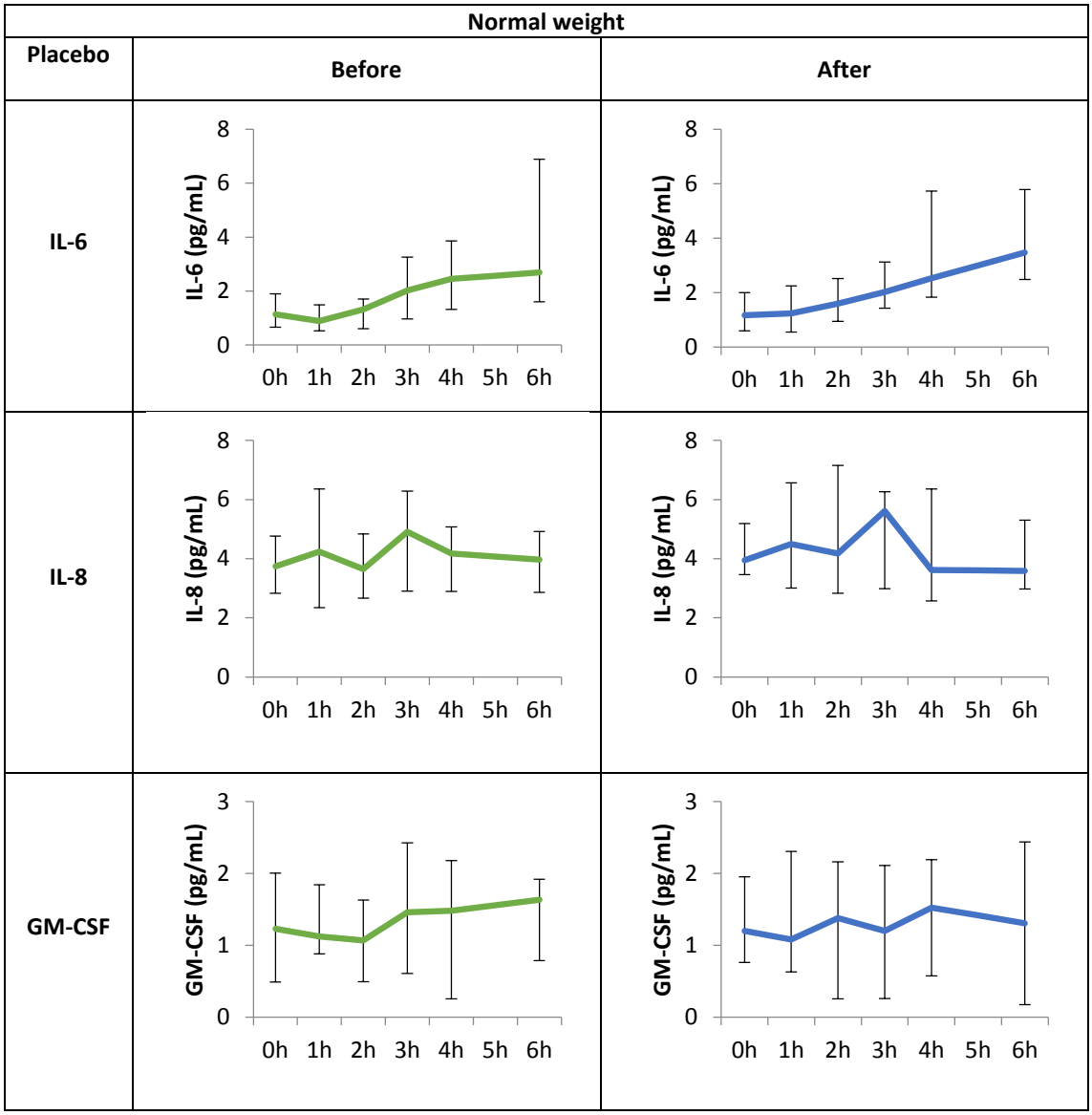


Figure 8.5a Cytokines response to a HFM challenge in normal weight subjects after 12 weeks placebo supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points.

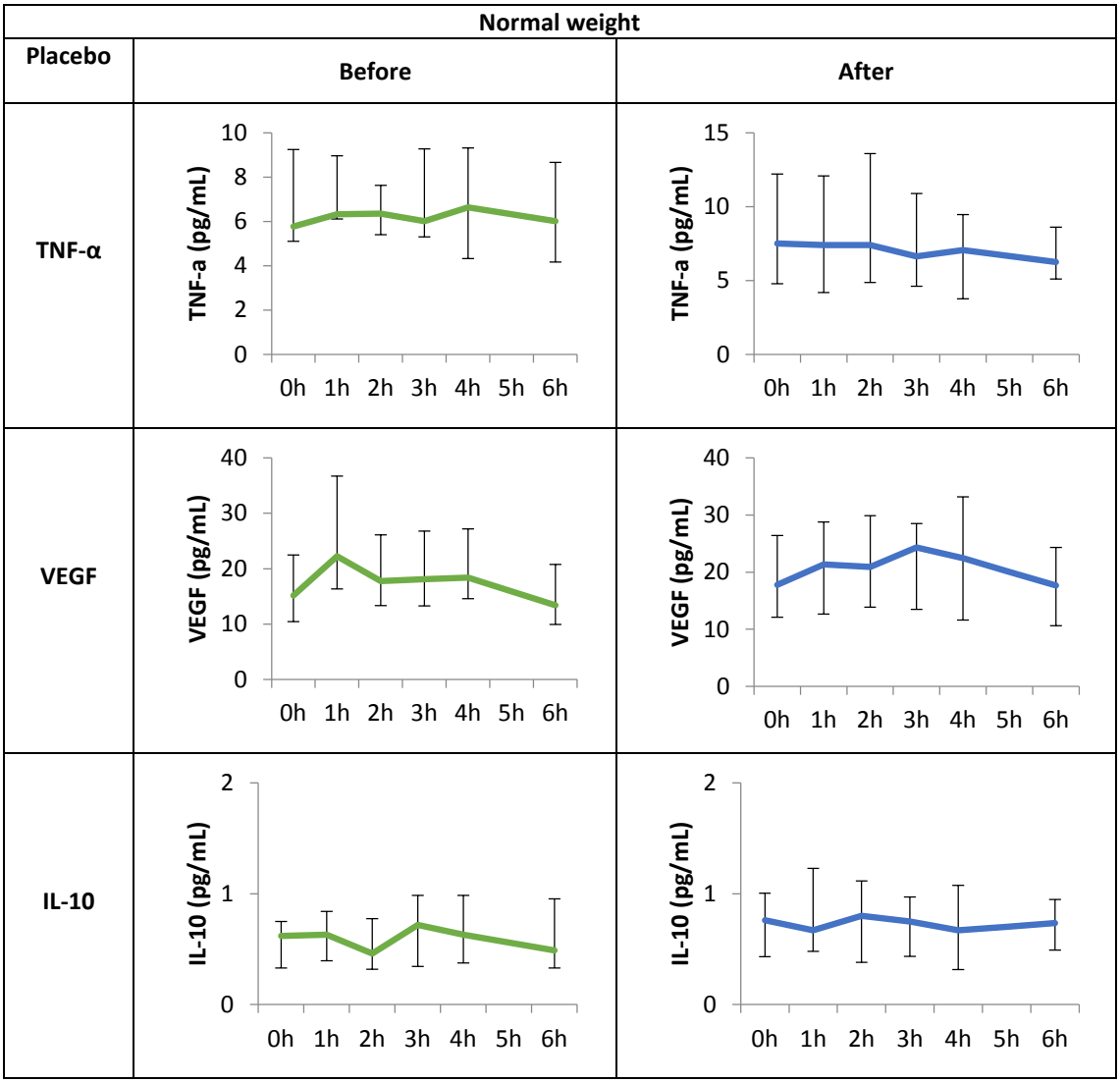


Figure 8.5b Cytokines response to a HFM challenge in normal weight subjects after 12 weeks placebo supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *ρ-value <0.05, comparison between before and after supplementation time points.

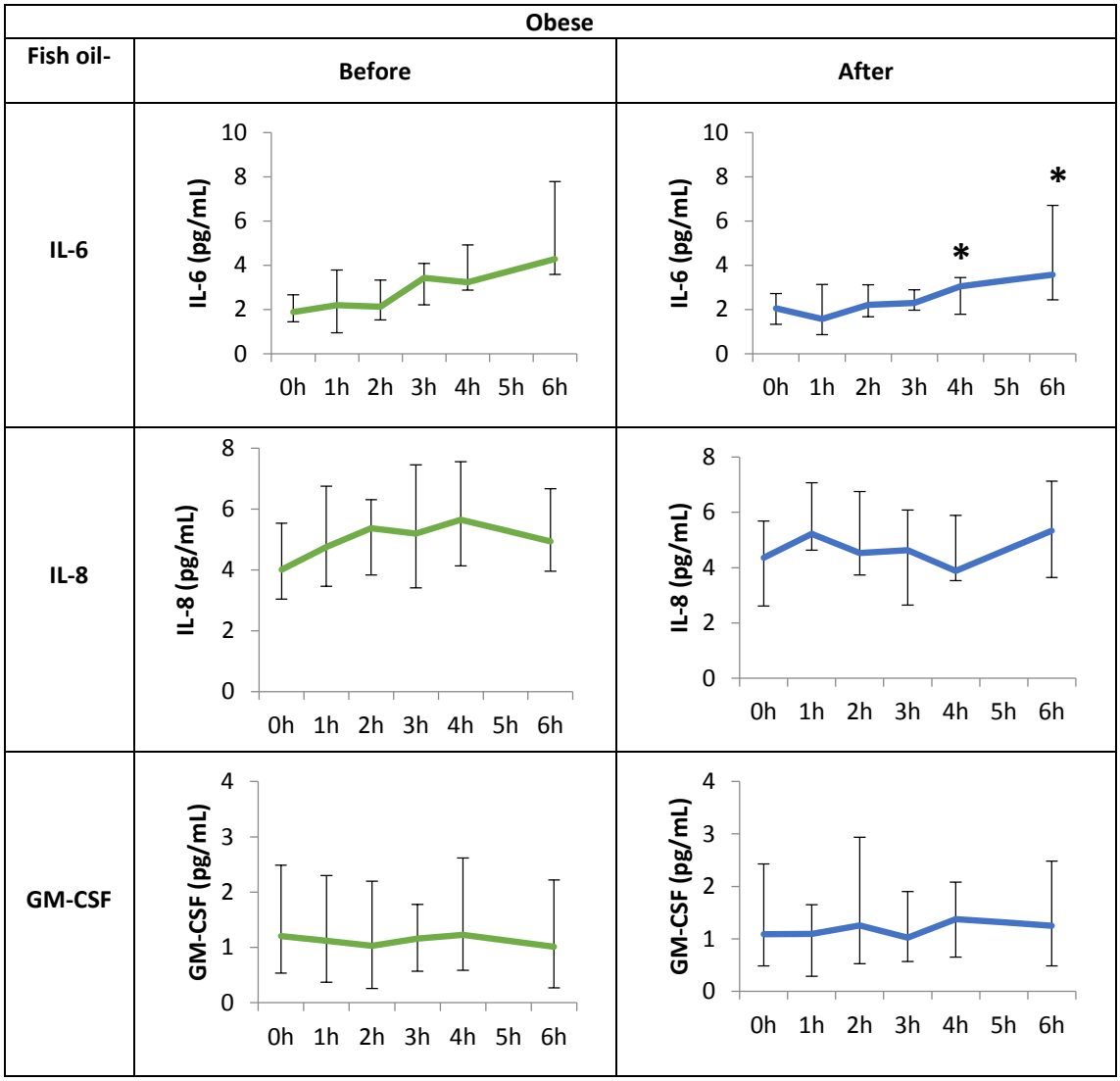


Figure 8.6a Cytokines response to a HFM challenge in obese subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points.

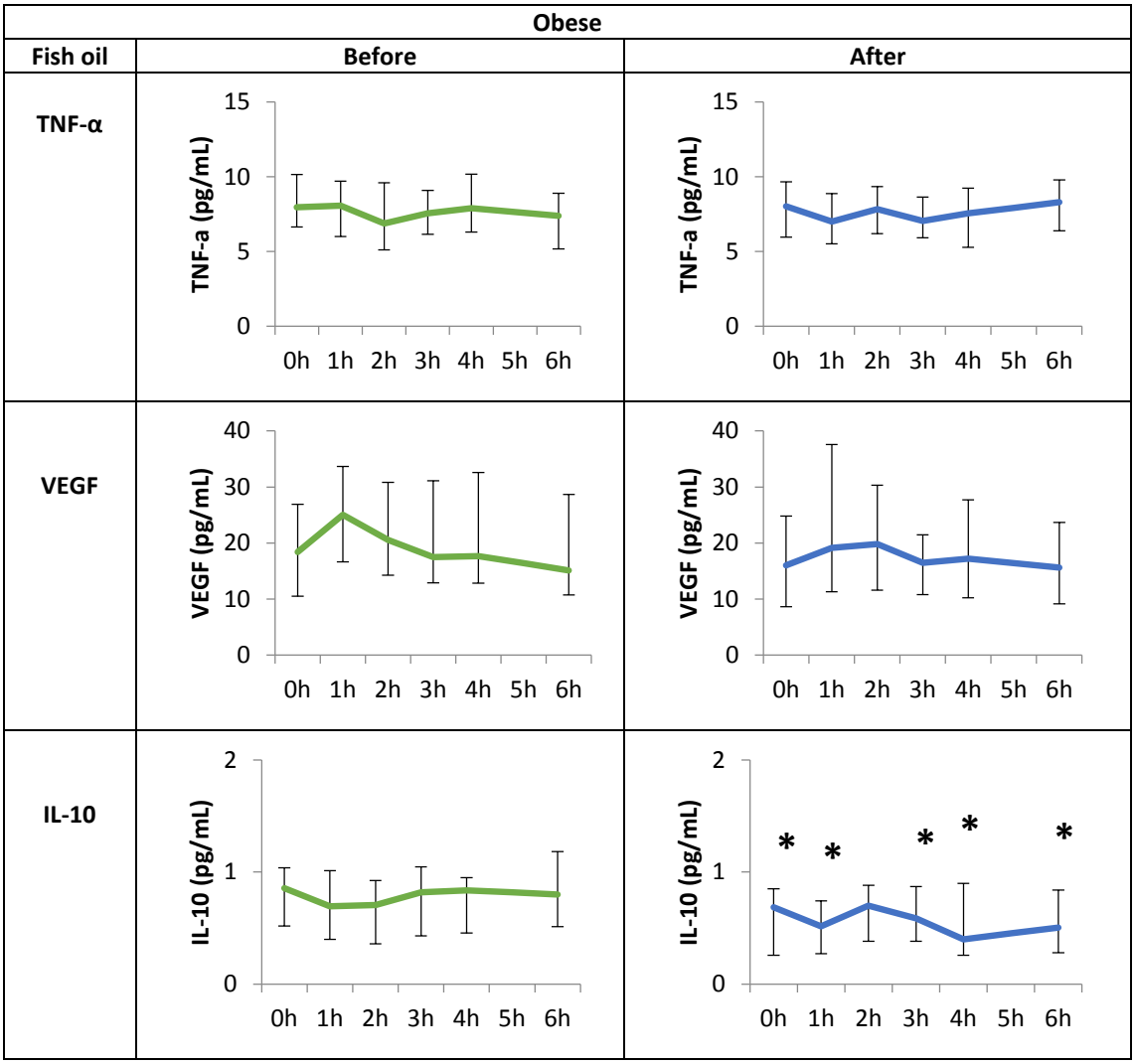


Figure 8.6b Cytokines response to a HFM challenge in obese subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *ρ-value <0.05, comparison between before and after supplementation time points

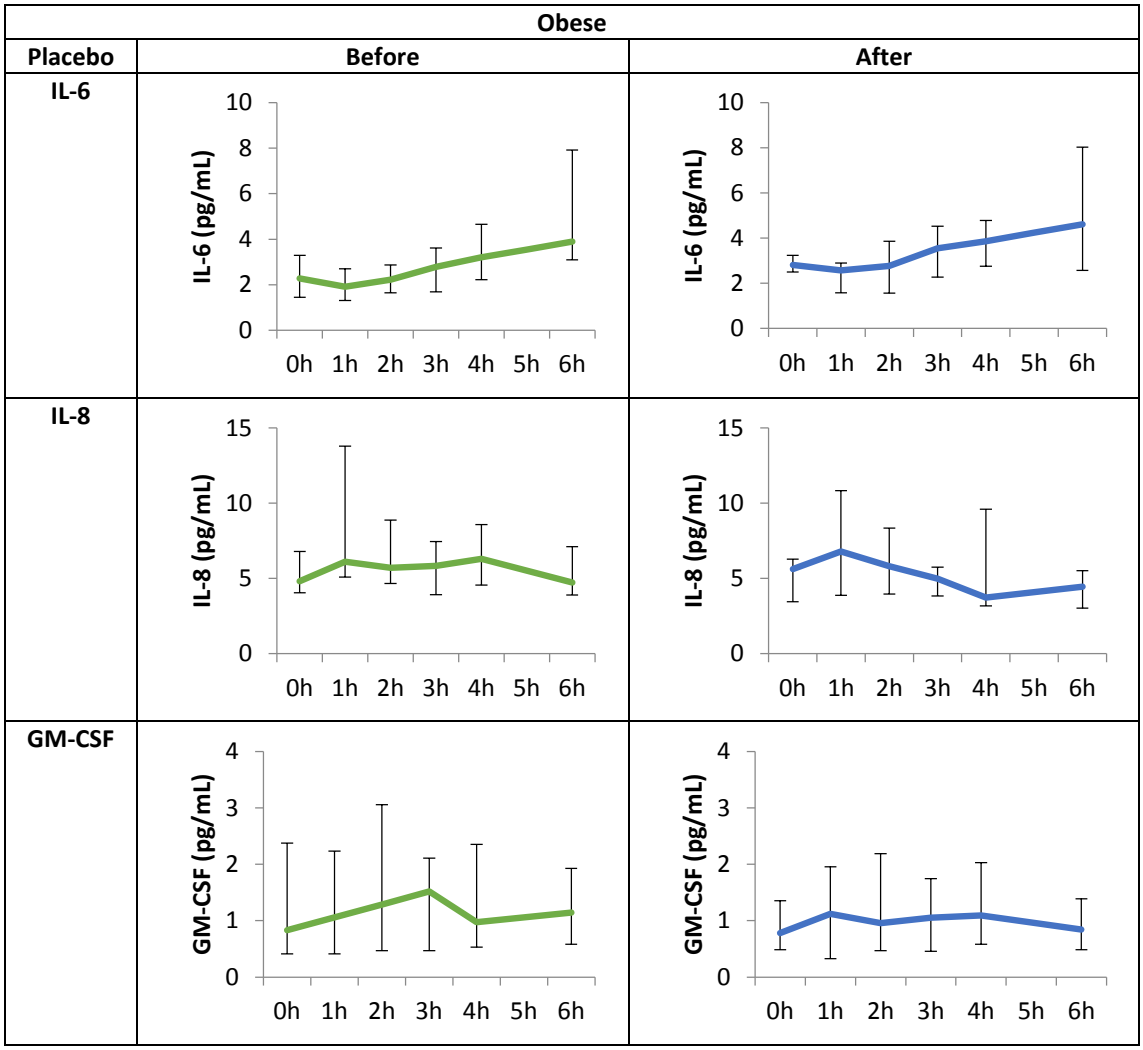


Figure 8.7a Cytokines response to a HFM challenge in obese subjects after 12 weeks placebo supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points

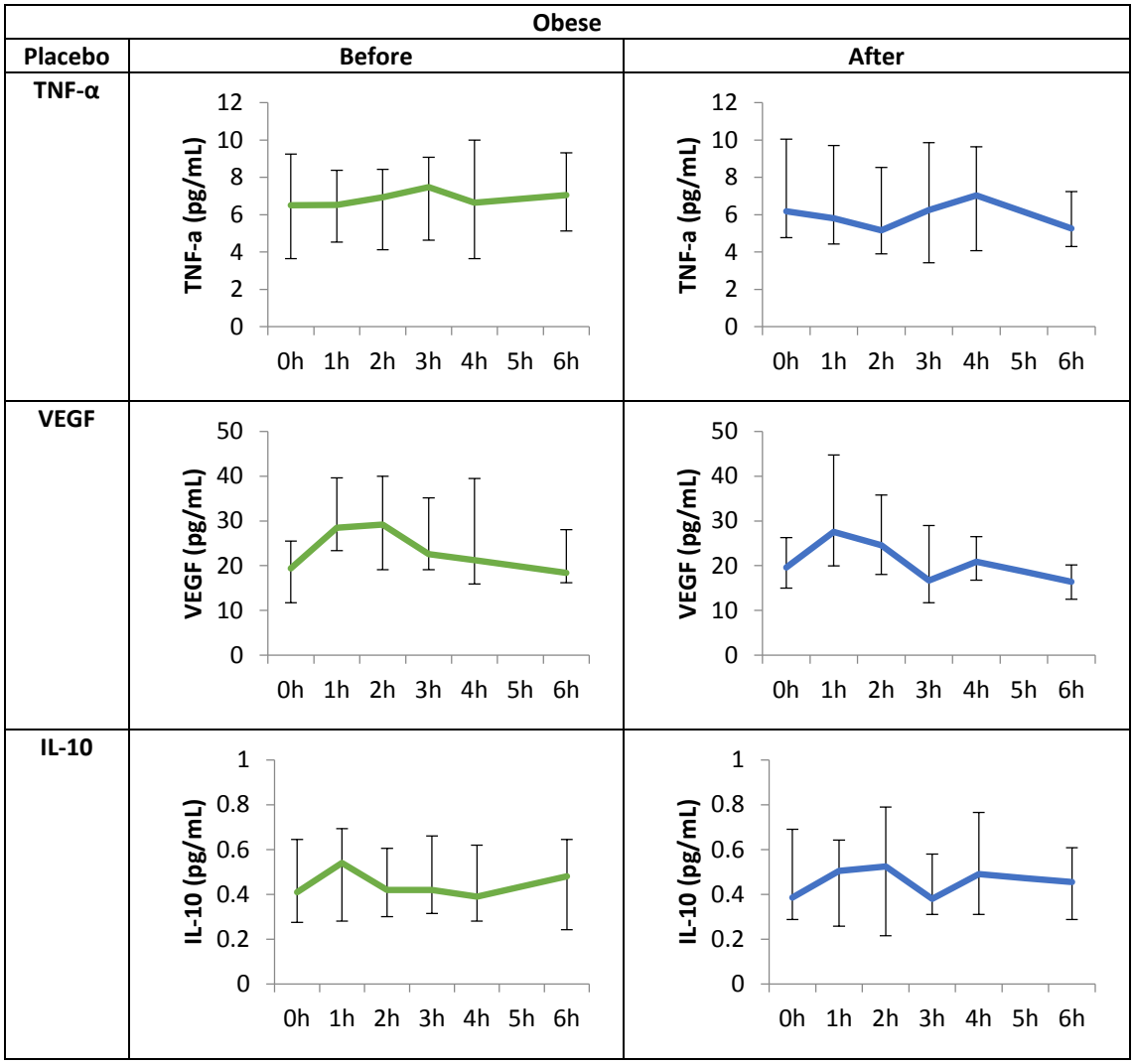


Figure 8.7b Cytokines response to a HFM challenge in obese subjects after 12 weeks placebo supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *ρ-value <0.05, comparison between before and after supplementation time points

		Fish oil			Placebo		
AUC		Before	After	**p-value**	Before	After	**p-value**
IL-6	**Lean**	19.51 (11.77-28.82)	14.07 (7.10-24.13)	0.06	13.34 (9.45-19.07)	15.37 (8.66-25.54)	0.33
	Obese	20.24 (15.86-30.88)	15.29 (12.13-24.17)	**<0.05**	16.98 (13.01-23.08)	20.75 (13.00-26.75)	0.29
IL-8	**Lean**	30.7 (18.54-39.09)	29.11 (15.37-33.09)	0.16	27.03 (17.56-33.21)	29.48 (18.24-38.37)	0.74
	Obese	32.53 (24.84-42.89)	29.67 (23.56-38.14)	0.44	37.04 (30.49-48.88)	31.78 (22.43-45.88)	0.16
GM-CSF	**Lean**	6.02 (2.19-10.02)	5.67 (2.21-10.36)	0.50	8.41 (4.50-12.06)	6.93 (3.16-12.71)	0.50
	Obese	7.51 (2.37-12.55)	7.25 (3.07-13.36)	0.35	7.05 (2.61-13.75)	6.63 (3.39-12.22)	0.29
TNF-α	**Lean**	54.97 (28.70-61.18)	47.63 (25.28-54.68)	0.20	41.07 (31.22-51.15)	45.16 (30.51-64.77)	0.88
	Obese	45.44 (39.41-58.47)	45.31 (38.26-53.29)	0.74	41.70 (26.42-52.62)	36.43 (24.94-54.16)	0.27
VEGF	**Lean**	95.44 (70.43-116.90)	77.01 (64.14-117.00)	0.35	109.40 (84.31-155.00)	136.60 (66.31-180.55)	0.61
	Obese	108.95 (83.92-179.72)	112.60 (67.19-167.97)	0.14	139.05 (112.85-201.25)	132.10 (104.06-165.73)	**<0.05**
IL-10	**Lean**	3.95 (2.72-5.130	3.85 (3.08-7.37)	0.16	4.15 (2.48-5.52)	4.16 (2.46-5.86)	0.85
	Obese	4.82 (2.35-5.73)	3.49 (2.19-4.98)	**0.01**	2.81 (1.75-3.68)	3.20 (1.84-3.78)	0.80

Data expressed as median and percentile 25th and 75th; differences before and after treatment were evaluated using paired t-test; all data were log transformed before analysis.

Table 8.6a AUC of cytokines following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects.

		Fish oil			Placebo		
iAUC		Before	After	**p-value**	Before	After	**p-value**
IL-6	Lean	7.05 (1.18-15.94)	3.90 (2.35-10.63)	0.48	5.24 (1.03-13.12)	7.27 (3.18-12.41)	**<0.05**
	Obese	5.62 (1.97-15.56)	3.22 (1.56-7.71)	0.38	2.85 (0.91-13.66)	4.71 (1.31-11.66)	0.16
IL-8	Lean	1.58 (0.43-7.75)	2.10 (0.21-4.77)	0.93	2.36 (1.08-5.61)	4.93 (2.42-9.90)	0.43
	Obese	4.33 (1.22-7.00)	2.98 (0.08-7.54)	0.24	8.41 (2.74-15.67)	4.25 (1.42-10.47)	0.35
GM-CSF	Lean	0.99 (0.36-2.44)	0.43 (0.18-1.96)	0.11	0.88 (0.00-4.01)	0.48 (0.00-2.91)	0.11
	Obese	0.34 (0.00-0.72)	0.89 (0.29-1.73)	0.72	0.42 (0.00-1.25)	0.52 (0.16-1.96)	0.64
TNF-α	Lean	0.89 (0.00-7.22)	2.34 (0.12-4.94)	0.95	2.37 (0.66-4.30)	0.97 (0.04-4.52)	0.67
	Obese	1.11 (0.21-2.33)	0.69 (0.04-3.61)	0.26	0.62 (0.09-4.06)	2.23 (0.07-3.63)	0.28
VEGF	Lean	10.74 (6.65-32.18)	17.26 (4.42-31.93)	0.91	16.82 (7.02-27.28)	35.18 (3.24-52.56)	0.72
	Obese	16.21 (3.32-35.34)	23.18 (6.78-38.29)	0.73	37.23 (26.11-58.33)	25.33 (7.55-34.49)	0.09
IL-10	Lean	0.26 (0.02-0.89)	0.25 (0.04-1.34)	0.91	0.36 (0.04-1.05)	0.70 (0.08-1.44)	0.63
	Obese	0.05 (0.00-0.52)	0.24 (0.07-1.03)	0.17	0.19 (0.07-0.29)	0.24 (0.00-0.43)	0.47

Data expressed as median and percentile 25th and 75th; differences before and after treatment were evaluated using paired t-test; all data were log transformed before analysis.

Table 8.6b iAUC of cytokines following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects.

AUC	Normal weight (n=34)		Obese (n=40)		Treatment	Obesity	Interaction
	Δ Fish oil	**Δ Placebo**	**Δ Fish oil**	**Δ Placebo**	**p-value**	**p-value**	**p-value**
IL-6	-3.44 (-6.97-0.96)	1.88 (-0.69-5.63)	-3.21 (-9.29-2.69)	1.92 (-3.01-7.25)	0.91	0.18	0.08
IL-8	-5.23 (-10.76-3.47)	1.85 (-4.04-9.16)	-1.29 (-4.76-2.54)	-5.47 (-14.57-6.94)	0.23	0.75	0.26
GM-CSF	-0.45 (-1.23-0.21)	0.03 (-0.47-1.38)	0.12 (-1.13-1.33)	0.09 (-1.0-0.58)	0.76	0.94	0.54
TNF-α	-3.26 (-6.05-(-1.32))	-0.16 (-4.65-8.75)	-4.38 (-8.79-4.27)	-1.25 (-3.75-1.15)	0.88	0.08	0.09
VEGF	-8.40 (-27.90-8.00)	25.90 (-22.75-54.54)	-14.82 (-43.15-9.43)	-27.99 (-58.85-9.80)	0.06	0.92	0.13
IL-10	-0.04 (-0.35-1.35)	0.42 (-0.05-1.11)	-0.25 (-2.52-0.18)	0.08 (-0.25-0.69)	**<0.05**	**0.00**	0.13

Data expressed as median and percentile 25th and 75th; the effect of treatment, group and interaction was evaluated using 2 way ANOVA; all data were adjusted for age and sex and were log transformed before analysis

Table 8.7a Changes in AUC for cytokines following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects.

iAUC	Normal weight (n=34)		Obese (n=40)		Treatment	Obesity	Interaction
	Δ Fish oil	**Δ Placebo**	**Δ Fish oil**	**Δ Placebo**	**p-value**	**p-value**	**p-value**
IL-6	-0.06 (-5.70-2.14)	0.53 (-2.57-3.36)	-1.73 (-8.15-0.43)	0.42 (-4.12-9.61)	0.81	0.12	0.10
IL-8	-0.26 (-2.53-1.89)	0.88 (-0.88-7.25)	-0.36 (-3.23-3.96)	-4.22 (-11.48-7.27)	0.46	0.91	0.68
GM-CSF	-0.17 (-1.45-0.42)	0.12 (-2.35-0.78)	0.47 (0.18-0.96)	0.20 (-1.12-1.14)	0.2	0.42	0.53
TNF-α	0.23 (-1.51-2.03)	-0.47 (-3.88-1.79)	-0.21 (-1.54-2.29)	0.59 (-1.38-2.99)	0.39	0.99	0.71
VEGF	-1.28 (-10.67-24.58)	10.34 (-13.45-31.94)	-1.37 (-14.00-12.84)	-20.71 (-49.35-(-0.59))	0.11	0.61	0.17
IL-10	-0.02 (-0.27-0.39)	0.00 (-0.82-0.64)	0.10 (-0.28-0.63)	0.01 (-0.21-0.28)	**0.01**	**0.01**	**0.01**

Data expressed as median and percentile 25th and 75th; the effect of treatment, group and interaction was evaluated using 2 way ANOVA; all data were adjusted for age and sex and were log transformed before analysis

Table 8.7b Changes in iAUC for cytokines following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects.

8.3.4 The effect of chronic fish oil supplementation on the postprandial adhesion molecule response in normal weight and obese subjects

The postprandial plasma concentrations of various adhesion molecules were evaluated after fish oil and placebo 12 weeks supplementation as described in **Figures 8.8**-**8.11** and **Tables 8.7 and 8.8**.

Fish oil supplementation reduced significantly the plasma concentration of P-selectin from 0 h to 4 h in normal weight subjects after a HFM ($\rho<0.05$) (**Figure 8.8**). Similarly, P-selectin AUC was reduced after fish oil supplementation compared with baseline in the normal weight subjects (**Table 8.7**).

ANOVA analysis revealed a significant reduction of AUC and iAUC for postprandial E-selectin in subjects receiving fish oil supplementation when compared with placebo ($\rho<0.05$) (**Table 8.8a and 8.8b**). Furthermore a similar effect was observed for ICAM AUC ($\rho<0.05$) (**Table 8.8a**).

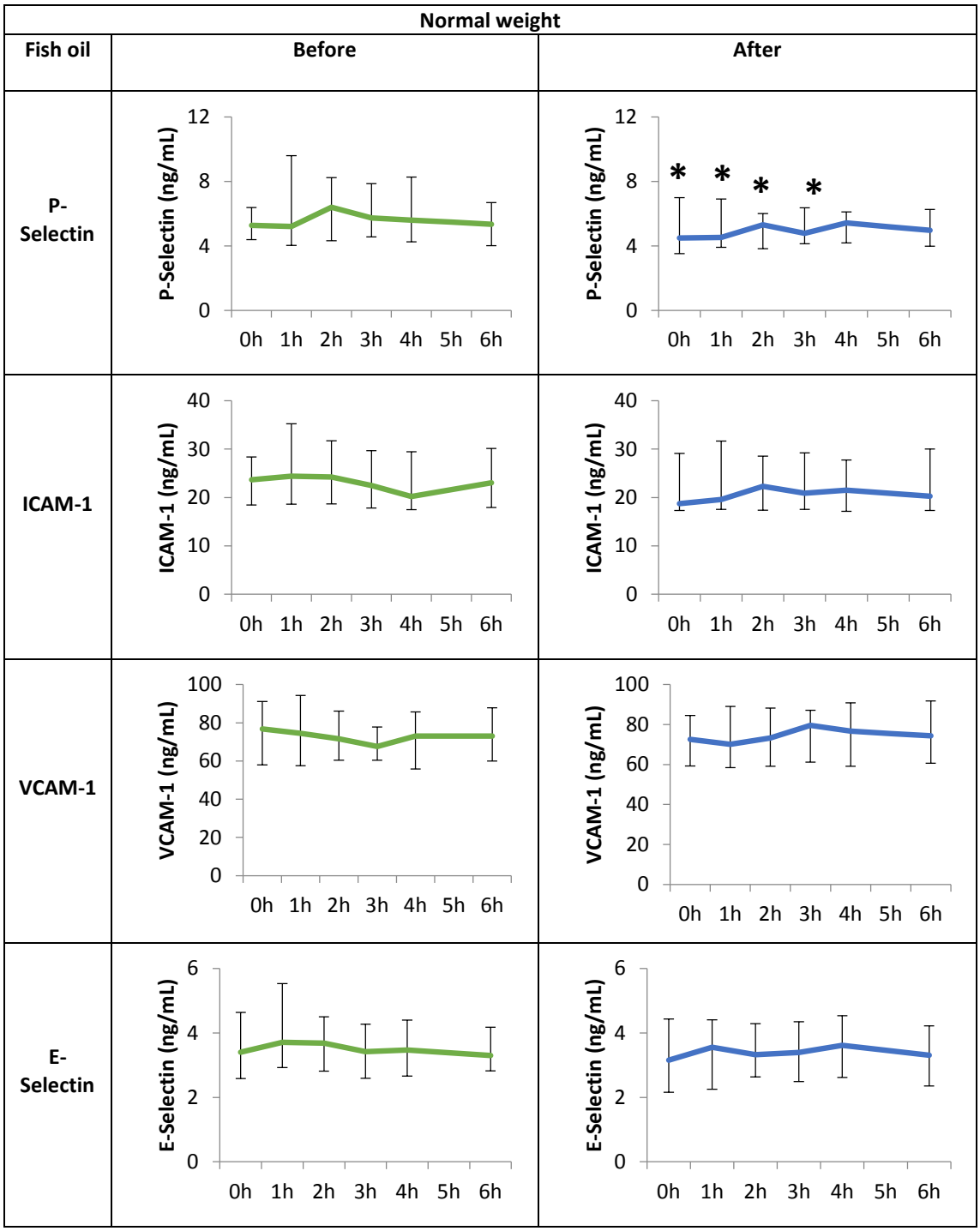


Figure 8.8 Adhesion molecules responses to a HFM challenge in normal weight subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points

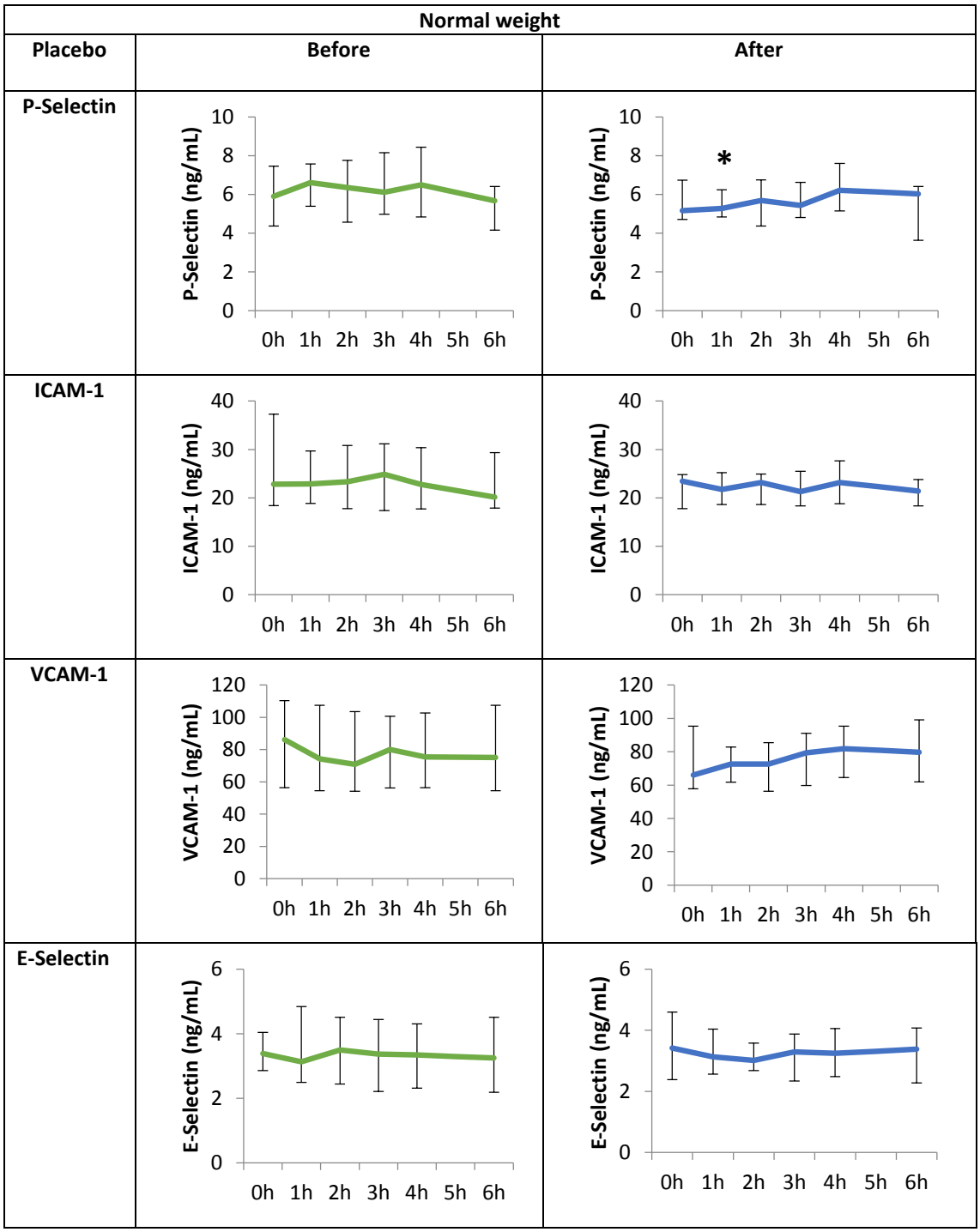


Figure 8.9 Adhesion molecules responses to a HFM challenge in normal weight subjects after 12 weeks placebo supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points

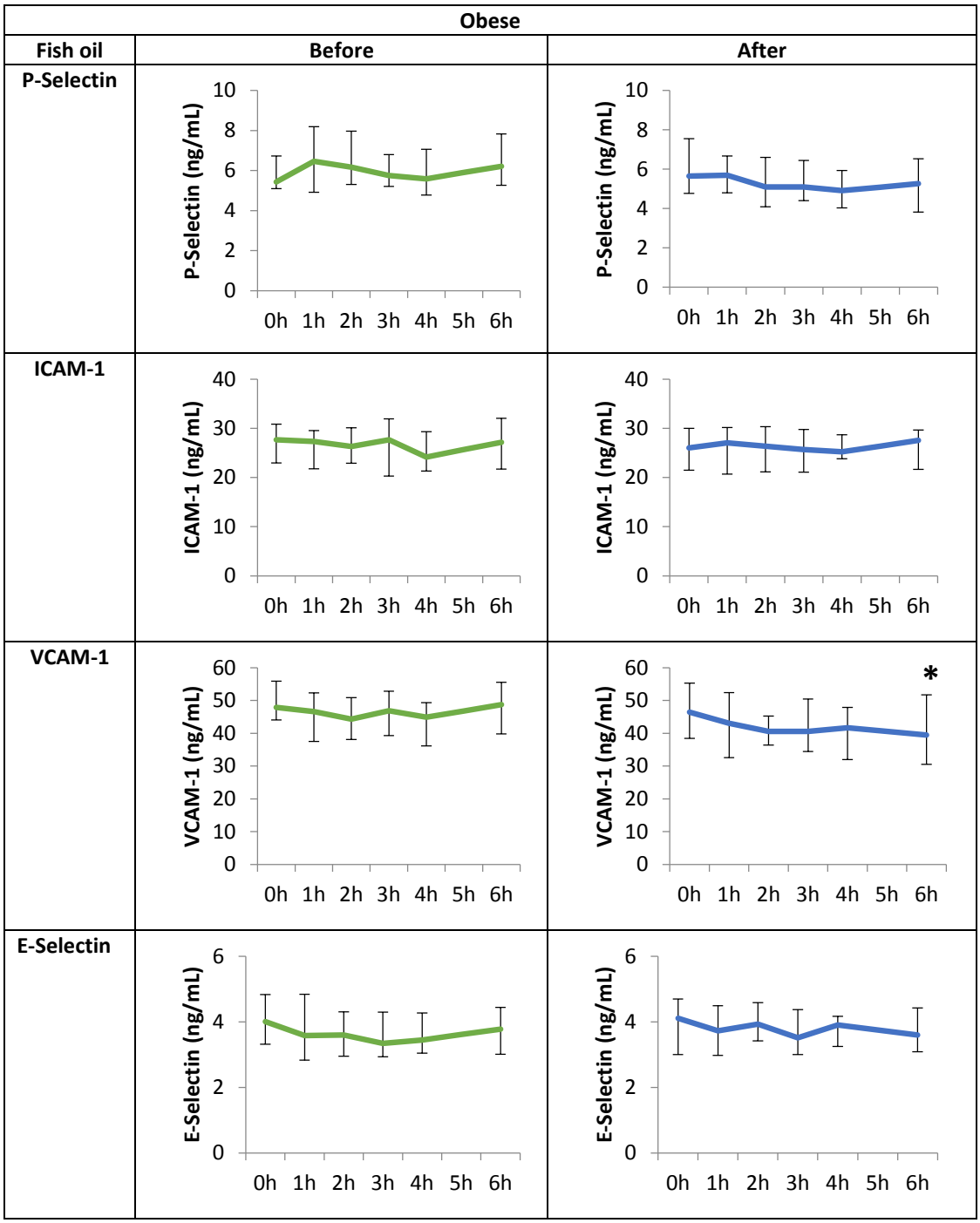


Figure 8.10 Adhesion molecules responses to a HFM challenge in obese subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *ρ-value <0.05, comparison between before and after supplementation time points

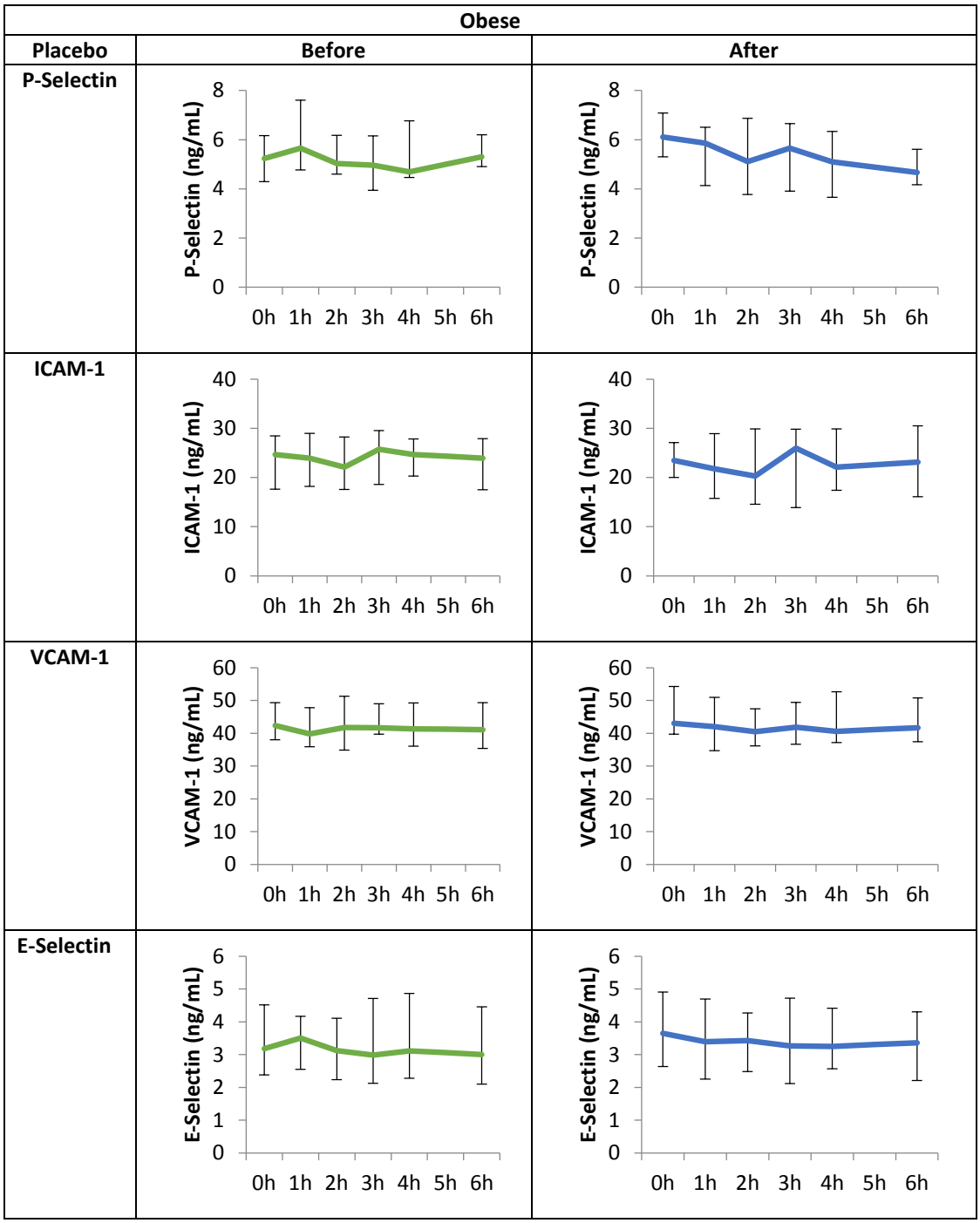


Figure 8.11 Adhesion molecules responses to a HFM challenge in obese subjects after 12 weeks placebo supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points

AUC		Fish oil			Placebo		
		Before	After	p-value	Before	After	p-value
P-Selectin	Lean	35.58 (26.89-49.56)	29.17 (25.80-37.80)	**<0.01**	40.28 (28.50-45.51)	36.19 (28.28-39.63)	0.07
	Obese	36.51 (31.66-44.59)	31.75 (26.78-38.03)	0.24	32.19 (22.26-37.30)	32.91 (24.24-37.06)	0.72
ICAM-1	Lean	143.90 (110.05-183.07)	124.20 (107.30-182.30)	0.23	140.80 (107.15-183.45)	131.50 (117.95-145.15)	0.30
	Obese	154.65 (137.53-183.00)	163.35 (129.60-174.40)	0.79	140.90 (114.15-165.85)	133.25 (92.85-177.25)	0.46
VCAM-1	Lean	449.90 (356.30-513.10)	434.80 (366.40-536.80)	0.83	457.00 (344.10-629.55)	473.20 (373.30-544.40)	0.56
	Obese	283.30 (222.83-309.50)	236.20 (210.45-303.18)	0.12	242.00 (219.77-289.60)	247.95 (224.72-287.20)	0.98
E-Selectin	Lean	20.36 (16.55-28.42)	20.45 (14.79-25.85)	0.59	20.65 (14.71-27.14)	19.58 (14.46-23.99)	0.75
	Obese	22.48 (18.06-25.96)	22.29 (19.03-27.00)	0.57	18.92 (13.61-27.38)	19.77 (13.43-27.51)	0.07

Data expressed as median and percentile 25th and 75th; differences before and after treatment were evaluated using paired t-test; all data were log transformed before analysis.

Table 8.8a AUC for adhesion molecules following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

iAUC		Fish oil			Placebo		
		Before	After	**p-value**	Before	After	**p-value**
P-Selectin	Lean	2.06 (0.57-6.46)	2.75 (0.21-5.63)	0.85	0.95 (0.29-3.98)	2.27 (0.47-5.15)	**<0.01**
	Obese	3.21 (0.81-6.81)	0.76 (0.00-3.23)	0.62	1.36 (0.62-3.06)	0.19 (0.00-2.71)	**<0.01**
ICAM-1	Lean	3.11 (0.74-11.79)	4.26 (0.73-11.32)	0.68	0.63 (0.06-3.06)	2.21 (0.17-12.91)	0.53
	Obese	2.81 (0.00-8.34)	3.83 (0.00-12.38)	0.54	2.01 (0.35-16.43)	4.68 (0.00-15.95)	0.43
VCAM-1	Lean	5.68 (0.00-35.61)	12.84 (0.19-58.17)	0.40	0.03 (0.00-22.43)	5.53 (0.09-41.63)	0.63
	Obese	1.27 (0.00-6.48)	2.57 (0.00-11.31)	0.28	0.32 (0.00-11.48)	8.03 (0.00-24.45)	0.23
E-Selectin	Lean	0.64 (0.03-1.47)	0.45 (0.08-1.33)	0.78	0.00 (0.00-0.36)	0.40 (0.01-1.64)	0.21
	Obese	0.01 (0.00-0.43)	0.12 (0.00-1.66)	0.14	0.64 (0.05-1.13)	0.11 (0.00-1.67)	0.35

Data expressed as median and percentile 25th and 75th; differences before and after treatment were evaluated using paired t-test; all data were log transformed before analysis.

Table 8.8b iAUC for adhesion molecules following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

AUC	Normal weight (n=34)		Obese (n=40)		Treatment	Obesity	Interaction
	Δ Fish oil	**Δ Placebo**	**Δ Fish oil**	**Δ Placebo**	**p-value**	**p-value**	**p-value**
P-selectin	-2.64 (-14.05-0.25)	-4.21 (-7.56-1.65)	-2.55 (-8.66-(-1.06))	-0.18 (-5.47-4.39)	0.08	0.45	0.27
ICAM-1	-4.40 (-31.55-11.12)	-4.10 (-15.55-4.25)	0.05 (-10.07-5.97)	2.82 (-16.66-11.25)	0.29	0.69	0.73
VCAM-1	4.40 (-44.05-39.70)	-20.30 (-72.25-29.95)	-14.00 (-67.42-14.13)	-4.35 (-34.85-5.07)	0.85	0.88	0.91
E-selectin	-0.38 (-4.32-2.16)	0.05 (-2.94-2.71)	0.55 (-0.75-1.63)	0.98 (-0.92-2.73)	**0.01**	0.23	0.46

Data expressed as median and percentile 25th and 75th; the effect of treatment, group and interaction was evaluated using 2 way ANOVA; all data were adjusted for age and sex and were log transformed before analysis

Table 8.9a Changes in AUC for adhesion molecules following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

iAUC	Normal weight (n=34)		Obese (n=40)		Treatment	Obesity	Interaction
	Δ Fish oil	**Δ Placebo**	**Δ Fish oil**	**Δ Placebo**	**p-value**	**p-value**	**p-value**
P-selectin	0.00 (-5.98-3.06)	-0.31 (-1.38-3.86)	-0.68 (-4.30-0.12)	-0.61 (-1.85-0.37)	0.98	0.76	0.99
ICAM-1	-0.82 (-5.47-2.42)	1.25 (-1.29-12.28)	0.63 (-0.88-7.29)	-0.63 (-2.12-12.99)	**<0.05**	0.31	0.69
VCAM-1	0.00 (-15.82-21.22)	1.67 (-20.38-17.88)	0.00 (-0.61-4.40)	7.83 (0.00-16.72)	0.33	0.94	0.78
E-selectin	0.06 (-0.45-0.67)	0.03 (-0.35-1.09)	0.02 (0.00-0.58)	-0.03 (-0.93-1.20)	**<0.05**	0.07	0.79

Data expressed as median and percentile 25th and 75th; the effect of treatment, group and interaction was evaluated using 2 way ANOVA; all data were adjusted for age and sex and were log transformed before analysis

Table 8.9b Changes of iAUC for adhesion molecules following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

8.3.5 The effect of chronic fish oil supplementation on the postprandial adipokine response in normal weight and obese subjects

The changes induced by fish oil and placebo in the postprandial adipokines response are shown in **Figure 8.12**- **8.15** and **Table 8.10** and **8.11**.

In normal weight subjects, the postprandial adipsin and PAI-1 concentrations were reduced at 0 h and 6 h, respectively, after chronic fish oil consumption (**Figure 8.12a** and **8.12b**) ($\rho<0.05$). In the obese group, postprandial CRP concentration were reduced between 1 h- 5 h after fish oil supplementation ($\rho<0.05$). Moreover, fish oil lowered postprandial resistin concentrations between 2 h – 4 h and PAI-1 at 6 h in the obese group ($\rho<0.05$) (**Figure 8.14a** and **8.14b**).

After fish oil treatment the AUC for CRP and resistin response was significantly smaller for the obese subjects ($\rho<0.05$) (**Table 8.10**). Furthermore, MCP-1 and adipsin AUC were reduced after fish oil intervention in normal weight subjects ($\rho<0.05$) (**Table 8.10**). The changes in the postprandial PAI-1 response induced by fish oil were lower than those observed following supplementation with the placebo (**Table 8.11**). Normal weight and obese subjects responded differently to the fish oil and placebo treatment as shown in **Table 8.11**.

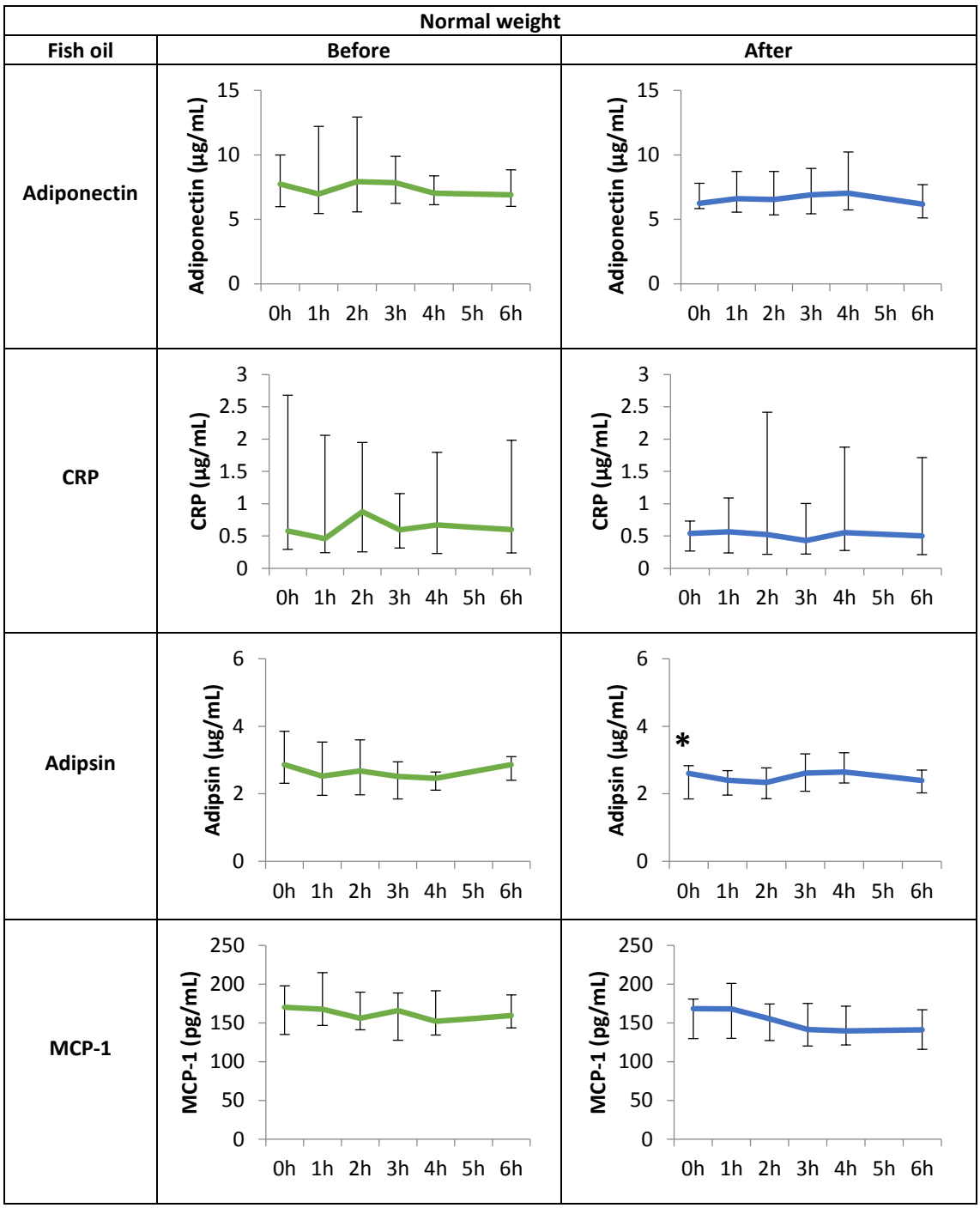


Figure 8.12a Adipokine responses to a HFM challenge in normal weight subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points

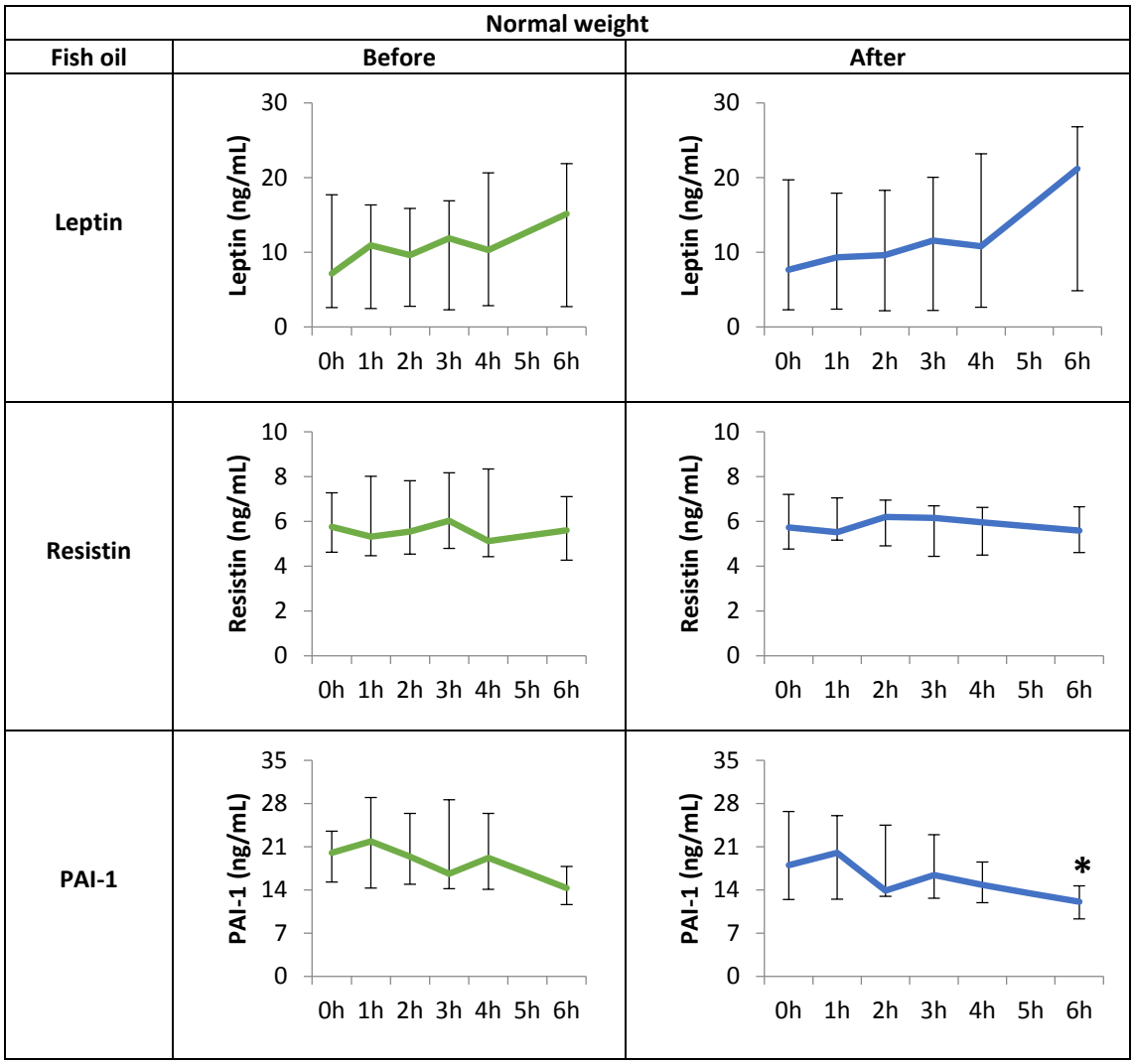


Figure 8.12b Adipokine responses to a HFM challenge in normal weight subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation.Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points

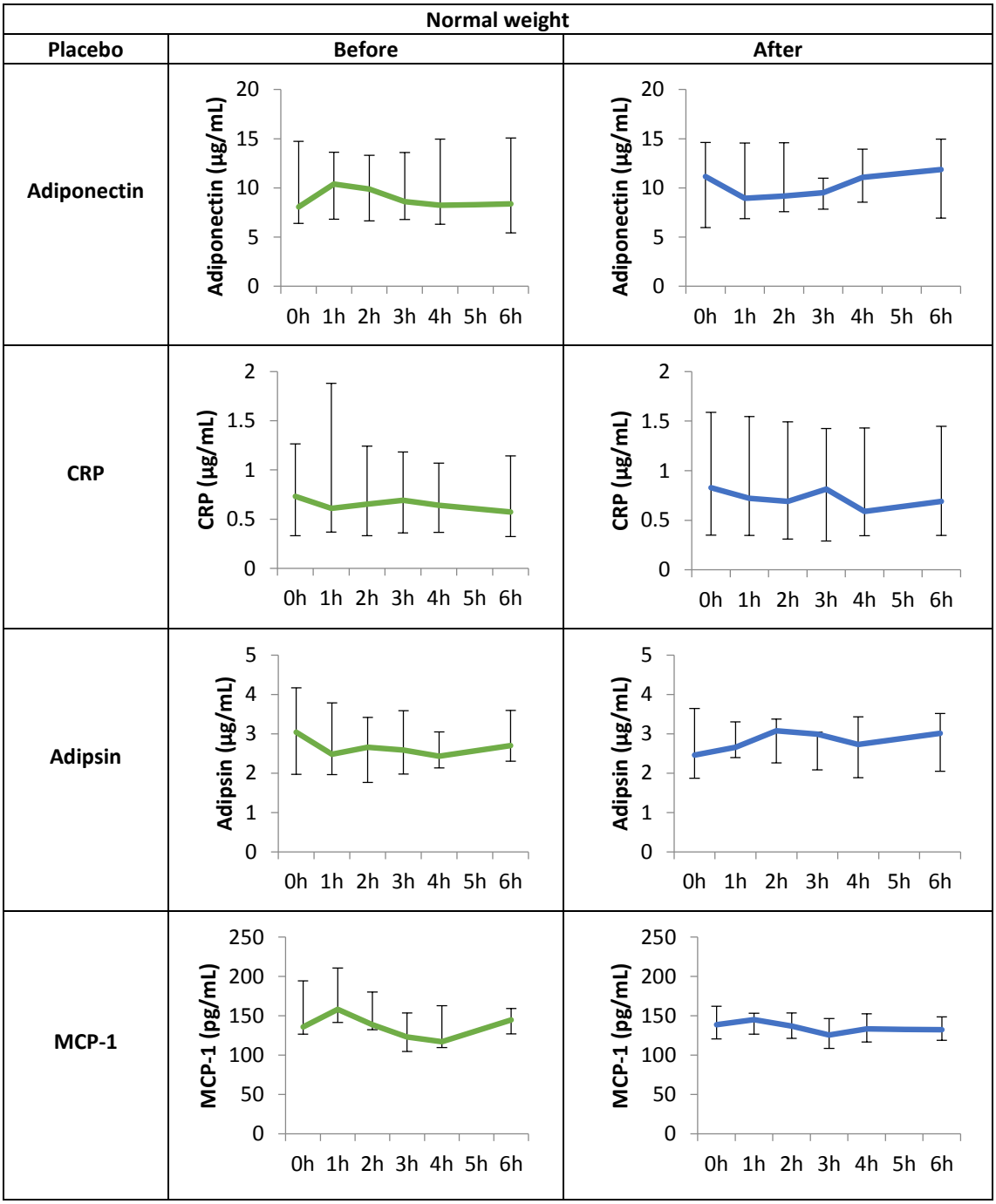


Figure 8.13a Adipokines responses to a HFM challenge in normal weight subjects after 12 weeks placebo supplementation Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points

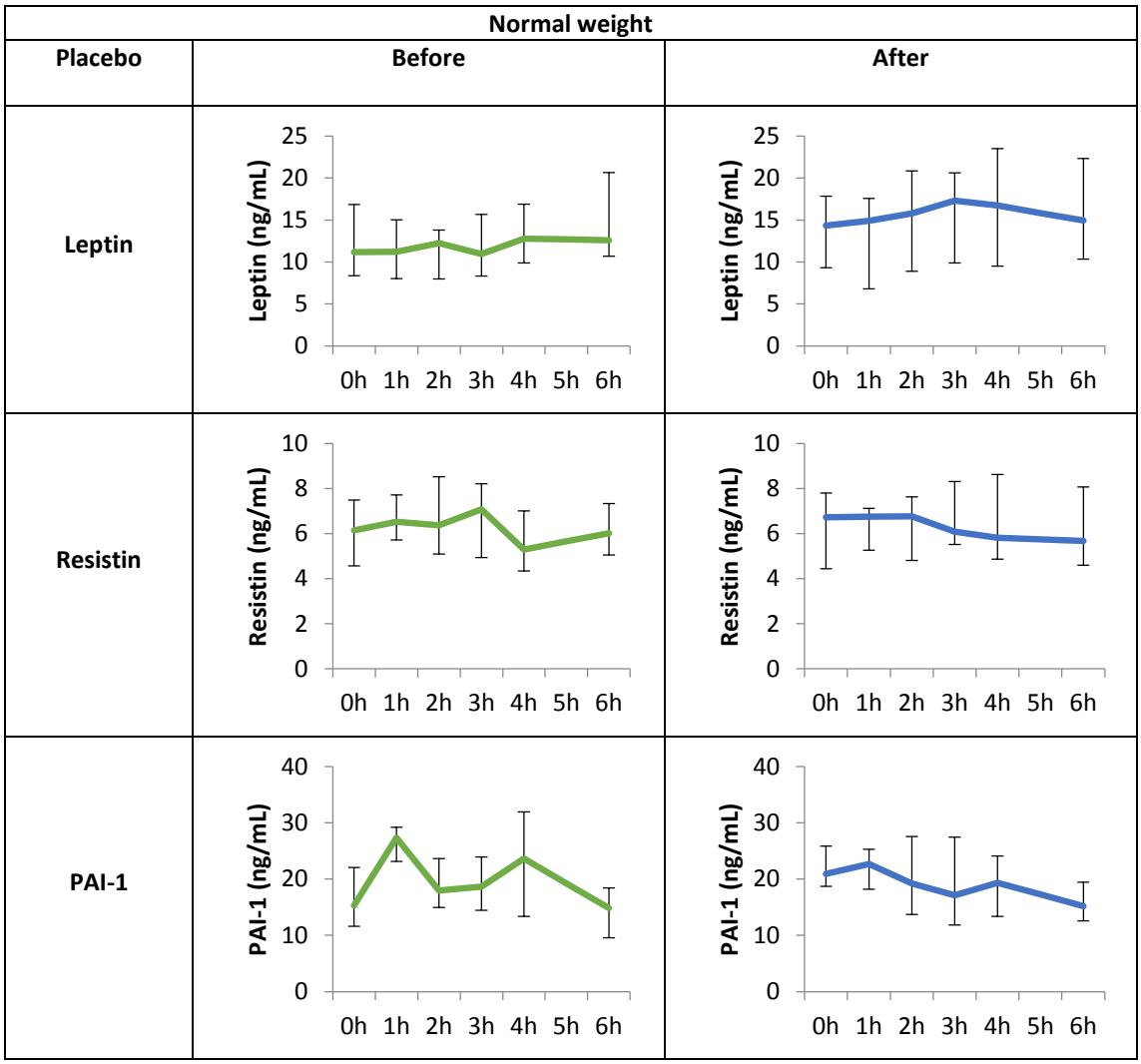


Figure 8.13b Adipokines responses to a HFM challenge in normal weight subjects after 12 weeks placebo supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *ρ-value <0.05, comparison between before and after supplementation time points

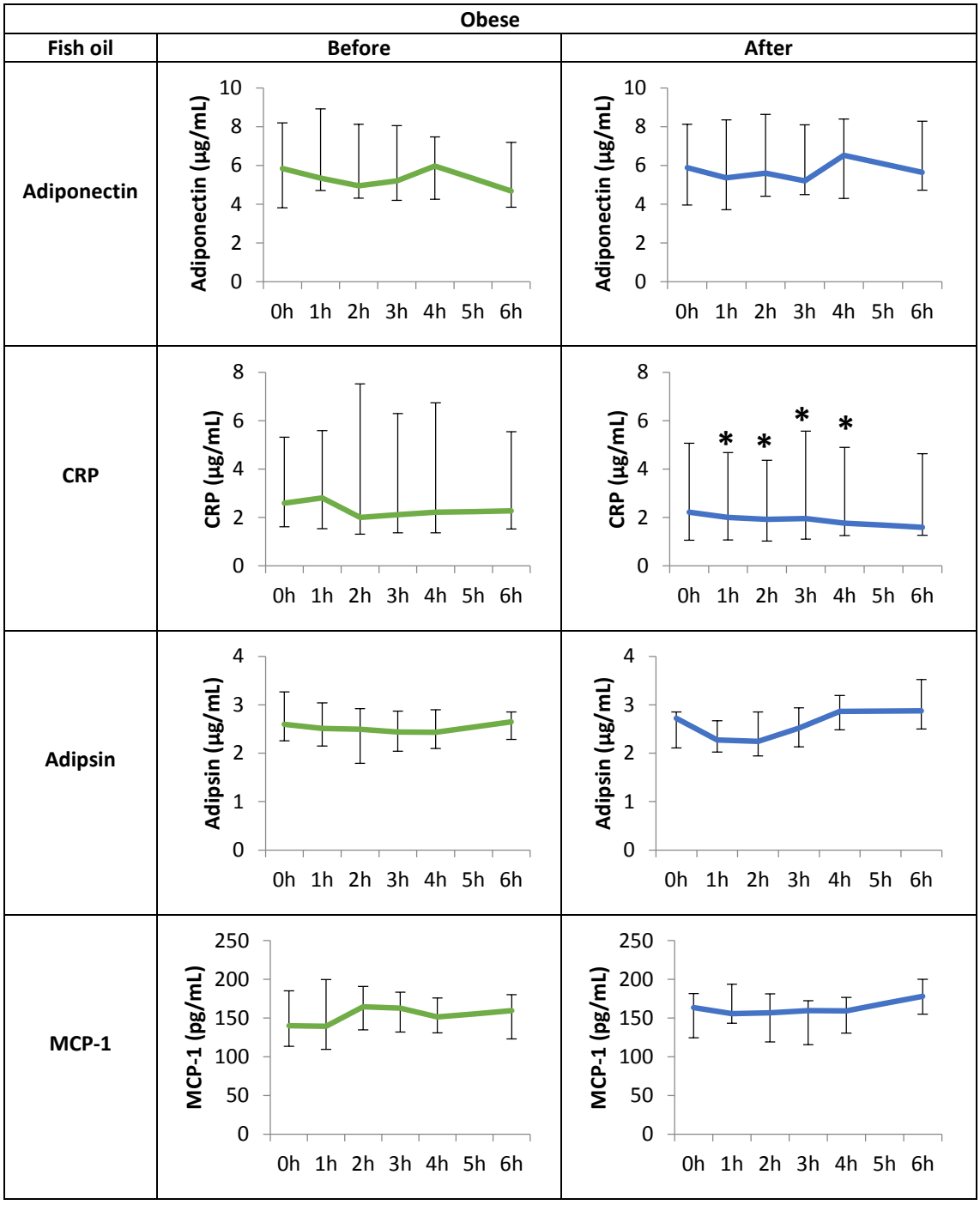


Figure 8.14a Adipokines responses to a HFM challenge in obese subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points

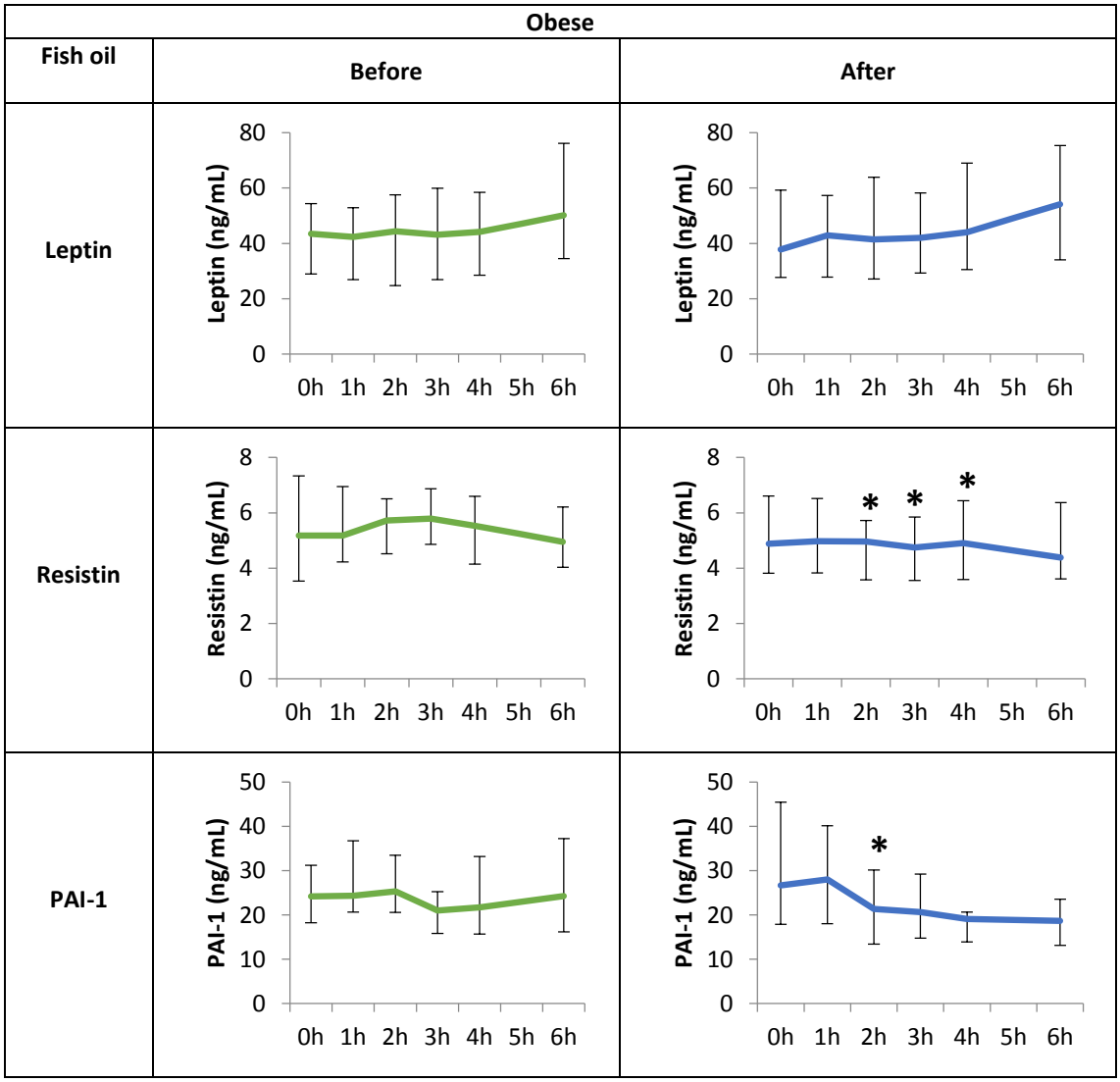


Figure 8.14b Adipokines responses to a HFM challenge in obese subjects after 12 weeks fish oil supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points

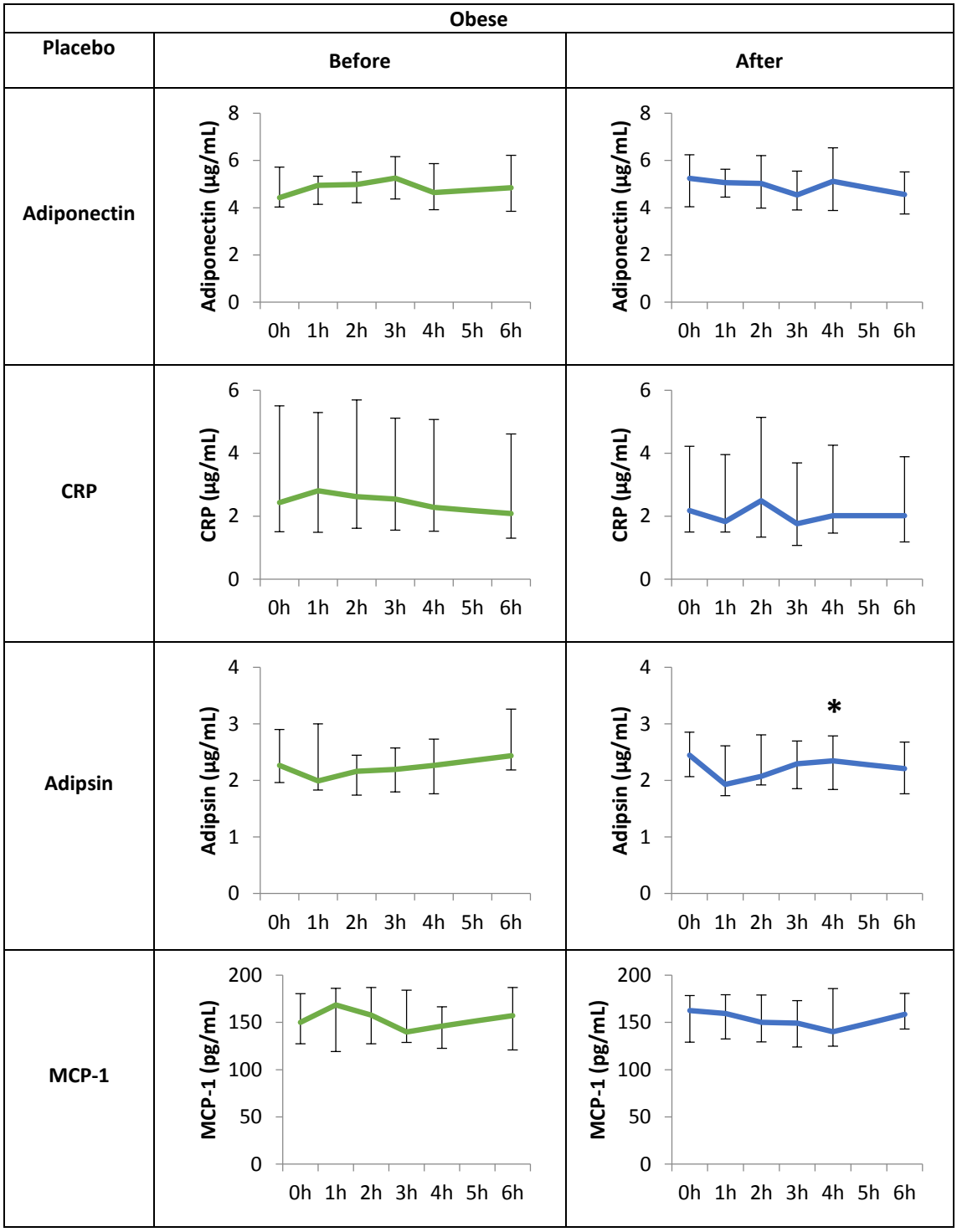


Figure 8.15a Adipokines responses to a HFM challenge in obese subjects after 12 weeks placebo supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *p-value <0.05, comparison between before and after supplementation time points

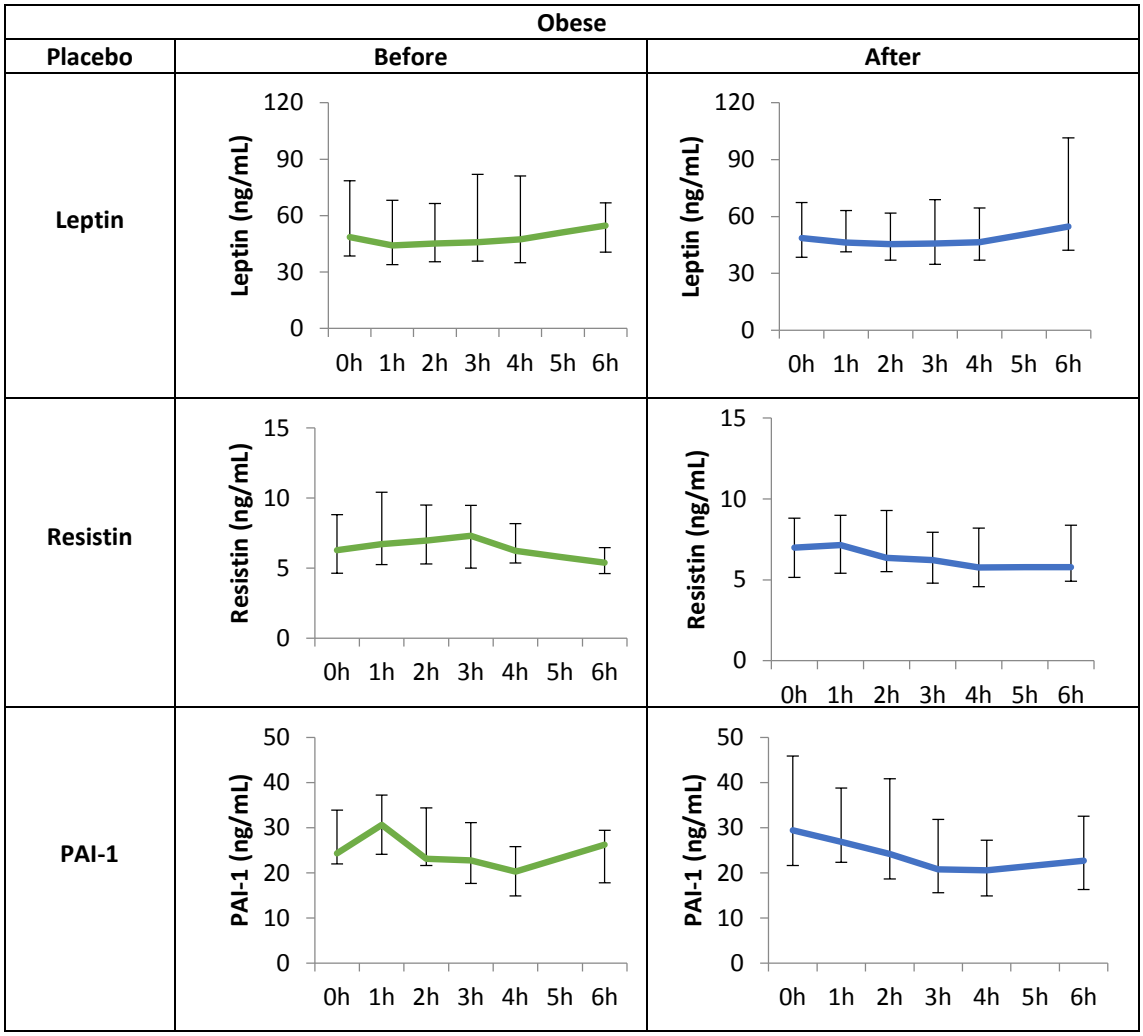


Figure 8.15b Adipokines responses to a HFM challenge in obese subjects after 12 weeks placebo supplementation. Before (-**green**-) or after (-**blue**-) fish oil or placebo supplementation. Data are expressed as median concentration with 25th and 75th centile bars; *ρ-value <0.05, comparison between before and after supplementation time points

AUC		Fish oil			Placebo		
		Before	After	ρ-value	Before	After	ρ-value
Adiponectin	Lean	4.39 (3.67-6.07)	4.11 (3.29-4.87)	0.13	5.31 (3.73-9.18)	6.84 (4.53-7.78)	0.20
	Obese	3.06 (2.62-4.64)	3.46 (2.62-4.97)	0.62	3.00 (2.53-3.37)	2.97 (2.49-3.57)	0.56
CRP	Lean	0.42 (0.15-1.05)	0.32 (0.14-0.94)	0.61	0.38 (0.21-0.81)	0.41 (0.19-0.89)	0.97
	Obese	1.38 (0.86-3.78)	1.10 (0.71-3.04)	**<0.05**	1.48 (0.90-3.15)	1.28 (0.78-2.58)	0.45
Adipsin	Lean	1.61 (1.26-1.93)	1.39 (1.28-1.64)	**<0.05**	1.46 (1.27-2.26)	1.66 (1.32-2.02)	0.72
	Obese	1.50 (1.27-1.74)	1.56 (1.34-1.81)	0.15	1.35 (1.19-1.65)	1.33 (1.14-1.66)	0.36
MCP-1	Lean	1015.00 (829.35-1117.00)	899.20 (735.70-1052.00)	**<0.05**	844.50 (763.45-1022.75)	782.00 (733.85-851.45)	0.19
	Obese	909.55 (805.83-1140.75)	962.75 (795.87-1099.00)	0.94	908.50 (769.82-1055.25)	885.70 (798.63-1034.75)	0.71
Leptin	Lean	67.60 (15.22-114.03)	64.29 (20.20-123.94)	0.44	77.93 (56.29-100.61)	98.01 (57.53-131.09)	0.19
	Obese	271.06 (170.36-358.08)	264.41 (188.68-384.31)	0.98	284.37 (212.34-486.06)	282.01 (257.93-395.48)	0.86
Resistin	Lean	33.91 (27.43-47.19)	35.78 (28.36-40.730	0.37	37.32 (30.71-45.81)	36.25 (31.27-47.39)	0.99
	Obese	30.60 (27.05-38.42)	29.66 (22.14-36.64)	**<0.05**	41.43 (31.18-49.99)	40.16 (30.44-52.35)	0.65
PAI-1	Lean	10.52 (8.84-16.13)	10.75 (8.06-12.00)	0.31	12.54 (8.57-14.58)	10.63 (8.89-14.71)	0.73
	Obese	14.53 (10.67-22.29)	14.42 (8.94-16.19)	0.17	15.41 (13.03-18.27)	14.13 (11.14-20.63)	0.40

Data expressed as median and percentile 25th and 75th; differences before and after treatment were evaluated using paired t-test; all data were log transformed before analysis.

Table 8.10a AUC for adipokines following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

iAUC		Fish oil			Placebo		
Adiponectin	Lean	0.11 (0.00-0.66)	0.32 (0.01-1.04)	0.54	0.21 (0.04-0.46)	0.36 (0.10-0.81)	0.17
	Obese	0.21 (0.00-0.56)	0.14 (0.03-0.48)	0.52	0.30 (0.09-0.59)	0.06 (0.00-0.40)	0.08
CRP	Lean	0.00 (0.00-0.03)	0.01 (0.00-0.04)	0.71	0.00 (0.00-0.09)	0.00 (0.00-0.01)	0.67
	Obese	0.11 (0.00-0.39)	0.08 (0.02-0.15)	0.98	0.12 (0.00-0.35)	0.05 (0.00-0.30)	0.08
Adipsin	Lean	0.00 (0.00-0.23)	0.06 (0.00-0.28)	0.19	0.02 (0.00-0.09)	0.04 (0.00-0.15)	0.06
	Obese	0.00 (0.00-0.08)	0.12 (0.00-0.23)	0.87	0.04 (0.02-0.14)	0.01 (0.00-0.08)	0.23
MCP-1	Lean	0.00 (0.00-74.26)	2.90 (0.00-68.87)	0.85	22.52 (4.89-75.30)	21.07 (0.39-52.34)	0.45
	Obese	41.65 (14.49-101.97)	42.78 (2.96-86.28)	0.66	7.97 (1.17-42.93)	23.02 (0.39-73.42)	0.72
Leptin	Lean	6.19 (0.25-15.25)	9.75 (0.93-22.85)	0.78	7.64 (1.01-10.93)	6.21 (2.51-20.17)	0.80
	Obese	12.51 (1.59-30.85)	29.81 (0.34-60.88)	0.64	21.12 (8.69-31.74)	9.21 (1.95-30.60)	0.89
Resistin	Lean	1.13 (0.58-4.41)	0.57 (0.00-3.63)	0.15	0.38 (0.23-2.55)	0.86 (0.00-8.44)	0.54
	Obese	1.23 (0.15-5.66)	0.56 (0.10-2.16)	0.90	2.71 (0.41-6.40)	1.49 (0.00-3.92)	0.09
PAI-1	Lean	0.83 (0.11-2.93)	0.71 (0.00-2.35)	0.12	1.25 (0.00-4.33)	0.22 (0.03-3.69)	0.32
	Obese	0.63 (0.00-3.25)	0.11 (0.00-1.39)	0.61	0.60 (0.13-3.29)	0.02 (0.00-1.83)	0.09

Data expressed as median and percentile 25th and 75th; differences before and after treatment were evaluated using paired t-test; all data were log transformed before analysis.

Table 8.10b iAUC for adipokines following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

AUC	Normal weight (n=34)		Obese (n=40)		Treatment	Obesity	Interaction
	Δ Fish oil	**Δ Placebo**	**Δ Fish oil**	**Δ Placebo**			
Adiponectin	-1.90 (-13.04-3.59)	0.32 (-8.12-10.22)	0.90 (-4.22-5.26)	-0.32 (-2.40-3.10)	0.07	0.18	0.07
CRP	0.57 (-1.66-3.01)	0.19 (-1.07-1.37)	-2.03 (-8.91-(-0.41))	0.94 (-7.27-4.83)	0.07	0.11	0.42
Adipsin	-0.27 (-2.81-0.28)	0.19 (-1.28-2.29)	0.49 (-0.75-1.36)	-0.54 (-1.24-0.96)	0.09	0.68	0.38
MCP-1	-148.50 (-214.05-24.40)	-24.00 (-116.60-53.65)	-19.00 (-78.37-74.70)	38.30 (-86.25-129.85)	0.06	0.44	0.06
Leptin	4.55 (-4.76-31.41)	4.33 (-7.84-30.02)	3.01 (-12.20-44.85)	3.17 (-42.19-58.65)	0.59	0.52	0.65
Resistin	0.48 (-8.72-2.94)	1.39 (-5.72-8.33)	-1.35 (-5.22-1.12)	0.81 (-5.93-3.31)	0.53	0.29	0.69
PAI-1	-12.55 (-48.97-5.81)	9.98 (-16.25-18.29)	-4.01 (-35.00-22.32)	-3.78 (-30.83-20.35)	**<0.05**	0.65	0.94
Data expressed as median and percentile 25th and 75th; the effect of treatment, group and interaction was evaluated using 2 way ANOVA; all data were adjusted for age and sex and were log transformed before analysis							

Table 8.11a Changes in AUC for adipokines following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

iAUC	Normal weight (n=34)		Obese (n=40)		Treatment	Obesity	Interaction
	Δ Fish oil	**Δ Placebo**	**Δ Fish oil**	**Δ Placebo**			
Adiponectin	0.10 (-2.03-5.51)	1.55 (-2.17-7.02)	0.15 (-4.09-2.19)	-0.89 (-1.53-0.56)	0.36	0.99	0.65
CRP	0.00 (-0.03-0.24)	0.00 (-0.89-0.04)	-1.01 (-2.63-0.71)	0.04 (-1.46-0.72)	0.50	**<0.05**	0.31
Adipsin	0.13 (-0.16-0.86)	0.47 (-0.16-1.13)	0.81 (0.00-1.67)	-0.09 (-0.84-0.22)	0.63	**<0.05**	0.16
MCP-1	0.00 (-62.43-37.78)	0.00 (-14.51-18.91)	0.12 (-52.61-50.63)	8.74 (-30.52-57.26)	0.68	0.93	0.98
Leptin	1.73 (-0.92-12.95)	0.84 (-0.59-12.27)	3.61 (-2.12-47.88)	-3.00 (-22.91-20.96)	0.26	0.38	0.42
Resistin	-0.63 (-1.37-1.08)	0.00 (-0.46-5.24)	-0.49 (-4.78-1.55)	-0.18 (-2.76-1.94)	0.29	0.22	0.36
PAI-1	-0.48 (-17.17-4.07)	0.00 (-26.85-14.52)	-2.91 (-27.54-0.00)	-1.46 (-20.29-8.41)	0.34	**<0.01**	**<0.01**
Data expressed as median and percentile 25th and 75th; the effect of treatment, group and interaction was evaluated using 2 way ANOVA; all data were adjusted for age and sex and were log transformed before analysis							

Table 8.11b Changes on iAUC for adipokines following a HFM after 12 weeks supplementation with fish oil or placebo in normal weight and obese subjects

8.4 Discussion

Some studies report that n-3 PUFA supplementation is associated with a reduction of fasting plasma concentrations of metabolic and inflammatory markers (triglycerides, adhesion molecules and inflammatory cytokine secretion). However, there is not sufficient evidence regarding the role of n-3 PUFA in the regulation of postprandial inflammation. In this section, the effects of chronic n-3 PUFA consumption in postprandial metabolism and inflammation were described. **Table 8.16** summarizes the results reported in this chapter.

Previous reports have shown a reduction in triglycerides during the postprandial response in healthy normal weight subjects; the results shown in this chapter are in agreement with these studies (Brown and Roberts,. 1991; Shirner *et al.* 2012; Jackson *et al.* 2012). Nevertheless these positive effects were not extended to the obese group. These differences are unexpected, as previous studies have shown a reduction of postprandial triglycerides in overweight and obese hypertriglyceridemic patients (Tinker *et al.* 1999; Maki *et al.* 2011). The doses used in this study are lower than those studies (1.89g/day vs 3.5-5 g /day), thus these contrasts may partly explain the differences in the treatment effect.

An increase in postprandial insulin concentration was also observed in obese subjects after fish oil supplementation. The role of n-3 PUFA in glucose metabolism regulation has not been clarified. Studies in obese rats reported an improvement in glucose tolerance after a n-3 PUFA diet (Luo *et al.* 1996; Yamazaki *et al.* 2011), while findings on glucose tolerance are inconsistent in human clinical trials of fish oil (Flachs, 2013). *In vitro*, long chain fatty acids (including n-3 PUFA) can bind G-protein coupled cell membrane receptors, including GPR40 and GPR120 (Itoh *et al.* 2003; Oh *et al.* 2010). GPR40 receptors are expressed in beta islets (rat tissue) at comparable levels to type A cholecystokinin receptors and glucagon like peptide 1 receptor (Itoh *et al.* 2003). Long chain fatty acids increases insulin secretion in MIN6 cells (mouse insulinoma cell line) through these receptors. Even though these observations were based in murine cells if translated to humans, the n-3 PUFA binding to GPRs may explain the increased postprandial insulin concentrations observed in this study.

After n-3 PUFA supplementation, the IL-6 postprandial response was reduced in obese subjects. This finding is consistent with a previous study documenting a similar response in hypertriglyceridemic subjects after fish oil supplementation (combined with a diet and exercise) (Derosa *et al.* 2012). Moreover, Jellema *et al* (2012) reported a similar trend in healthy obese subjects after a shorted period of fish oil supplementation (6 weeks). In parallel to the reduction of postprandial IL-6 concentrations, a reduction in CRP and resistin responses was observed in obese subjects in this study. As described in **Chapter 4**, CRP is produced by hepatocytes under IL-6 regulation and its synthesis is activated rapidly in the presence of an inflammatory stimulus. Resistin is synthesized by adipose tissue and expressed at high levels in human liver (Szalowska *et al.* 2009). Moreover, *in vitro* studies in a human hepatocyte cell line (HEPG2) show that EPA and DHA treatment (100 μM) prior to IL-6 stimulation cause a reduction in CRP expression (Wang *et al.* 2013). In human studies, the postprandial resistin response was lowered by fish oil supplementation (Derosa *et al.* 2011). Together these results suggest a down regulation of inflammatory signals after fish oil supplementation, either provided by changes in signalling pathways (NF-κB) in circulating monocytes, or a reduction of inflammation in the liver or adipose tissue.

Normal weight subjects		Obese subjects		Both groups
Reduced after FO	**Increased after FO**	**Reduced after FO**	**Increased after FO**	**No changes**
Metabolism				
iAUC: Triglycerides	-	-	**AUC:** Insulin **iAUC:** Insulin	**AUC**: NEFA, Glucose, Triglycerides **iAUC**: NEFA, Glucose
Time points: Triglycerides: 4 h & 6 h.	**Time points:** Glucose: 4 h	-	-	-
TLR expression on monocytes				
-	-	**AUC:** MFI $CD14^+TLR2^+$	-	**AUC**: All except AUC MFI $CD14^+TLR2^+$; **iAUC**: All
Time points: $\%CD14^+TLR2^+$: 4 h; $\%CD14^+TLR4^+$: 1 h, 2 h ;MFI $CD14^+TLR4^+$: 2 h, 3 h & 4 h	-	-	-	-
Cytokines				
-	-	**AUC**: IL-6 & IL-10	-	**AUC**: VEGF, TNF-α, GM-CSF & IL-8; **iAUC**: All
-	-	**Time points:** IL-10: 0 h, 1 h, 3 h, 4 h & 6 h; IL-6: 4 h & 6 h	-	-
Adhesion Molecules				
AUC: P-selectin	-	-	-	**AUC:** all except P-selectin; **iAUC**: all
Time points: P-selectin: 0 h, 1 h, 2 h & 3 h; ICAM-1: 0 h	-	**Time points:** VCAM: 6 h	-	-
Adipokines				
AUC: Adipsin & MCP-1	-	**AUC:** CRP & Resistin	-	**AUC**: Leptin, adiponectin & PAI-1; **iAUC**: all
Time points: Adipsin: 0 h; PAI-1: 1 h	-	**Time points:** CRP: 1 h, 2 h, 3 h & 4 h Resistin: 2 h, 3 h, & 4 h PAI-1: 2 h; Adipsin: 4 h	-	-

Table 8.12 Summary of the main effects of fish oil supplementation on postprandial metabolism and inflammation

In addition to the changes in cytokines and adipokines, the postprandial % of TLR-2 monocytes was found at significantly lower levels after fish oil supplementation. The reduction in the postprandial TLR-2 positive cells, in addition to the lower postprandial IL-6 and IL-10 response reported in this chapter, implies a down regulation of proinflammatory signalling pathways. Nevertheless, the underlying causes of the differences in TLRs in obese and normal weight subjects and the specific role of TLR-2 in the regulation of metabolism remains to be elucidated.

The postprandial P-selectin and MCP-1 responses were lower after a chronic fish oil intervention in normal weight subjects. In addition, fish oil treatment induced a significant reduction in ICAM-1 and E-selectin concentrations when compared with supplementation with the placebo. A reduction of postprandial MCP-1 has been previously reported in healthy elderly obese men (Plat *et al*. 2009). Results from this study demonstrated that fish oil lowers postprandial MCP-1 concentrations in normal weight but not in the obese subjects. Compared with the Plat *et al* (2009) study, the obese group in this study have similar baseline anthropometric, metabolic and inflammatory characteristics, but in contrast the study population was not elderly and included both male and female subjects. Previous studies have reported the effect of chronic fish oil supplementation on postprandial VCAM-1, ICAM-1 and E-selectin and findings have been contradictory (Jellerma *et al.* 2004; Plat *et al.* 2009; Derosa *et al.* 2012). The discrepancy in the literature may be associated with the protocol differences (fish oil alone or fish oil + diet intervention), the dose of n-3 PUFA used and the supplementation period (6 weeks or 6 months). The results presented in this chapter suggest that 12 weeks fish oil supplementation (without a dietary intervention) is sufficient to reduce the circulating concentrations of adhesion molecules in healthy normal weight subjects.

8.5 Conclusion

Chronic fish oil supplementation lowered postprandial inflammation in both normal weight and obese subjects. Furthermore, fish oil induced changes in triglyceride and insulin responses in both groups. Some of the markers modulated by these fatty acids are well-established predictors of cardiovascular disease. Thus, moderate fish oil consumption may be protective against cardiovascular conditions.

CHAPTER 9

Final discussion

CHAPTER 9

Final discussion

9.1 Summary of the main results

The “postabsorptive” or fasted state, describes the phase where the components of the most recent meal have been absorbed from the intestinal tract; thus metabolic and inflammatory activities in this state occur in the absence of food as an exogenous factor. The postprandial state reflects the alterations induced by components from the diet and the ability of the body to handle these challenges. There is abundant evidence regarding metabolic markers in the fasted state and their relationship with cardiovascular disease and insulin resistance. Similarly several studies have described the postprandial lipemic response, characterising its components and dynamics. Fasting levels of inflammation have recently been associated with metabolic disorders. In addition to the typical markers of inflammation (e.g. CRP), some adipose tissue derived molecules have been related to inflammation and insulin resistance, including leptin and PAI-1. Some components from the diet, especially fatty acids, have been shown to induce postprandial inflammation. Given that humans may remain in a postprandial state for much of the day, the levels of inflammation induced by acute and chronic exposure to some components from the diet may contribute to or prevent the development of chronic degenerative disease.

Fish oil, a source of bioactive n-3 fatty acids, has been associated with anti-inflammatory properties and has been shown to have protective effects on cardiovascular health. Studies have reported effects of fish oil on human metabolism and inflammation at both high pharmacological doses and at concentrations similar to dietary recommendations. Nevertheless, there is limited evidence regarding the role of the acute and chronic consumption of fish oil in the modulation of postprandial inflammation. The work described in this thesis aimed to describe in detail the metabolic and inflammatory responses of normal weight and obese subjects, and how these can be modulated by acute and chronic consumption of fish oil. In addition, a macrophage model was used to study the *in vitro* regulation of inflammatory pathways by different fatty acids commonly found in the diet including those n-3 PUFA found enriched in fish oil.

High levels of fasting plasma lipids were observed in obese subjects compared with normal weight. In parallel, fasting plasma levels of well-established inflammatory markers, including CRP and IL-6, were higher in obese subjects compared with normal weight. Moreover, in the basal state monocytes from obese subjects were in a more inflammatory state, evidenced by a high proportion of $TLR4^+$ and $TNF\text{-}\alpha^+$ cells. However, monocytes from obese subjects were less responsive to an endotoxin challenge *ex vivo*. This may be because the cells from obese subjects are "exhausted" from chronic low grade stimulation *in vivo*, and so they respond poorly when re-stimulated in vitro. In addition to clear differences in metabolic and inflammatory markers between obese and normal weight subjects in the fasted state, there were differences also during the postprandial state. Obese subjects presented a higher metabolic response to a high fat meal compared with normal weight subjects showing elevated triglyceride, glucose and insulin responses. In addition, a higher AUC and iAUC for plasma concentrations of CRP, leptin, VEGF and an increased proportion of TLR4 positive monocytes were observed in obese subjects compared with normal weight. Furthermore, higher concentrations of IL-6 and IL-8 were detected at some postprandial time points in obese subjects compared with normal weight. Together this evidence suggest an up regulation of inflammation in both the fasting and postprandial states in obese compared with normal weight subjects.

Acute exposure to fish oil induced modest changes in the postprandial state compared with placebo. In normal weight subjects, a HFM including fish oil resulted in a decrease of postprandial triglycerides. This could be an important effect in lowering cardiovascular risk, since elevated postprandial triglyceridemia has been shown to be a risk factor for future disease. This effect was not observed in obese subjects, although, postprandial concentrations of P-selectin and the TLR4 expression were reduced at some time points in the fed state in obese subjects.

Similarly to results from the acute study, chronic consumption of fish oil had contrasting effects on metabolic and inflammatory markers between obese and normal weight subjects. After 12 weeks supplementation, plasma concentrations of P-selectin, ICAM-1, CRP, adiponectin and adipsin decreased while HDL and insulin sensitivity (HOMA %S) is increased in normal weight subjects. These changes were not extended to the obese group, although a reduction in inflammatory cytokines

production *ex-vivo* by monocytes and whole blood, including IL-1β, IL-6 and IL-8, was found.

The effect of fish oil on postprandial lipid metabolism was similar following acute and chronic supplementation in normal weight subjects. Fish oil induced a consistent reduction in postprandial triglyceride concentrations in normal weight subjects, while in obese subjects, only an increase in plasma insulin concentrations was detected after chronic supplementation. This may be an undesirable effect of n-3 PUFA in obese subjects.

Chronic fish oil supplementation had a higher impact on postprandial inflammatory markers than acute treatment. In normal weight subjects, a reduction in the net postprandial P-selectin, MCP-1 and adipsin concentrations was detected. Moreover, TLR4, E-selectin and ICAM-1 concentrations were lower at some time points after fish oil supplementation compared with the baseline observations. In agreement with the reduction of IL-6 synthesis *ex-vivo* after fish oil supplementation, postprandial concentrations of IL-6 and CRP were reduced after fish oil treatment. Moreover, TLR2, IL-10 and resistin concentrations were observed at significantly lower concentrations compared with baseline.

In parallel to the human study, a molecular study was performed to determine the effect of various fatty acids on the activation of NF-κB, a pivotal transcription factor involved in the production of inflammatory cytokines and other inflammatory markers. In contrast with the human study findings, no changes in the basal cytokine profile were observed in THP-1 macrophages pre-treated with n-3 PUFA. Nevertheless, a reduction of LPS-induced p65 activation was observed in cells pre-treated with EPA prior to LPS stimulation. In parallel, total IκB-α protein was higher in the cytoplasm and it was less phosphorylated in response to LPS in cells pre-treated with DHA and EPA, although this result requires more replicates to be confirmed. Moreover, THP-1 macrophages enriched with DHA and EPA produced less TXB_2 and PGE_2 in response to LPS than those pre-treated with AA and control.

In summary, the results presented in this thesis extend the knowledge regarding differences in inflammation and some adipose tissue derived molecules between normal weight and obese subjects in the fasting and postprandial states. The

consumption of a HFM with fish oil capsules had modest effects on postprandial inflammation in normal weight and obese subjects while chronic supplementation of fish oil resulted in a reduction of fasting and postprandial inflammatory markers in both normal weight and obese subjects. Moreover, the experiments in THP-1 cells showed that incorporation of EPA into cell membranes has the ability to reduce p65 NF-κB activity and both DHA and EPA pretreatment reduced the synthesis if TXB_2 and PGE_2 in response to LPS compared with AA and control.

Taking all the evidence together, the molecules affected by fish oil supplementation are directly or indirectly synthesized in response to NF-κB activation. Thus, the results observed *in vitro* and in the human study suggest that the inhibition or changes in the modulation of this signalling pathway may be one of the pivotal mechanisms of action involved in the anti-inflammatory effects of fish oil. Furthermore, the enrichment of cell membranes with n-3 PUFA was shown to down regulate the production of pro-inflammatory lipid mediators. Thereby, this is another mechanisms of action to be studied in detail.

9.2 Integration with the literature

As discussed in previous chapters some of the results described in this study are in agreement with the literature while some are contrasting with previous reports. Some of the similarities with previous studies include the differences in fasting and postprandial metabolic profile between normal weight and obese subjects, although this was studied more extensively here than in most previous reports. In terms of inflammatory markers, fasting concentrations of CRP, IL-6, PAI-1 and leptin were found at significantly higher levels in obese while adiponectin concentrations were lower in this group compared with normal weight subjects (Landin *et al.* 1990; Arita *et al.* 1999; Pradhan *et al.* 2001; Engeli *et al*. 2003; Skurk *et al.* 2004; Lucas *et al.* 2013). Some discrepancies and unexpected results compared with the literature include the lower concentrations of MCP-1 and VCAM-1 observed in obese compared with normal weight subjects. These molecules have been observed at higher concentrations in obese subjects and are believed to play a role in development of atherosclerosis (Lemos *et al.* 2000; Zicarddi *et al.* 2002; Bosanska *et al.* 2010).

In agreement with previous reports studying the effect of meals containing different proportions of MUFA, PUFA and SFA, acute fish oil consumption induced reductions in postprandial triglyceride concentrations, however the effect on postprandial inflammation was modest (Jackson *et al*. 2012; Esser *et al.* 2013).

The evidence regarding the chronic effect of fish oil on fasting and postprandial plasma cytokines, adipokines and adhesion molecules is limited. The reduction of fasting concentrations of adhesion molecules observed in normal weight subjects following fish oil supplementation are consistent with previous studies exploring similar adhesion molecules (Miles *et al.* 2001). However, other studies have reported no effects of fish oil supplementation on circulating concentrations of adhesion molecules (Damsgaard *et al*. 2008; Din *et al.* 2008 Pot *et al.* 2009; Bragt et al. 2012). As suggested in **Chapter 7**, the duration and doses of fish oil supplementation appear to be an important determinant of the anti-inflammatory effect of fish oil.

Another important observation is that acute exposure to fish oil reduced postprandial P-selectin concentrations in obese subjects while this effect was observed in normal weight subjects only when fish oil was supplemented chronically. P-selectin is constitutively expressed in endothelial cells and is synthesized in response to agonists including inflammatory cytokines (TNF-α, IL-1) and thrombin. Thus, it would be expected to observe a similar effect in both groups. A possible explanation to this discrepancy may be related to the baseline inflammation differences between groups or differences related to the incorporation of fatty acids in different compartments.

In addition to the changes in adhesion molecules after chronic exposure to fish oil, some changes in adipokines and cytokines were detected. CRP plasma concentrations were significantly reduced in normal weight subjects and this finding is consistent with studies in overweight and hypertriglyceridemic subjects (Browning *et al.* 2007; Kelley

et al. 2009). Even though chronic fish oil supplementation did not affect fasting concentration of CRP in obese subjects, a reduction in the postprandial concentration of this acute phase protein, IL-6 and resistin was observed. Moreover, fish oil supplementation reduced the basal production of IL-1β, IL-6 and IL-8 in mononuclear cells and whole blood. These novel findings extend the knowledge regarding to the anti-inflammatory effect of fish oil supplementation during a postprandial response and suggest that a fish oil background diet reduces inappropriate inflammatory response to exogenous challenges.

9.3 Study strengths and limitations

In comparison with previous clinical trials, this study presents several advantages. 1) The study design for this trial allowed the comparison of the baseline characteristics of normal weight and obese subjects, and at the same time the effect of acute and chronic supplementation of fish oil in these groups. 2) Recently inflammation has been accepted to play an important role in metabolic homeostasis, however few studies have documented both aspects together. This study provided information about the regulation of a wider number of molecules related to inflammation and metabolism compared with previous reports. 3) In contrast with other studies, the sample size employed was adequate to detect the differences between groups (obese and normal weight) and the treatment effects (fish oil supplementation). Moreover, as inflammatory markers vary considerably between subjects, sample size is a pivotal aspect to consider when evaluating a treatment effect. 4) The subject compliance was high (greater than 90%). 5) this study described novel findings, including the effect of obesity in TLRs protein expression in the postprandial response and some other molecules including VEGF and adipsin. Furthermore, this is the first study to describe the effect of chronic supplementation of fish oil in the postprandial inflammatory response (including cytokines, adipokines and adhesion molecules analysis) of normal weight and obese subjects and to show a reduction of postprandial P-selectin, MCP-1, adipsin, IL-6, CRP and resistin after treatment.

In addition to the study strengths, this study has some limitations. At the start of this clinical trial normal weight subjects were rapidly recruited from the Southampton Hospital and University population, these included members of the staff and mainly students. Given the inclusion criteria required to recruit healthy obese subjects, the recruitment of these individuals was challenging and slow. A change in recruitment strategy was employed (including advertisement wording), and the advertisement was extended beyond Southampton area, including Salisbury, Winchester, Portsmouth and other locations. Moreover, given the rapid recruitment of young subjects in the normal weight group (many aged 20-30 y), the advertisement to recruit obese subjects was focused in young populations (clinics, gyms, societies, etc.). Nevertheless, at the end of the study the mean age of normal weight and obese subjects was significantly different. To deal with this issue, statistical analysis was performed correcting data for volunteers' age and sex. Another limitation of this study is that some data regarding to the subjects' physical activity was not recorded, this variable may explain some of the results and provide relevant information regarding to the volunteers health status. Moreover, the *ex-vivo* experiments were not performed in all subjects from the start of the study, because these work was not initially planned and due to optimization process (including antibody selection and titiration, cell permeabilization protocol). Thus, some data regarding to the *ex-vivo* production of inflammatory cytokines by monocytes and whole blood, mainly in normal weight subjects, was not recorded, and this may affect the results.

Another factor that may have an impact in the results presented in this document is seasonal variation. During the year there are changes in photoperiods that influence various physiological processes in humans, including immunological responses. Pineal melatonin and vitamin D are some examples of molecules regulated by exposure to sun light that have an impact on adaptative and innate immunity.

Some of the molecules evaluated in this study, including IL-6 and TNF-α, have also been associated with the circadian rhythm (24 h cycle). Moreover, these changes have been directly linked with variations in plasma cortisol (an endogenous immunosuppressant) (Petrovsky *et al.* 1998). To minimize the effect of the cirdadian cycle in the postprandial response outcomes, the postprandial interventions started at approximately the same time for all visits for all volunteers (8:30-9:30 am).

In terms of the *in vitro* work, one of the main limitations of the study is the lack of time to obtain more information about the expression of some proteins activated in response to NF-κB, including LOX. Furthermore, in addition to the chronic effect of n-3 PUFA on inflammatory signalling pathways, some experiments were performed to evaluate these aspects in response to acute fatty acid treatment. Nevertheless this set of data was not completed and preliminary results are included in **Appendix I.**

9.4 Future work

The results described herein provided relevant information regarding the effect fish oil on human metabolism and some elements of inflammation. Additionally, this study provided novel evidence associated with the modulation of some membrane receptors and adipokines during the postprandial response in obese and normal weight subjects, while the molecular work described a reduction of proinflammatory lipid mediators when cells were enriched with n-3 PUFAs.

In addition to the changes in cytokine profile, fatty acids are substrates for lipid mediators. As described in **Chapter 1**, lipid mediators are derived from the metabolism of long chain fatty acids, including AA, DHA and EPA. Studies by Serhan and colleagues described the role of n-3 PUFA derived mediators, named resolvins, protectins and maresins, in the initiation of the resolution of inflammation (Serhan *et al.* 2008; Fredman and Serhan, 2011; Serhan *et al*. 2012). These studies based in animal models, documented the effect of these mediators in the resolution of acute inflammation.

Recently, n-3 PUFA derived lipid mediators have been detected in plasma and serum from healthy subjects receiving fish oil capsules supplementation for 3 weeks (Mas *et al*. 2012). Additionally, studies in severely obese subjects observed an increment in resovin D1 in adipose tissue following fish oil supplementation (Itariu *et al.* 2012). Thus, fish oil supplementation itself may switch the production of these mediators to a more regulatory profile, and this may be a mechanism underlying some of the effects observed in this study. Future work should focus on the evaluation of plasma and adipose tissue concentrations of these mediators to investigate the effects of fish oil supplementation, and the relationship of these novel lipid-derived modulatory mediators with the changes in the inflammatory molecules observed in this study.

Adipose tissue is an important organ involved in the regulation of metabolic homeostasis and recently it has been identify as a source of proinflammatory mediators. Some studies have reported differences in the fatty acid profile in different adipose tissue depots from human (Calder *et al.* 1992; Garaulet *et al.* 2006). N-3 PUFA and n-6 PUFA concentrations are similar in both omental and subcutaneous adipose tissue. However, the concentrations of n-3 and n-6 in adipose tissue have been associated with adipocyte size (Garaulet *et al.* 2006). After fish oil supplementation, a reduction in fasting and postprandial adipokines was detected. Thus, it would be interesting to look at the differences in adipose tissue physiology and inflammation between normal weight and obese subjects and investigate how these aspects may be modulated by fish oil supplementation.

APPENDIX

APPENDIX I

Reagent and material

Chapter 2	Name	Provider	Cat No
Cell culture	THP-1 cells	ECACC	88081201
	RPMI 1640 medium	Gibco	21875-091
	Foetal bovine serum (heat inactive)	10500-064	10500-064
	L-glutamine	Gibco	
	Penicillin/ Streptomycin	Gibco	15070-063
	HEPES Buffer (IM) 1 M, pH 7.0-7.6, sterile-filtered, BioReagent, suitable for cell culture (100mL)	Sigma	H0887
	PMA	Sigma	P1585
	PBS	Gibco	14190-094
	Standard lipopolysaccharide from E. coli K12 strain	InvivoGen	tlrl-eklps
Flow cytometry			
	Cell dissociation solution	Sigma	C5914-100ML
	BD Pharmingen™, CD14	BD	555397
	BD Pharmingen™, CD11c	BD	561355
	BD Pharmingen™, Mouse IgG_1, κ, FITC	BD	555748
	BD Pharmingen™, CD235a, IgG_{2b}, κ	BD	555570
	BD Pharmingen™ CD206 Macrophage mannose receptor	BD	555953
	BD Sheath Solution (FACS Flow)	BD	336911

	BD CellWASH	BD	349524
IHC			
	Glass cover 16mm 0.13-0.17 thick	Fisher	12313138
	Microscope slides-superfrost plues 25x75x1.0mm	Thermo Scientific	J1800AMNZ
	Monoclonal Mouse Antihuman CD68, Clone PG-M1	Dako	M0876
	ABC kit	Vector	PK-6100
	Avidin/Biotin blocking kit	Vector	SP-2001
	AEC substrate	Vector	SK-4200
	Hematoxylin Solution, Mayer's	Sigma	MHS16-500ML
	Polyglycine	Sigma	P8791-100MG
	Pertex	Leica Biosystems	3808706E
Kits			
	Human Th1 Th2 11-plex Ready to use kit FlowCytomix	eBioscience	BMS810FFRTU
	Prostaglandin E_2 Express EIA Kit	Cayman Chemicals	500141
Sample collection			
	TRI Reagent	Sigma	T9424
	Nuclear Extract kit	Active Motif	40010
Fatty acids			
	Bovine Serum Albumin Fatty acid free, low endotoxin, lyophilized powder, BioReagent, suitable for cell culture, ≥96% (agarose gel electrophoresis)	Sigma	A8806
	DHA	Sigma	D2534
	EPA	Sigma	E2011
	Linoleic acid	Sigma	L1376
	Arachidonic acid	Sigma	A9673
	Ethyl palmitate (palmitic acid)	Sigma	P9009
	Ethyl stearate (stearic acid)	Sigma	S8269
	Ethyl dodecanoate (lauric acid)	Sigma	61630
	All solvents	Fisher	-
	All salts	Sigma	-

Chapter 3	Name	Provider	Cat No
Flow cytometry			
	Mouse Anti Human CD282-RPE	AbSerotech	MCA2152PE
	Anti-Human CD284 (TLR4) PE	eBiosciences	
	FITC Mouse Anti-Human CD14	BD	555397
	Lysis Solution	BD	349202
Kits			
	Human C-Reactive Protein/CRP Quantikine ELISA Kit	R&D	DCRP00

PROTEIN EXPRESSION ANALYSIS IN THP-1 CELLS[1]

The expression of NFκB signalling molecules was evaluated in the nuclear and cytosolic fraction from THP-1 macrophages after different treatments. Nuclear and cytoplasmic fraction was freshly isolated using Active Motif kit (5 x 10^6 cells per samples) as described below.

SAMPLES PREPARATION

Nuclear/Cytoplasmic fractions extraction

Buffer preparation and recommendations

Reagent to prepare	*Component*	*60mm plate or 3.2 x* 10^6 cells
PBS/Phosphatase inhibitors	*10x PBS*	*0.4mL*
	Distilled water	*3.4mL*
	Phosphatase inhibitor	*0.2mL*
	Total required	*4.0mL*
1x Hypotonic buffer	*10x Hypotonic buffer*	*25.0µL*
	Distilled water	*225.0µL*
	Total required	*250 µL*
Complete Lysis buffer	*10 mM DTT*	*2.5 µL*
	Lysis Buffer AMI	*22.25 µL*
	Protease Inhibitor cocktail	*0.25 µL*
	Total required	*25.0 µL*

Cell collection

Aspirate media out of dish. Was with 2.5 mL ice cold PBS/Phosphatase inhibitors. Aspirate solution out and add 1.5 mL ice-cold PBS/Phosphatase inhibitors.

Remove cells from dish by gently scraping with cell lifter. Transfer cells to a pre-chilled 15 mL conical tube.

[1] Updated in May 2014 by Carolina Paras Chavez

Centrifuge cell suspension for 5 min at 300 g in a centrifuge pre-cooled at 4°C.

Discard supernatant. Keep cell pellet on ice.

Cytoplasmic Fraction collection

Gently resuspend cells in 250 µL of 1X hypotonic buffer by pipetting up and down several times. Transfer to a pre-chilled microcentrifuge tube incubate for 15 min on ice.

Add 12µL detergent and vortex 10 sec at highest setting.

Centrifuge suspension for 30 sec at 8,000 x g (or hold "play" bottom in micro centrifuge) in a micro centrifuge pre-cooled at 4°C.

Transfer supernatant (cytoplasmic fraction) into a pre-chilled micro centrifuge tube. Store at -80°C.

Nuclear Fraction collection

Resuspend nuclear pellet in 25µL complete lysis buffer by pipetting up and down. Vortex 10 seconds at highest setting.

Incubate suspension for 30 min on ice on a rocking plataform set at 150rpm (**for this step , samples were sonicated instead**).

Vortex 30 sec at highest setting. Centrifuge for 10 min at 8 000 x g in a micro centrifuge pre-cooled at 4°C.

Transfer supernatant to a pre-chilled micro centrifuge tube. Aliquot and store at -80°C.

PROTEIN CONCENTRATION DETERMINATION

Bradford assay

Samples were prepared as described in the previous section. Standard curve was prepared as described in Table 1.

Dilution	Final [g-globulin] (mg/mL)	µL H2O miliQ	µL g-globulin stock (1.38mg/mL)	Sample Volume (µL)	Final volume (µL)	Bradford reagent volume (µL)
P0	2.78	0	10	-	10	245
PI	1.39	0	5	-	5	250
PII	0.834	2	3	-	5	250
PIII	0.278	4	1	-	5	250
Blank	0	5	0	-	5	250
Samples	-	-	-	5	5	250

Bradford reagent dilution = 1 : 4

Read at 595nm within 50 min

Calculations example:

Calculate total protein concentration based in standard curve (standards)

Calculate amount per µL (if sample was diluted- consider dilution factor)

Determine amount of protein needed to detect protein of interest (between 20-50 µg is generally a good amount).

ELECTROPHORESIS- POLYACRILAMIDE/SDS GEL

Sample preparation

Samples at a determined concentration (20-50 µg) we mixed with Laemly 1x buffer and boiled for 10min at 95°C.

Sample name	Volume for 20µg	Laemly (µg)	Total volume (µg)
Sample 1	10	10	20
Sample 2	7	14	20

Running and stacking gel preparation

Gel buffers were prepared as described in "Reagent section". The gel concentration depends on the molecular weight of the protein of interest. See table below to determine gel concentration required.

Protein density	Gel SDS %
4-40kDa	20%
12-45kDa	15%
10-70kDa	12.5%
15-100kDa	10%
25-200kDa	8%

Gels were set in BIORAD "gel platform" using short and large gel glasses.

Gel preparation

Running gel buffer was placed between glasses- leaving 2cm for stacking gel. 1mL of miliQ water was poured at the top to improve polymerization and obtain a flat surface. Once gel has set, water is removed carefully, making sure there are not residues.

Stacking gel buffer was poured on top of running gel, until it overflow the glasses. A comb was placed on top- to create wells for each sample. *make sure you use appropriate combs, depending in final ps/laemly volume.

Gels were placed in BIORAD tank and the space between gel platforms was filled with Electrophoresis buffer. *If only one gel is going to be run, make sure there is a pair of empty glasses or a spare gel in the opposite side**

Electrophoresis

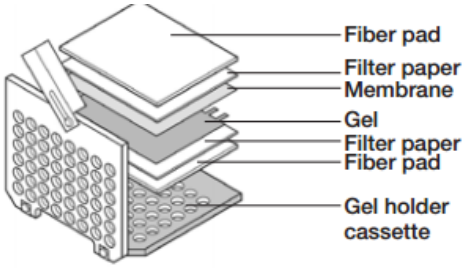
Samples (containing Laemly buffer, previously denaturalized) and markers were load on each well.

Tank was connected to electrode and electrophoresis was ran at 70 V for 10min approximately (or until samples passed through the stacking gel and reached running gel. After this gel was run 150 V over 40 min or until samples are near (1cm) to reach the bottom of the gel.

In the meantime, Whatman paper, nitrocellulose membranes and sponges are cut and soaked in transference buffer.

Protein transference

Once electrophoresis has finished, " transference sandwich" is prepared as shown in the following figure. **make sure gels do not dry out, and avoid bubbles in each sandwich layer**



An ice container is placed in one of the tank sides. Transference tank is filled with "transference buffer" and run at 100V during 60-90 min (in the cold room- or in an ice container).

To double check transference, membranes can be stained for 10sec with red ponceau solution, wash with PBS buffer afterwards

Membrane blocking and antibody incubation

After transference, membrane is blocked with "blocking solution" for 1h on a rocking platform.

After blocking, membranes are treated with primary antibodies and blocking solution for different times. See "Antibody optimization section" for more information about antibody treatment times and conditions.

Membranes are washed with solutions C, B and PBS for 5-15min each solution.

After last wash, membranes are treated during 1-2h with secondary antibody at room temperature

Step 3 is repeated.

Protein detection- Blotting

After last wash, membranes are covered with 0.7-1mL of blotting solutions and visualized.

For stripping membranes

Incubate membranes with stripping solution for 30min.

Wash twice with PBS Tween (10min each incubation)

Block 30min with blocking solution.

Ab	uL/5mL blocking solution
COX-2	5µL
COX-1	5µL
IkB-α	5µL
LOX-5	*to optimize
iNOS	*to optimize
Actin	1.4µL
Anti rabbit	2 µL

BUFFERS AND SOLUTIONS PREPARATION

<u>General use buffers</u>

Tris 1M pH 6.8/8.8 (1x)

Trizma base	12.11g
H2O	100mL
*Adjust pH to 6.8 or 8.8 with HCl 12N. Store at room temperature	

SDS 20% v/v

SDS	20g
H2O	100mL
Store at room temperature	

PSA 10% (ammonium persulfate)

PSA	100mg
H2O	1mL
Store at 4°C, max 1 week	

Sample Preparation

Laemly 1x

Glycerol	1mL
SDS 10% (10g/100mL)	2mL
Bromophenol blue	2mg
Tris HCl 1M pH 6.8	1.25mL
H2O	c.s.p. 10mL
*Before use add 1 μL β-mercapbetanol per 50 μL Laemly 1x	

Gels preparation

10% SDS Running Gel (1x)

H2O	2.75 mL
Tris HCl 1M pH 6.8	3.75 mL
Bis Acrilamide 30%	3.3 mL
SDS 20%	50 μL
PSA 10%	100 μL
TEMED	6.66 μL

Use order indicated

Staking Gel (1x)- 10%

H2O	2.3 mL
Tris HCl 1M pH 6.8	420 µL
Bis Acrilamide 30%	560 µL
SDS 20%	16.6 µL
PSA 10%	33.3 µL
TEMED	3.3 µL

Use order indicated

Staking Gel (1x)- 7%

H2O	2.42 mL
Tris HCl 1M pH 6.8	420 µL
Bis Acrilamide 30%	420 µL
SDS 20%	16.6 µL
PSA 10%	33.3 µL
TEMED	3.3 µL

Use order indicated

<u>Protein electrophoresis and transference</u>

Electrophoresis buffer (10x) pH 8.8

Trizma base	30.2g
Glycine	188g
Disolve in 600 mL of H_2O then add …	
SDS 20%	50mL
Adjust to 1000mL and store at 4°C	

Transfer Buffer (1x)

Trizma base	3g
Glycine	14g
Methanol	200mL
Adjust to 1000mL and store at 4°C	

Red Ponceau Solution

Red Ponceau	0.2g
10% Tricloruro acetico	30mL
H2O	100mL
Store at room temperature	

Solution B (NP-40 0.5%)

NP-40	5mL
PBS 1X	1000mL

Solution C

NP-40	5mL
PBS 1X	1000mL
NaCl 5M	50.83mL
29.22g NaCl in 100mL H2O	

Blocking Solution

Marvel skimmed milk	5g
Solution B	100mL

PBS 10X pH 7.2-7.4

Na2HPO4	10.7g
NaH2PO4 2H2O	3.9g
NaCl	85g
H2Od	(to reach 1L)
Store at 4°C	

*For PBS 1x mix 100mL of PBS 10x WITH 900mL of distilled water.

Striping Solution

Glycine	750.7mg
Distilled water	400mL
SDS	4g
Adjust pH to 2	

PBS Tween

Tween	100µL
PBS 1x	200mL

CELL CULTURE FATTY ACID COMPOSITION

%

%

	V	DHA	EPA	STEA	PALM	LAU	AA
	0	0	0	0.115508	0.04609	0.4238	0.054738
14:00	1.525215	0.758762	0.814905	0.81792	1.047748	0.711439	0.937702
16:00	26.14418	24.01398	24.66351	25.90349	25.8533	24.3901	23.41814
16:1n-7	1.747659	1.657522	2.018101	1.786351	1.909376	2.055268	1.754352
18:00	17.98711	16.09393	16.41306	19.7745	17.04421	17.86303	15.72334
18:1n-9	21.96139	20.30133	20.46509	21.61779	22.001	20.19809	19.44027
18:1n-7	5.514732	5.294441	5.226366	5.736663	6.124948	5.19403	5.49577
18:2n-6	3.810134	4.151532	7.891571	4.215581	4.578869	10.01061	3.958982
18:3n-6	0.503738	0.20627	0.375592	0.244607	0.204669	0.289775	0.203234
18:3n-3	0.806319	0.571918	0.57164	0.35237	0.341663	0.611122	0.366862
20:00	0.563341	0.453095	0.395489	0.31056	0.415402	0.45581	0.386859
20:1n-9	0.483129	0.413	0.347348	0.455436	0.45135	0.424331	0.308693
21:0 std							
20:2n-6	0.664431	0.658459	0.711348	0.607578	0.620241	0.485354	0.543214
20:3n-6	2.442642	2.051175	2.549016	2.167425	2.212322	1.9461	2.10069
20:4n-6	5.811206	5.757488	6.779653	6.251871	6.749492	6.409638	15.7173
20:4n-3	0.195061	0.398249	0.303877	0.36895	0.399042	0.426053	0.30693
20:5n-3	1.26243	1.16632	2.30448	1.232283	1.191469	1.05665	1.130734
24:00:00	0.250434	0.140504	0.284165	0.154317	0.160025	0.233719	0.200426
24:1n-9	0.263219	0.177062	0.32935	0.163492	0.13909	0.1797	0.155437
22:5n-3	3.307333	3.375175	3.166718	3.467095	3.742044	2.964494	3.458006
22:6n-3	4.756296	12.3598	4.388708	4.256204	4.767692	3.670905	4.338285
total	100	100	100	100	100	100	100

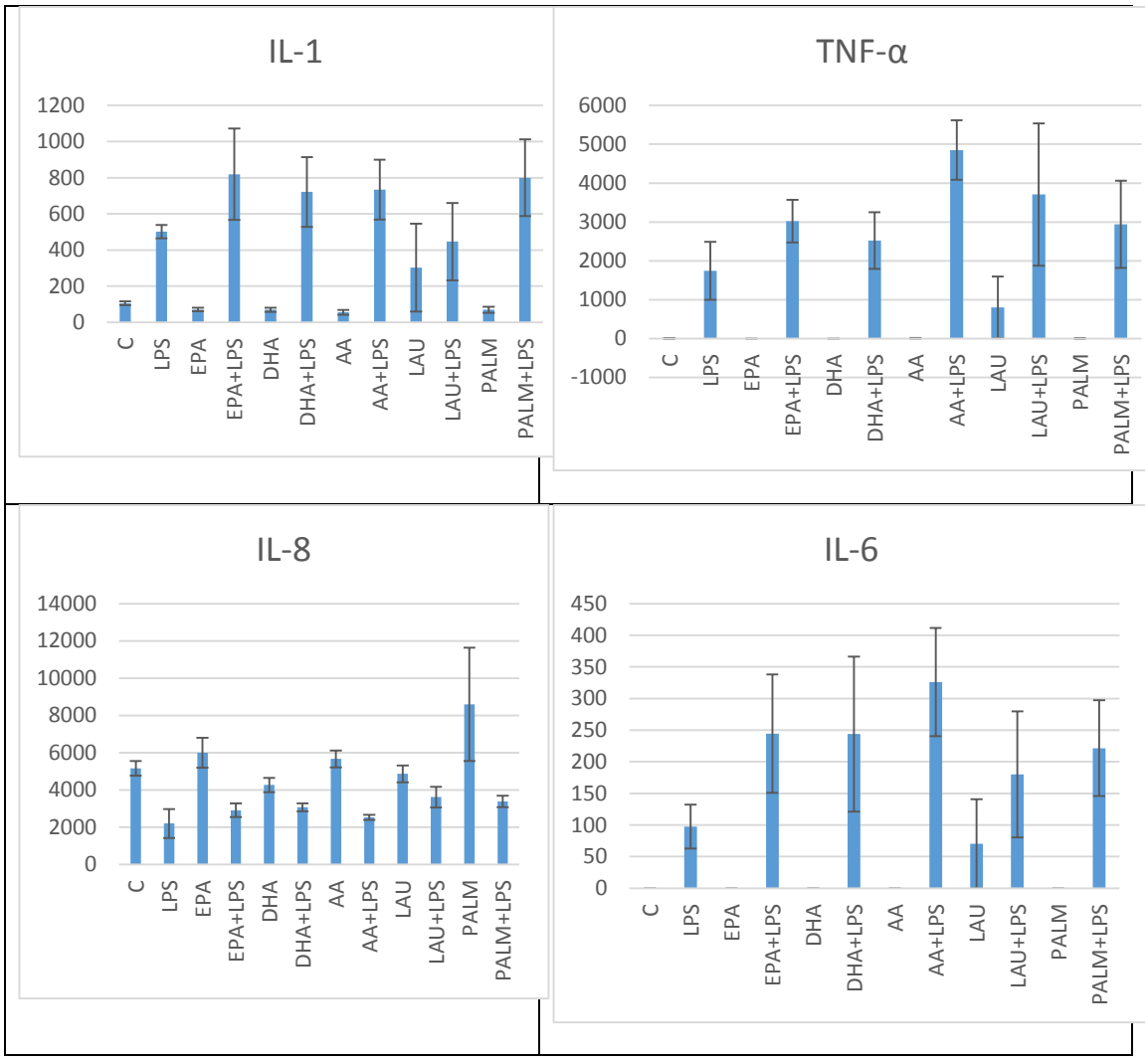
pg/mL

	V	DHA	EPA	STEA	PALM	LAU	AA
12:00	0	0	0	0.037452	0.012418	0.115863	0.017111
14:00	0.528949	0.281104	0.257388	0.233243	0.278236	0.203347	0.293141
16:00	9.010539	8.723145	8.226455	7.054748	6.865429	7.073409	7.320842
16:1n-7	0.611856	0.605692	0.675151	0.491222	0.506998	0.601454	0.548436
18:00	6.209185	5.849315	5.537623	5.374997	4.524593	5.223439	4.91533
18:1n-9	7.603217	7.371458	6.803532	5.864244	5.838454	5.859548	6.077295
18:1n-7	1.900691	1.922191	1.731203	1.55934	1.625482	1.483767	1.718068
18:2n-6	1.304308	1.515821	2.816688	1.151605	1.215787	3.130613	1.237634
18:3n-6	0.174922	0.075008	0.11638	0.066591	0.054224	0.085546	0.063537
18:3n-3	0.272509	0.202323	0.176019	0.095719	0.090774	0.187703	0.114683
20:00	0.204022	0.161969	0.127911	0.090436	0.110369	0.137256	0.120936
20:1n-9	0.17085	0.148273	0.116069	0.123561	0.119699	0.126304	0.0965
21:0 std							
20:2n-6	0.235247	0.238753	0.223941	0.162004	0.164376	0.136424	0.169815
20:3n-6	0.852542	0.745821	0.854495	0.585133	0.587132	0.562202	0.6567
20:4n-6	2.000201	2.095206	2.295839	1.69314	1.790595	1.872662	4.913436
20:4n-3	0.068303	0.144145	0.098869	0.099645	0.105817	0.126856	0.095948
20:5n-3	0.441622	0.424333	0.756529	0.331123	0.316014	0.308024	0.353484
24:00:00	0.078627	0.05142	0.092591	0.042888	0.042504	0.067789	0.062654
24:1n-9	0.09217	0.064592	0.114714	0.044851	0.036764	0.052825	0.048593
22:5n-3	1.143435	1.229133	1.038981	0.946996	0.992875	0.845095	1.081018
22:6n-3	1.647093	4.489222	1.45658	1.155111	1.264492	1.044639	1.356207
total	34.55029	36.33892	33.51696	27.20405	26.54303	29.24477	31.26137

Preliminary results

Acute effect of fatty acids on cytokines production (50 uM)

12h treatment FA+LPS



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APPENDIX II

	Normal weight			Obese		
	Men (n=10)	**Women (n=25)**	**p-value**	**Men (n=11)**	**Women (n=29)**	**p-value**
P-Selectin	65.8 (51.7-82.2)	55.0 (41.3-66.0)	**0.03**	55.4 (46.2-72.5)	54.0 (49.4-64.8)	0.76
ICAM-1	242.2 (210.4-382.7)	232.2 (184.8-309.8)	0.28	243.9 (223.2-303.1)	257.6 (202.6-288.0)	0.34
VCAM-1	713.9 (650.8-870.5)	802.4 (644.4-995.9)	0.17	457.8 (415.4-590.1)	436.8 (391.6-506.9)	0.32
E-Selectin	40.3 (37.8-50.4)	31.3 (26.2-37.8)	**0.04**	38.8 (24.8-62.6)	36.5 (26.7-43.8)	0.75
Adiponectin	5.7 (5.5-6.7)	8.5 (7.3-13.2)	**0.02**	5.1 (3.7-6.1)	4.7 (4.1-6.9)	0.33
CRP	1.2 (0.3-1.6)	0.9 (0.5-1.3)	0.83	2.1 (1.2-6.5)	2.6 (1.8-4.7)	0.79
Adipsin	2.8 (2.4-3.4)	2.9 (2.6-3.8)	0.48	2.9 (2.3-3.2)	2.3 (2.2-2.9)	0.19
MCP	202.9 (176.4-231.8)	150.6 (128.3-180.3)	**0.001**	179.3 (147.6-199.2)	141.4 (122.9-173.8)	**0.03**
Leptin	2.9 (1.8-9.3)	12.7 (10.2-19.8)	**0.001**	23.2 (16.5-32.1)	51.4 (41.7-78.2)	**0.001**
Resistin	6.1 (5.3-6.8)	6.1 (4.8-7.1)	0.79	5.2 (4.0-6.6)	6.2 (4.7-7.5)	0.23
PAI-1	23.1 (19.1-29.7)	18.6 (14.8-24.4)	0.85	28.0 (23.0-44.6)	26.4 (21.7-38.2)	0.48
IL-6	2.1 (1.2-2.5)	1.5 (0.7-2.0)	0.06	2.7 (1.8-3.5)	2.1 (1.4-3.0)	0.85
IL-8	6.5 (4.2-7.4)	4.2 (2.3-5.5)	0.08	5.1 (4.1-6.6)	5.0 (3.5-6.4)	0.36
GM-CSF	1.4 (0.3-2.1)	1.2 (0.6-1.8)	0.61	1.4 (0.6-2.8)	1.1 (0.5-2.3)	0.99
TNF-α	7.6 (5.4-10.5)	7.6 (5.3-9.2)	0.24	8.8 (5.6-9.9)	7.6 (4.9-9.9)	0.43
VEGF	14.2 (10.5-23.2)	13.9 (9.6-17.0)	0.23	23.8 (13.8-29.8)	18.9 (11.4-27.3)	0.45
IL-10	0.7 (0.4-1.1)	0.6 (0.4-0.8)	0.48	0.6 (0.4-0.9)	0.5 (0.3-0.9)	0.65
All data were adjusted for age and log transformed for multiple linear regression analysis. Data expressed as median and percentiles 25 and 75						

Table. Spearman correlations between metabolic, anthropometric and inflammatory markers

	Age	BMI	% Fat mass	W / H ratio	% Lean mass	Glucose	Insulin	TG	NEFA	HOMA %B	HOMA %S	HOMA-IR	QUICKI
IFN-Y N	0.059	0.110	0.057	0.241	-0.013	0.183	.304*	0.151	0.095	.259*	-.296*	.304*	-.288*
IFN-Y LPS	-0.121	-0.009	-0.141	0.064	0.091	-0.022	0.076	0.040	-0.028	0.234	-0.137	0.114	-0.116
IL-2 N	-0.208	-.386**	-0.191	-.347**	.299*	-.255*	-.330**	-.322*	-0.216	-0.234	.404**	-.363**	.417**
IL-2 LPS	-0.089	-.428**	-.269*	-0.174	.352**	-0.180	-0.226	-0.127	-0.218	-0.154	.298*	-0.251	.308*
IL-10 N	0.160	0.140	-0.019	.256*	-0.047	0.026	0.101	0.157	0.143	0.051	-0.082	0.081	-0.079
IL-10 LPS	-0.079	0.224	.318*	0.138	-0.236	0.235	0.250	-0.003	0.075	0.181	-.276*	0.250	-.274*
IL-8 N	0.044	.263*	0.196	.329*	-0.240	.298*	0.254	.260*	0.187	0.087	-.347**	.325*	-.353**
IL-8 LPS	-0.061	-.307*	-0.154	-.356**	.352**	-0.172	-.262*	-0.229	-0.033	-0.239	.302*	-.271*	.304*
IL-6 N	0.150	.328*	0.148	.404**	-.287*	.348**	.255*	.296*	0.153	0.003	-.362**	.323*	-.373**
IL-6 LPS	-0.028	0.103	0.123	0.164	-0.082	0.088	0.051	0.034	0.064	0.034	-0.013	0.036	-0.036
IL-4 N	-0.187	-.448**	-.308*	-.328*	.400**	-0.240	-.359**	-.329*	-0.193	-.304*	.436**	-.385**	.430**
IL-4 LPS	-.262*	-.530**	-.406**	-.270*	.487**	-.276*	-.345**	-0.178	-0.210	-.260*	.414**	-.368**	.416**
IL-5 N	0.095	0.023	0.014	0.081	0.050	0.152	0.040	-0.087	-0.129	-0.048	-0.036	0.036	-0.051
IL-5 LPS	0.129	0.009	0.066	0.169	0.036	0.164	0.242	0.032	0.080	0.127	-0.199	0.232	-0.202
IL-1β N	0.098	.263*	0.012	.365**	-0.106	.307*	.416**	.293*	0.157	0.209	-.446**	.422**	-.442**
IL-1β LPS	-0.168	-0.156	-0.123	-0.094	.297*	-0.088	-0.080	-0.071	-0.146	0.031	0.099	-0.055	0.085
TNF-α N	0.024	0.254	0.061	0.239	-0.108	.257*	.318*	0.216	-0.066	0.179	-.369**	.325*	-.369**
TNF-α LPS	-0.136	-0.019	-0.056	0.186	0.166	0.077	0.019	0.098	-0.032	-0.016	0.032	-0.004	0.007

N=basal production of cytokines and LPS=cytokines production in response to lipopolysaccharide in whole blood
BMI=body mass index; TG=triglycerides; NEFA=non esterified fatty acids, HOMA=homeostasis model assessment;
QUICKI=quantitative Insulin sensitivity check index
**p-value<0.05, ** p-value<0.001*

Table. Spearman correlations between metabolic, anthropometric and intracellular production of IL-1β, IL-6 and TNF-α in $CD14^+$ monocytes

	Age	BMI	% Fat mass	W / H ratio	% Lean mass	Glucose	Insulin	TG	NEFA	HOMA %B	HOMA %S	HOMA-IR	QUICKI
IL-1β N	.371*	0.164	0.153	0.030	-0.188	0.113	0.183	0.022	-0.171	0.169	-0.116	0.145	-0.128
IL-1β LPS	0.058	0.134	-0.023	0.159	0.021	-0.033	-0.081	0.173	0.030	-0.032	0.119	-0.133	0.094
IL-6 N	0.034	-0.016	-0.076	0.083	0.149	0.013	-0.081	0.207	-0.315	-0.050	0.075	-0.083	0.074
IL-6 LPS	0.195	0.206	0.001	0.182	-0.093	0.072	0.030	0.066	0.025	0.073	0.026	-0.022	0.005
TNF-α N	.343*	0.227	0.103	0.212	-0.241	0.220	.322*	.475**	-0.036	0.123	-.351*	.315*	-.331*
TNF-α LPS	0.029	0.116	-0.245	0.081	0.074	0.080	0.003	0.100	-0.092	-0.137	0.073	-0.061	0.018

N=basal production of cytokines and LPS=cytokines production in response to lipopolysaccharide in $CD14^+$ monocytes
BMI=body mass index; TG=triglycerides; NEFA=non esterified fatty acids, HOMA=homeostasis model assessment;
QUICKI=quantitative Insulin sensitivity check index
**ρ-value<0.05, ** ρ-value<0.001*

	Normal weight			Obese		
	Men (n=10)	Women (n=25)	p-value	Men (n=10)	Women (n=25)	
BMI	23.52 ± 1.39	21.79 ± 1.74	0.001	34.44 ± 2.84	34.79 ± 2.60	0.71
Waist circumference (cm)	80.42 ± 30.38	73.12 ± 5.34	0.01	117.63 ± 9.93	104.21 ± 10.61	0.001
W/H ratio	0.83 ± 0.04	0.77 ± 0.05	0.1	1.00 ± 0.05	0.88 ± 0.07	0.001
Body fat (%)	11.27 ± 4.92	26.61 ± 4.13	0.001	26.62 ± 13.91	43.10 ± 8.83	0.001
Fat mass (kg)	8.23 ± 3.54	15.55 ± 3.36	0.001	29.67 ± 16.26	40.19 ± 10.07	0.016
Lean mass (%)	88.71 ± 4.99	73.39 ± 4.13	0.001	55.19 ± 27.64	53.46 ± 10.72	0.08
Lean mass (kg)	64.68 ± 6.93	42.51 ± 3.82	0.001	60.94 ± 30.86	49.45 ± 10.32	0.77
Glucose	4.87 ± 0.45	4.73 ± 0.31	0.31	5.98 ± 1.54	5.45 ± 0.65	0.13
Insulin	5.44 ± 2.09	5.68 ± 2.51	0.81	14.52 ± 6.69	12.88 ± 6.21	0.47
Triglyceride	0.83 ± 0.26	0.75 ± 0.28	0.48	1.48 ± 0.66	1.26 ± 0.65	0.35
NEFA	371.53 ± 123.11	529.65 ± 113.74	0.001	577.27 ± 149.47	621.13 ± 193.38	0.51
HOMA %B	78.35 ± 32.11	85.52 ± 24.62	0.49	108.42 ± 34.04	114.48 ± 43.76	0.68
HOMA %S	194.41 ± 100.90	171.33 ± 66.36	0.43	64.61 ± 29.56	79.77 ± 47.79	0.33
HOMA IR	0.70 ± 0.37	0.74 ± 0.31	0.74	1.94 ± 0.94	1.68 ± 0.78	0.38

*Univariate linear regression model, data adjusted for age

You are invited to take part in a research study. Before you decide whether or not you wish to take part it is important that you understand what the research will involve. Please read this information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear, or if you would like more information. Please take as much time as you would like.

What is the purpose of this study?

Fish oil type supplements contain omega-3 fats. These are believed to be good for health. One effect of omega-3s is to dampen inflammation, which can harm the body and lead to disease. Researchers have discovered that inflammation within adipose (fat) tissue plays a role in obesity. We do not know if omega-3s have the same anti-inflammatory effects in normal weight and in obese people. Nor do we know if omega-3s can influence inflammation within fat tissue. We want to find out about the effects of omega-3s in normal weight and obese people. This is important in identifying whether dietary supplements will be equally effective in people with different body fatness. We plan to compare the effects of an omega-3 supplement in normal weight and obese individuals. A dummy called a placebo will be used in half of the people enrolled in the study in order to make a comparison. The dummy will contain vegetable oil. The fish oil supplement to be used is available in pharmacies, supermarkets, health food shops etc. Since the supplement contains fish oil and has a gelatin coating it is not suitable for

vegetarians and vegans. In the study we will measure the amounts of inflammatory chemicals in the bloodstream and in adipose tissue.

Why have I been chosen?

You have been contacted because you have expressed an interest in our research and you appear to meet the inclusion criteria. We need people aged 18 to 65 years. We will be recruiting 100 subjects in total.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. However, you will still be free to withdraw at any time, and without giving a reason. If you do withdraw, you will be asked whether you want us to destroy any samples you have given as part of the study.

What will happen to me if I take part?

If you decide to take part you will asked to attend 4 study appointments at the Clinical Research Facility at Southampton General Hospital. The first of these appointments will last about an hour and will be at any time of the day depending upon your availability. The other three study appointments will begin in the morning, before your breakfast, and each of these will last about seven hours.

At the first visit the amount of blood taken will be about 9 ml (two teaspoons full). We will use this blood to look at your DNA to identify whether there are any

features that relate to body weight, to the way you handle fat from your diet or to inflammation.

On study visits two, three and four you will be asked to fast (no food or drink apart from water) from 9 pm the night before. When you arrive for these visits a cannula will be placed into one of your arm veins and a small blood sample (20 ml, equivalent to just over a tablespoonful) will be taken. On visit four an additional 9 ml of blood will be taken, again for analysis of your DNA. After the initial blood sample is taken you will be given a breakfast and asked to take some omega-3 ("fish oil") or dummy capsules while you eat your breakfast. You will remain in the clinic for a further six hours and a series of blood samples will be taken over this time. Each sample will be 5 ml, so that over the entire visit you will provide 45 ml (about 3 tablespoons) of blood (54 ml at visit four). After the last blood sample is taken you will be provided with some food and a drink.

At the end of the second and fourth visits a small fat sample will be taken from your lower abdomen. Prior to this you will get a small injection to make your skin numb. Then a small cut up to an inch long will be made in the skin and a small piece of fat (about a teaspoon full) will be taken from under the skin. Then the cut will be stitched (usually two stitches will be required). You will need to come back to the Clinical Research Facility at Southampton General Hospital about a week later to have the stitches removed; this visit will be short lasting less than 15 minutes and will be at your convenience.

You will be given a supply of fish oil or dummy capsules with instructions on how to take these. You will take the capsules daily for 3 months between your third and fourth visits.

All samples are treated as a gift from you, and we will respect your wishes when using them. They will be stored for five years before being destroyed, and with your consent we may use them for future studies.

What do I have to do?

As described in the previous section, you will attend the Clinical Research Facility at SGH for 4 study visits. The first visit will last about one hour and visits two, three and four will each last about seven hours. All the visits will take place over four to five months. The second and third visits will be two or three weeks apart. The fourth visit will be about 3 months after the third visit.

For the period between the third and fourth visits, you will be asked to take some omega-3 ("fish oil") or dummy capsules each day.

What are the possible benefits of taking part?

You will not receive any direct benefit from taking part in the study. Knowledge gained from this study will help our research and will ultimately be of use to other researchers and to regulators, consumers and the supplements industry.

What are the possible disadvantages and risks of taking part?

There is a very small chance of bruising at the site of insertion of the needle for collection of blood samples. There is a very small chance of infection and a chance of bleeding and bruising at the site of the cut made in your skin for collecting the fat tissue. These risks will be minimised by using sterile techniques and trained members of staff.

What will happen if anything goes wrong?

If something goes wrong during an investigation day any procedures will be stopped and you will be seen by a doctor. Your involvement in the rest of the study may be stopped.

If you have a concern about any aspect of the study you should speak to the researcher involved who will do their best to answer your questions (telephone 023 8079 5252; email: p.noakes@soton.ac.uk). If you remain unhappy and wish to complain formally you should contact the Welcome Trust Clinical Research Facility in the first instance (telephone 023 8079 4989; email: wtcrf@suht.swest.nhs.uk). Any

complaint about the way that you have been dealt with during the study or any possible harm you might suffer will be addressed.

Will my taking part in this study be kept confidential?

Your GP will be notified that you are participating in this study, but any information that is collected about you during the course of the research will be kept strictly confidential.

What will happen to the study results?

We will inform you of the overall study results. As the samples from the study will not be processed until the end of the study, your results will not be available for several months. The overall results may be presented at scientific meetings or published in a scientific journal. You will not be identified in any of these presentations or publications. We will be happy to discuss the results with you when the study is completed, and will let you know where you can obtain a copy of the published results.

Will I be reimbursed for my time?

In recognition of your time commitment, you will be paid an honorarium of £150. Reasonable travel expenses will also be paid.

Who is organising and funding the study?

This study is being organised by The Institute of Human Nutrition at The University of Southampton. The European commission is funding the research. The legal sponsor of the study is the Southampton University Hospitals NHS Trust.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people called a Research Ethics Committee, to protect your interests. This study has been reviewed and given a favourable opinion by the NRES Committee South Central – Berkshire.

Contact for further information

If you have any further questions then please contact Paul Noakes at The Institute of Human Nutrition on 023 8979 5252 or e-mail p.noakes@soton.ac.uk

And finally…

Thank you for having taken the time to read this information sheet and your interest in the study. If you do decide to take part in the study, you will be given a copy of this information sheet and a signed consent form for you to keep.

Southampton NHS
University Hospitals NHS Trust

UNIVERSITY OF Southampton

Omega-3 fatty acids and inflammation in normal weight and obese subjects

Information Sheet for Study Participants

Ethical Approval number 11/SC/038
Chief Investigator: Prof P C Calder Mailpoint 887
Southampton General Hospital
Southampton SO16 6YD
Researchers: Dr Paul Noakes
Dr James Hopkins
Miss Annette West
Miss Carolina ParasChavez
Telephone: 023 8079 5252
Email: A.West@soton.ac.uk
Version 4a, 27th June 2012

CONSENT FORM

Southampton NHS
University Hospitals NHS Trust

Omega-3 fatty acids and inflammation in normal weight and obese subjects

LREC Reference Number: 11/SC/0384

Name of Chief Investigator: Professor Philip Calder

Please initial box

I confirm that I have read and understand the information sheet (version …. dated …….) for the above study and have had the opportunity to ask questions ☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason, without my medical care or legal rights being affected ☐

I consent to my general practitioner being notified of my participation in this research ☐

I consent to give blood samples according to the schedule described in the information sheet ☐

I consent to giving adipose tissue biopsies according to the schedule described in the information sheet ☐

I consent to my DNA being used for the purposes described in the information sheet ☐

I agree that samples taken as part of the protocol of this study may be stored for 5 years and used in further research studies during that period. Samples will only be used in studies that have been approved by the appropriate Ethics Committee ☐

I understand that I can withdraw this consent at any time by notifying the study team

I agree to take part in the above study

Volunteer: Print_____ Date_____
Signature_____

Member of

research

team taking

consent: Print_____ Date_____
Signature_____

3 copies required: 1 for researcher, 1 for volunteer, Original to be kept with investigator site file

Version 2: 1st August 2011

	Normal Weight			Obese		
	Fish oil	**Placebo**	**ρ- value**	**Fish oil**	**Placebo**	**ρ- value**
Age (y)	31 ± 15	31 ± 13	0.92	45 ± 11	33 ± 123	0.28
Sex (F/M)	12 / 6	13 / 2		8 / 4	8 / 2	
BMI	22.07 ± 1.73	22.41 ± 1.98	0.61	33.61 ± 2.50	36.26 ± 3.30	0.07
Waist circumference (cm)	74.65 ± 5.58	75.28 ± 6.51	0.77	105.53 ± 10.46	103.61 ± 13.93	0.44
W/H ratio	0.79 ± 0.05	0.79 ± 0.06	0.92	0.92 ± 0.08	0.92 ± 0.12	0.95
Body fat (%)	22.41 ± 8.31	23.93 ± 6.78	0.57	39.33 ± 7.38	42.93 ± 7.60	0.27
Lean mass (%)	77.59 ± 8.29	76.09 ± 6.78	0.58	60.80 ± 7.42	57.09 ± 7.60	0.26
*Data expressed as mean ± SD; *ρ-value<0.05; BMI=body mass index; W/H ratio= waist/hip ratio*						

Comparison between baseline anthropometric measurements in group fish oil and placebo (chronic intervention randomization) from normal weight and obese subjects.

	Normal weight			Obese		
	Fish oil	**Placebo**	**ρ- value**	**Fish oil**	**Placebo**	**ρ- value**
Glucose	4.80 ± 0.39	4.73 ± 1.45	0.68	5.26 ± 0.51	5.12 ± 0.35	0.54
NEFA	411.59 ± 142.66	495.07 ± 225.02	0.01	461.74 ± 106.19	344.18 ± 197.55	0.8
Triglycerides	0.83 ± 0.20	0.76 ± 0.26	0.48	1.35 ± 0.63	1.35 ± 0.82	0.99
Total Cholesterol	4.51 ± 1.14	4.57 ± 1.70	0.89	5.66 ± 2.04	5.14 ± 0.60	0.24
HDL	1.57 ± 0.51	1.59 ± 0.20	0.94	1.65 ± 0.54	1.33 ± 0.18	0.19
LDL	3.12 ± 0.95	2.45 ± 0.83	0.08	3.74 ± 0.90	3.54 ± 0.48	0.62
*Data expressed as mean ± SD *ρ-value<0.05, ; all units= mmol/L except NEFA= µmol/L.*						

Comparison between baseline lipid levels in group fish oil and placebo (chronic intervention randomization) from normal weight and obese subjects.

	Normal weight			Obese		
	Fish oil	**Placebo**	**ρ- value**	**Fish oil**	**Placebo**	**ρ- value**
IL-12	0.00 (0.00 - 9.08)	0.00 (0.00 - 20.67)	0.83	0.00 (0.00 - 9.08)	0.00 (0.00 - 4.19)	0.53
IFN-γ	29.49 (10.36 - 57.57)	15.48 (0.00 - 54.14)	0.71	0.00 (0.00 - 53.69)	65.86 (21.51 - 69.51)	0.03
IL-2	52.37 (48.29 - 100.97)	52.37 (25.08 - 80.91)	0.34	0.00 (0.00 - 75.88)	72.25 (0.00 - 86.35)	0.15
IL-10	23.96 (0.00- 33.83)	0.00 (0.00 - 27.48)	0.58	0.00 (0.00 - 27.55)	35.60 (33.1 - 36.40)	0.06
IL-8	91.71 (45.13 - 316.52)	136.39 (39.72 - 231.08)	0.63	164.77 (128.84 - 354.48)	99.71 (39.83 - 573.12)	0.49
IL-6	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)	0.41	0.00 (0.00 - 0.25)	0.00 (0.00 - 0.00)	0.49
IL-4	70.68 (65.48 - 74.59)	69.06 (57.05 - 74.27)	0.41	36.24 (0.00 - 73.60)	77.24 (67.78 - 80.95)	0.15
IL-1	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)	0.64	0.00 (0.00 - 0.00)	0.00 (0.00 - 11.99)	0.14
IL-5	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)	0.99	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)	0.99
TNF-α	2.38 (0.00 - 20.94)	7.1 (0.00 - 18.72)	0.91	0.00 (0.00 - 22.87)	19.01 (5.32 - 20.23)	0.21
CRP	5.31 (2.30 -7.01)	4.60 (3.44 - 8.79)	0.94	24.04 (18.90 - 34.70)	20.08 (18.84 - 23.98)	0.86
*Data expressed as median and 25th and 75th percentiles *ρ-value <0.05, cytokines unit= pg/mL; CRP units=mg/L*						

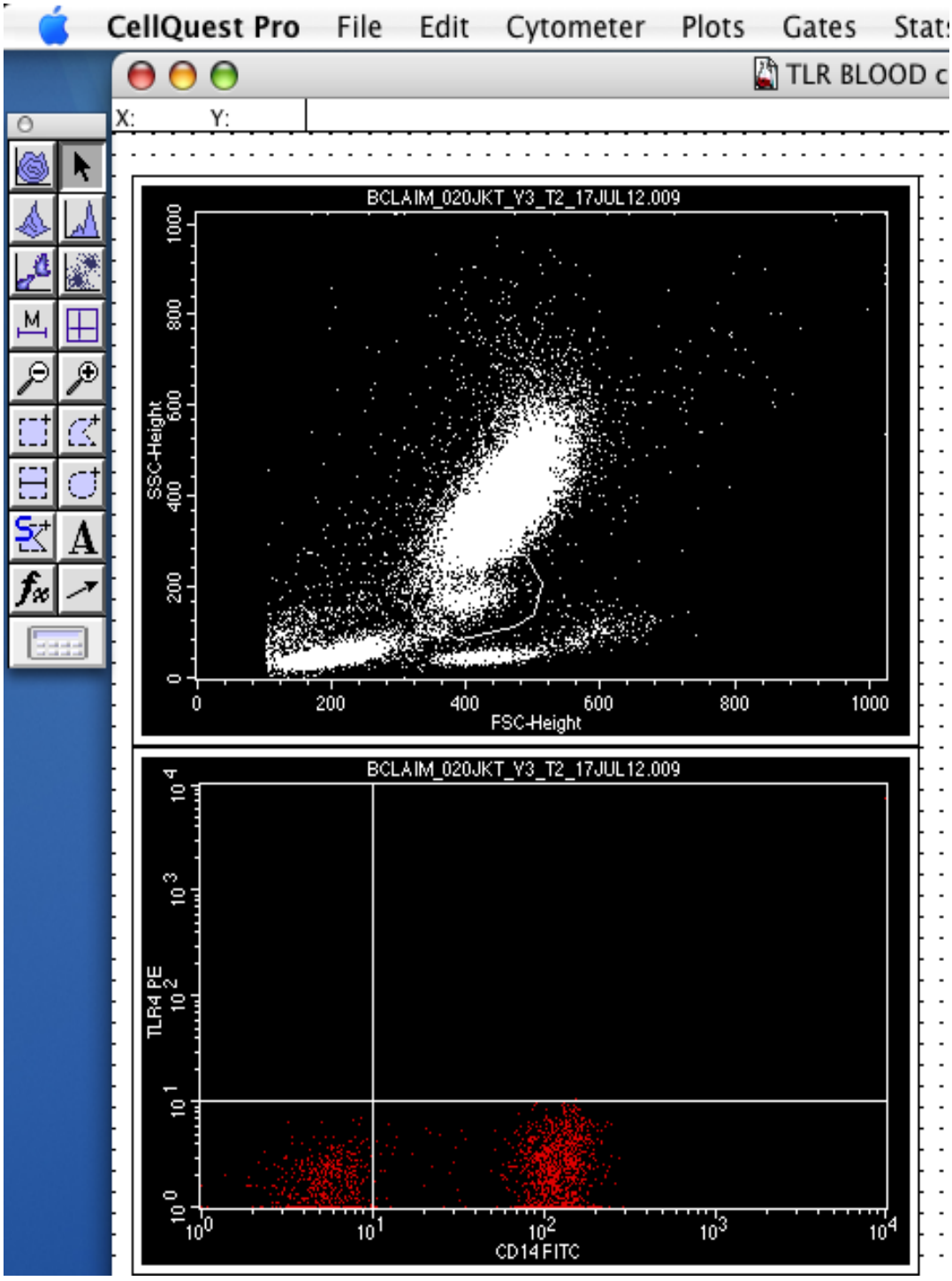
Comparison between baseline cytokine levels in group fish oil and placebo (chronic intervention randomization) from normal weight and obese subjects.

		Normal weight			Obese		
		Fish oil	**Placebo**	**ρ- value**	**Fish oil**	**Placebo**	**ρ- value**
CD14+TLR2+	**% gated**	19.08 (9.32-37.54)	15.03 (7.09-26.41)	0.41	14.52 (7.98-22.43)	10.87 (6.69-19.24)	0.22
	Geo Mean	59.83 (57.93-66.03)	61.71 (58.30-68.35)	0.56	59.75 (56.55-62.63)	57.37 (54.96-60.89)	0.15
CD14+TLR4+	**% gated**	1.58 (0.35-6.20)	2.11 (0.42-5.75)	0.69	6.59 (3.87-11.11)	6.25 (3.95-12.96)	0.64
	Geo Mean	88.53 (59.31-186.43)	106.13 (58.07-221.07)	0.75	214.58 (103.09-504.01)	232.67 (81.42-527.44)	0.66
*Data expressed as median and percentile 25TH and 75TH ; *ρ-value <0.05, MFI= mean fluorescence intensity*							

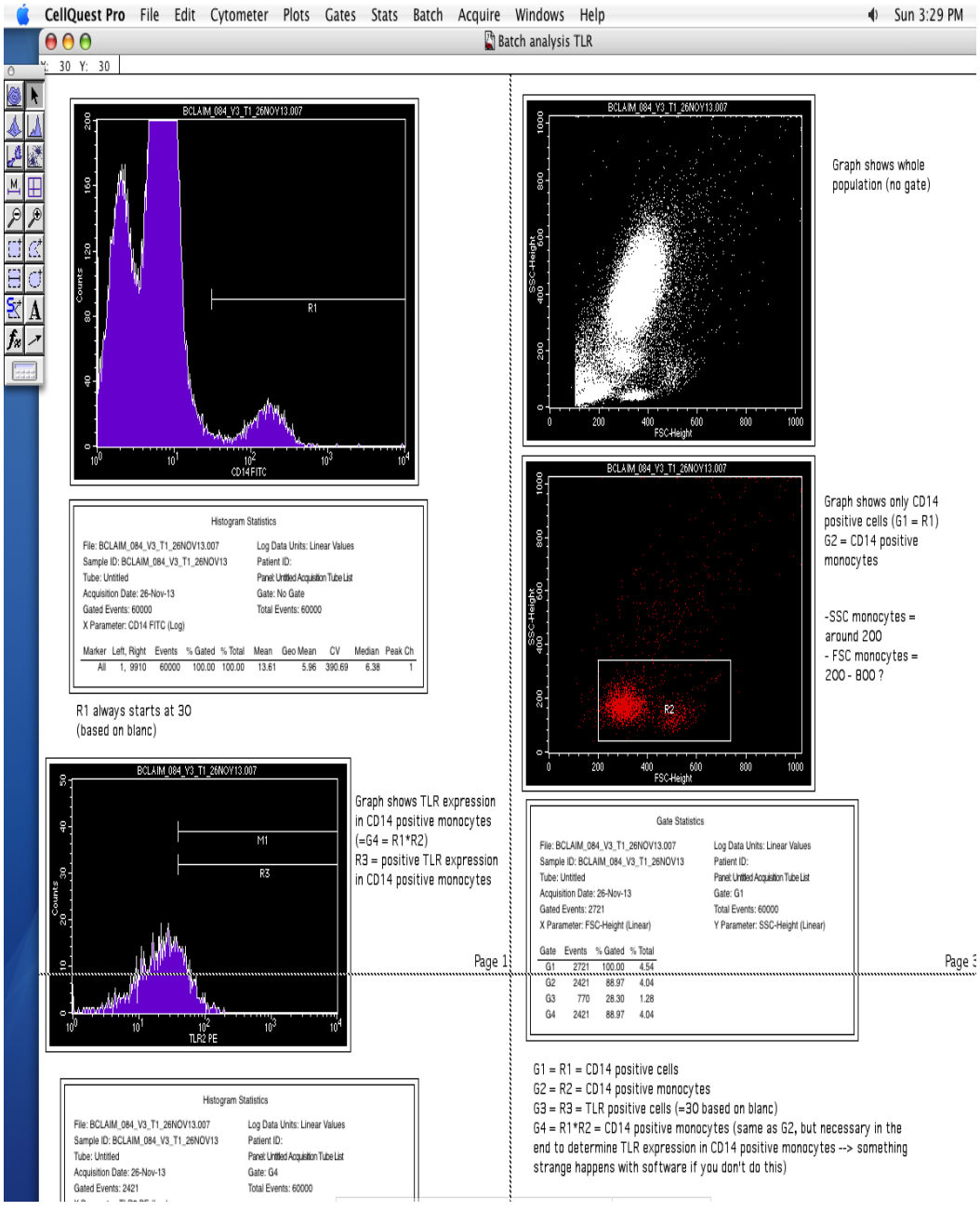
Comparison between baseline TLR2 and 4 levels in group fish oil and placebo (chronic intervention randomization) from normal weight and obese subjects.

TLR analysis

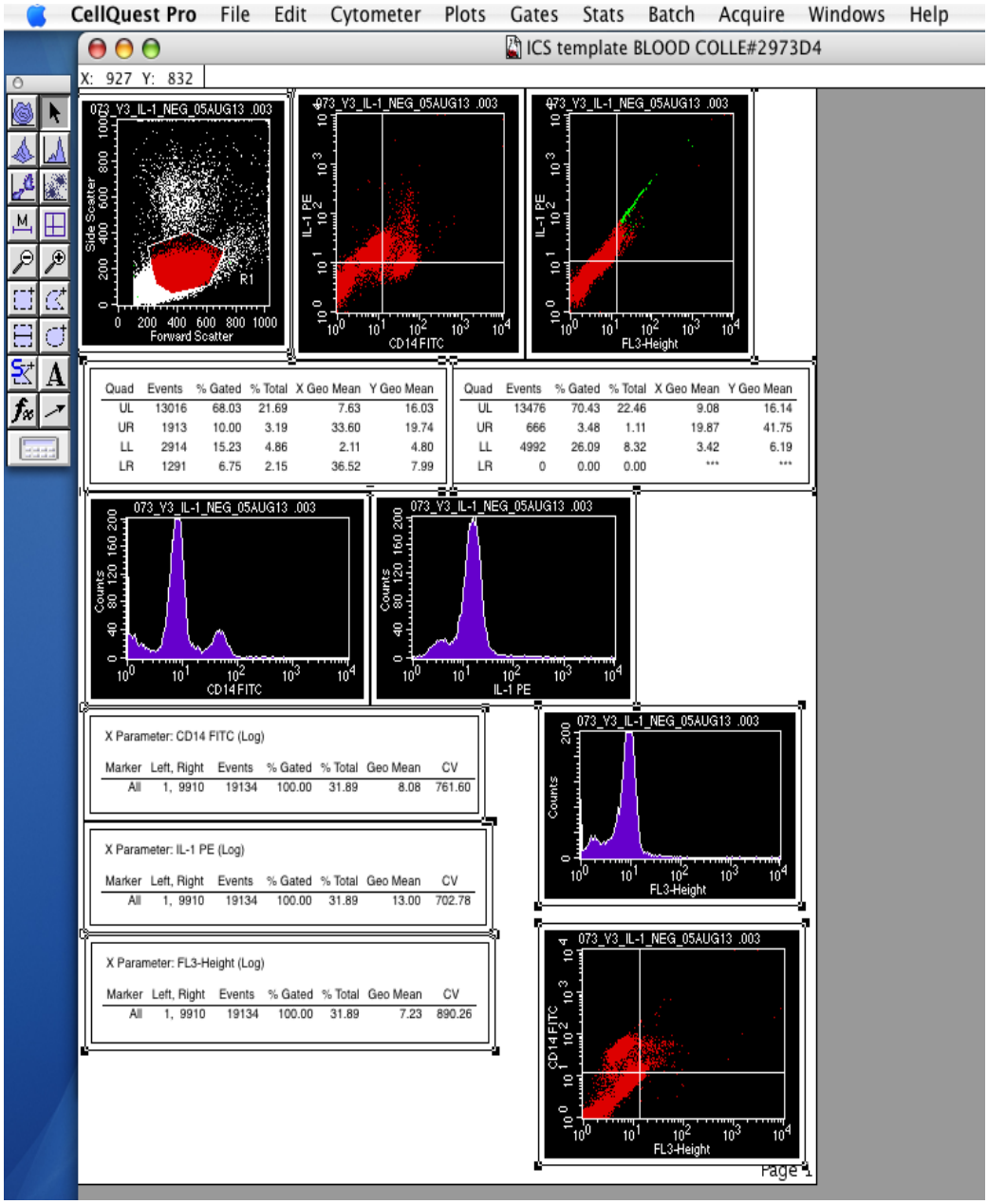
Collection template



TLR DATA ANALYSIS TEMPLATE

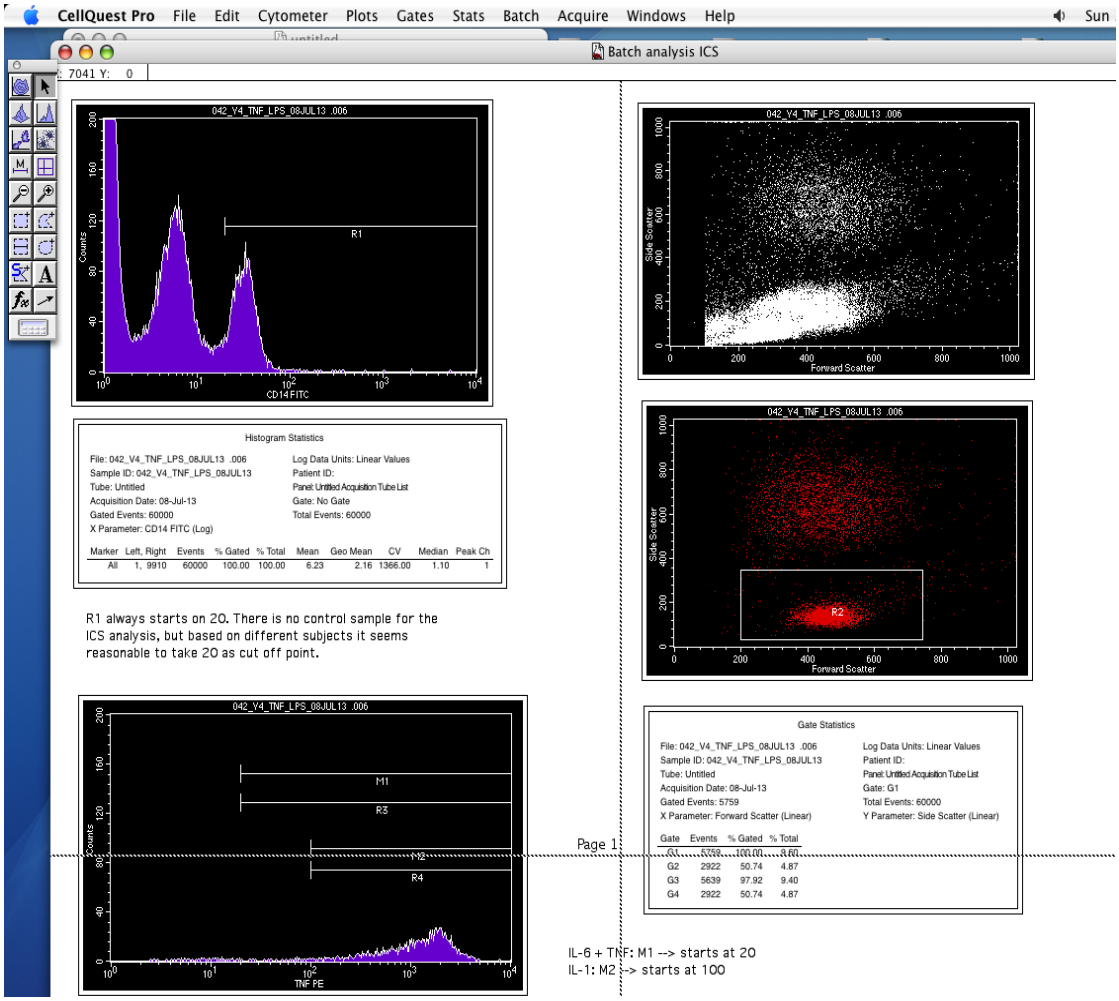


ICS DATA COLLECTION TEMPLATE



DATA ANALYSIS TEMPLATE

ICS



FLOW CYTOMETRY COMPENSATION

TOLL LIKE RECEPTOR EXPRESSION AND CD14

0.4%	FL2
37.5%	FL1
0.0%	FL3
23.5%	FL2

ICS CYTOKINE PRODUCTION COMPENSATION

0.6%	FL2
22.0%	FL1
0.0%	FL3
0.0%	FL2

FOR BOTH

200	FSC
407	SSC
492	FL1
630	FL2
760	FL3

**CALIBRATION WAS DOUBLE CHECKED BEFORE ANALYSIS

APPENDIX III

Luminex reagents and other buffers were prepared based on kit recommendations.

	Company	Cat no.
Human Adhesion Molecules Multiplex kit	R&D	LKT007
Human Obesity Base kit	R&D	**depends on analytes**
Human Cytokine kit	R&D	

Final flow cytometer set up for BD FACSCalibur. Cell population was adjusted if required

Detector	Voltage	Amp	Mode
FS	E00	5.4	Lin
SSC	350	2	Lin
FL1	600	1	Lin
FL2	650		Log
FL3	610		Log
Compensation			
FL1	0.0	FL2	
FL2	0.0	FL1	
FL2	0.0	FL3	
FL3	7.5	FL2	

Assay limit of detection (eBioscience)

Analyte	**Sensitivity (pg/mL)**
H IFN-Y	1.
H IL-1β	4.2
H IL-2	16.4
H IL-4	20.8
H IL-5	1.6
H IL-6	1.2
H IL-8	0.5
H IL-10	1.9
H TNF-α	3.2

LUMINEX Performance assay

Analyte	**Catalog number**	**Sensitivity (pg/mL)**
Adiponectin	Lob1065	19.8
CRP	Lob1707	1.9
Adipsin	Luh279	0.47
IL-6	Lob1824	3.5
IL-10	Luh206	1.11
Leptin	Luh217	0.3
Resistin	Lub398	20.2
PAI-1	Lob1359	7.3
TNF-a	Lob1786	0.29
IL-8	Luh210	1.5
GM-CSF	Lhsc208	0.07
VEGF	LHSC215	0.13
ICAM-1	LHSC293	1.35
E-Selectin	LKT007	30.3
P-Selectin	LKT007	7.4
VCAM-1	LKT007	12.2

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