The effect of *apolipoprotein E* (*APOE*) genotype and long chain omega 3 fatty acids on body weight and inflammation

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Abstract

Obesity-associated adipose tissue (AT) inflammation is characterised by macrophage (ATM) infiltration and polarisation from anti-inflammatory (M2) towards pro-inflammatory (M1) phenotype. Long chain (LC) *n-3* PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), exert various beneficial effects on inflammatory and metabolic parameters in relation to obesity. The *apolipoprotein Epsilon 4* (*APOE4*) genotype is associated with a pro-inflammatory state, lower adiposity yet greater metabolic dysfunction in obesity and an altered LC *n-3* PUFA status compared to *APOE3* genotype. This PhD project aimed to investigate the relationships between *APOE* genotype, adiposity, LC *n-3* PUFA status and inflammation.

In an existing data set of 312 participants involved in a fish oil intervention trial [Caslake et al., 2008, Kofler et al., 2012] we demonstrated that age, BMI and habitual oily fish intake, but not *APOE Epsilon* genotype, determined plasma LC *n-3* PUFA status. *APOE Epsilon* genotype interacted with -219G/T polymorphism (rs405509) to determine plasma apoE and select inflammatory marker concentrations.

In human *APOE* targeted replacement (TR) mice fed a high-fat diet (HFD) without or with 30 g EPA+DHA / kg of diet (HFD+FO) for 8 weeks, *APOE3*, but not *APOE4*, mice develop obesity and impaired glucose tolerance upon HFD feeding that is prevented by dietary fish oil. ATM infiltration and phenotype was similar between *APOE3* and *APOE4* mice. Fish oil increased the number of ATM without influencing their phenotype. LC *n-3* PUFA enrichment of AT was similar, although EPA and DHA levels tended to be 10 – 20% lower in *APOE4* mice compared to *APOE3* mice on HFD+FO. In primary murine bone marrow-derived macrophages the *APOE4* genotype was associated with higher prevalence of the anti-inflammatory M2 phenotype compared to *APOE3* genotype.

In conclusion, our findings contribute to the current understanding of the potential mechanisms through which the *APOE Epsilon* genotype modulates adiposity and its metabolic impact.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification at this or any other university or other institute of learning.

Statement of originality

I certify that this thesis, and the research to which it refers, is my own work, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged.

List of publications

- **Slim K.** and Minihane A.M.: *How Fatty Acids and Common Genetic Variants Together Affect the Inflammation of Adipose Tissue*. Current Cardiovascular Risk Reports, 2014, **8**(411): p. 1-11.

- **Slim K.**, Vauzour D., Tejera N., Cassidy A., Minihane A.M.: *The anti-obesity and insulin sensitising effects of dietary fish oil dependent on APOE genotype in human APOE3 and APOE4 TR mice*. International Journal of Obesity. In preparation June 2015

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List of abbreviations

95% CI	95% confidence interval
AA	arachidonic acid
Actb	beta actin
AD	Alzheimer's disease
ALA	alpha linolenic acid
ALP	alkaline phosphatase
ALT	aminotransferase
AMPK	5' AMP-activated protein kinase
apoB	apolipoprotein B
apoE, APOE	apolipoprotein E
Arg	arginine
Arg1	arginase 1
ASP	aspartate aminotransferase
AT	adipose tissue
ATM	adipose tissue macrophage
Atp5b	ATP synthase H+ transporting mitochondrial F1 complex beta subunit
BMI	body mass index
BMM	bone marrow-derived macrophage
C/EBP	CCAAT-enhancer-binding protein
CD11b	integrin alpha X (Itgax)
CD11c	integrin alpha M (Itgam)
CD206	mannose receptor C type 1 (Mrc1)
CHD	coronary heart disease
CHOP	transcription factor homologous to CCAAT-enhancer binding protein
CE	cholesterol ester
CVD	cardiovascular disease
CLS	crown-like structure
CM	chylomicron
COX	cyclooxygenase
CPT1	carnitine palmitoyltransferase 1
CRP	C-reactive protein
Cys	cysteine
DHA	docosahexaenoic acid
DIO	diet-induced obesity
DPA	docosapentaenoic acid
DRI	dietary reference intake
EPA	eicosapentaenoic acid

ER	endoplasmic reticulum
E-sel	E-selectin
F4/80	EGF-like module containing mucin-like hormone receptor-like sequence 1
FABP	fatty acid binding protein
FATP	fatty acid transport protein
FFQ	food frequency questionaire
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
GLUT4	solute carrier family 2 (facilitated glucose transporter) member 4 (Slc2a4)
GPR	G-protein coupled receptor
GTT	glucose tolerance test
HDL-C	high-density lipoprotein cholesterol
HFD	high-fat diet
HFD+FO	high-fat diet containing 30 g EPA+DHA per kg of diet
HOMA-IR	homeostasis model assessment – insulin resistance
ICAM-1	intercellular cell adhesion molecule 1
IGT	impaired glucose tolerance
IKKβ / NF-κB	IκB kinase β - nuclear factor kappa-B
Il1b, IL-1β	interleukin 1 beta
Il6, IL-6	interleukin 6
Il10, IL-10	interleukin 10
iNOS	inducible nitric oxide synthase
IPGTT	intra-peritoneal glucose tolerance test
IR	insulin resistance
IS	insulin sensitivity
JNK / AP-1	c-Jun N-terminal kinases – activator protein 1
LA	linoleic acid
LC *n-3* PUFA	long chain omega 3 polyunsaturated fatty acids
LDL-C	low-density lipoprotein cholesterol
LOX	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene
M1	proinflammatory macrophage phenotype
M2	anti-inflammatory macrophage phenotype
MAF	minor allele frequency
MCP-1	monocyte chemoattractant protein 1
M-CSF	macrophage colony-stimulating factor
MetS	metabolic syndrome
Mgl1	C-type lectin domain family 10 member A
Mgl2	macrophage galactose N-acetyl-galactosamine specific lectin 2
MHO	metabolically healthy obese

MUFA	monounsaturated fatty acid
MUO	metabolically unhealthy obese
NEFA	non-esterified fatty acids
NF-κB	nuclear factor kappa-B
Nos2, NOS2	inducible nitric oxide synthase 2
Nrf1	nuclear respiratory factor 1
Nrf2	nuclear factor (erythroid-derived 2)-like 2
OR	odds ratio
PC	phosphatidylcholine
PG	prostaglandin
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
PL	phospholipid
PPAR	peroxisome proliferator-activated receptor
PR	prevalence ratio
P-sel	P-selectin
PUFA	polyunsaturated fatty acid
RCT	randomized controlled trial
RR	relative risk
RT-QPCR	real-time quantitative polymerase chain reaction
SAT	subcutaneous adipose tissue
SD	standard deviation
SEM	standard error of the mean
SFA	saturated fatty acid
SMD	standard mean difference
SNP	single nucleotide polymorphism
SREBP	sterol regulatory element-binding protein
SVF	stromal vascular fraction
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TC	total cholesterol
TG	triglycerides
Tnfa, TNF-α	tumor necrosis factor alpha
TR	targeted replacement
UCP	uncoupling protein
UK	United Kingdom
VAT	visceral adipose tissue
VCAM-1	vascular cell adhesion molecule 1
VLDL	very low-density lipoprotein

VLDL-C	very low-density lipoprotein cholesterol
WMD	weighted mean difference
Ym1	chitinase 3-like 3

Chapter 1. Literature review: Obesity, adipose tissue inflammation, *APOE* genotype and fish oil fatty acids

A literature review of studies in humans and mice

Obesity and metabolic health

Obesity – a world-wide epidemic

Excess body weight has become a worldwide epidemic with approximately 1 billion overweight (BMI 25 – 29.9 kg/m²) and a further 475 million obese (BMI ≥ 30 kg/m^2) adults and is considered one of the most important public health problems in the world today [World Obesity Federation, 2014]. Obesity increases the risk of various diseases, including cardiovascular disease (CVD), Type 2 Diabetes Mellitus (T2DM), selected cancers and premature death [World Obesity Federation, 2014]. The WHO estimates from 2004 indicated that overweight and obesity was the fifth leading cause of death in the world accounting for 2.8 million deaths and 36 million disability-adjusted life years – a measure of the burden of disease or lost years of healthy life [WHO, 2009]. Furthermore, the number of overweight or obese infants and young children (0 - 5 yr) has increased dramatically over the last two decades to 42 million in 2013 and if the current trend continues will increase to 70 million by 2025 [WHO, 2014]. Without intervention, the obese infants and young children are likely to continue to be obese during childhood, adolescence and adulthood, which heightens their risk for T2DM, CVD and a variety of other co-morbidities before or during early adulthood [Lobstein et al., 2004]. Thus, overweight and obesity is becoming a worldwide problem with a major health impact and financial burden.

Adipose tissue and obesity

An excess of body weight is associated with adipose tissue (AT) expansion and dysfunction. AT was recognised centuries ago as the primary tissue for the storage of lipids and energy, but over the past decades it has also been identified to function as an endocrine organ producing various bioactive compounds [Frayn et al., 2003, Galic et al., 2010]. It is composed of primarily mature adipocytes, though it also contains a stromal vascular fraction (SVF) comprising of mesenchymal stem cells, pre-adipocytes, monocytes, macrophages, lymphocytes, fibroblasts and endothelial cells that contribute to adipocyte growth, adipocyte function and AT homeostasis [Ouchi et al., 2011]. AT produces various bioactive compounds, collectively called adipokines, which have an important role in controlling whole-body metabolism. They include the hormones leptin and adiponectin, growth factors (e.g. vascular endothelium growth factor), and chemokines and cytokines, such as monocyte

chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 [Calder et al., 2011A, Frayn et al., 2003, Kalupahana et al., 2011]. Most of these adipokines are increased in obesity, with the exception of adiponectin whose levels decrease with increasing adiposity (i.e. AT fat mass). There are two different types of AT, brown and white. Brown AT contains a large number of mitochondria and uncoupling protein 1 (UCP1) that is responsible for the dissipation of energy as heat (thermogenesis). Brown AT is prominent in the newborn where it is important for maintaining body temperature, but it descreases with increasing age and is only present to small degree in aldult humans. White AT is the most abundant type of fat that can account for up to 50 % of body weight in severe obesity. It is dispersed throughout the whole body and classified into subcutaneous and visceral AT. White AT is specialised in the storage of lipids and consist of approximately 80 % triglycerides (TG). The white AT fat stores reflect the imbalance between energy intake and energy expenditure over a long period of time. Excess energy is stored as TG in adipocytes predominantly through a pathway involving lipoprotein lipase hydrolysis of circulating lipoprotein-TG followed by fatty acid uptake and re-esterification to glycerol 3-phosphate [Frayn et al., 2003]. Fat mobilisation involves the action of hormone-sensitive lipase which hydrolyses TG releasing non-esterified fatty acids (NEFA). Insulin is an important hormone stimulating fat deposition and suppressing fat mobilisation pathways [Frayn et al., 2003]. In obesity structural, functional and cellular changes take place in the AT; adipocytes become hypertrophic by intracellular TG accumulation, various immune cells start to infiltrate the AT resulting in AT inflammation, and plasma concentrations of various adipokines, hormones and acute-phase proteins are altered with up to a 10-fold increase in various markers of chronic low-grade inflammation [Calder et al., 2011A, Dalmas et al., 2011, Frayn et al., 2003]. The adipocyte hyperthrophy and AT inflammation in obesity are believed to be an underlying cause of the metabolic complications of obesity, which is discussed in more detail in the following paragraphs. Adipocyte hyperthophy in obesity could be ameliorated by short term strategies aimed at increasing energy expenditure (i.e. physical activity), reducing energy intake (i.e. caloric restriction), and/or improving adipocyte function using bioactive food or pharmacological compounds. Furthermore, strategies using pharmacological or dietary food compounds with anti-inflammatory effects, such as long chain *n-3* polyunsaturated fatty acids, could ameliorate AT inflammation in obesity. The resolution of adipocyte hypertrophy and/or AT inflammation will improve AT function and plasma adipokine, hormone and acute-phase protein profiles. In summary, AT is an important metabolically active and endocrine organ with several dramatic changes occuring in obesity which have various health implications that will be discussed in more detail in the following paragraphs.

Obesity and metabolic health

Obesity is associated with the development of metabolic complications such as metabolic syndrome (MetS) and insulin resistance (IR), which increase the risk for T2DM and

cardiovascular disease (CVD) [Maury and Brichard, 2010, Van Gaal et al., 2006]. For example, obese individuals display dyslipidemia (characterised by high plasma TG and low plasma high-density lipoprotein cholesterol (HDL-C)), reduced insulin sensitivity and chronic low-grade inflammation. The relation between obesity and metabolic abnormalities has been well established, yet a proportion of obese people, termed metabolically healthy obese (MHO), do not develop metabolic abnormalities and may not be at an increased risk for disease [Karelis et al., 2004, Wildman et al., 2008]. These MHO individuals have a normal metabolic profile characterised by a low visceral fat (VAT) content, high plasma HDL-C levels, low plasma TG levels and high insulin sensitivity despite their high fat mass and BMI. MHO can account for up to one-third of obese people [Karelis et al., 2004, Primeau et al., 2011]. The prevalence of MHO appears to decrease with age suggesting that it is a temporary condition during which the body is still able to 'cope' with obesity [Primeau et al., 2011], with physical activity and cardiovascular fitness being two important determinants of the MHO phenotype [Ortega et al., 2012].

In addition, AT inflammation appears to be an important determinant of the metabolic consequences of obesity. For example, Barbarroja *et al.* showed that MHO insulin-sensitive individuals with a mean BMI of 56 ± 1 (SD) kg/m^2 lacked the inflammatory response in VAT that characterized the metabolically unhealthy obese (MUO) insulin-resistant individuals [Barbarroja et al., 2010]. Finally, the expansion capacity of AT has been postulated as a potential underlying mechanism connecting obesity to its metabolic complications, which is referred to as the adipose tissue expandability hypothesis [Virtue and Vidal-Puig, 2010]. According to this hypothesis, each individual has a defined maximum AT expansion capacity, which is determined by genetic and environmental factors, and as an individual gains weight it eventually reaches its maximum AT expansion capacity. After this point, AT cannot expand any further and ectopic lipid deposition occurs in liver, muscle and pancreas leading to IR, inflammation and cardiovascular complications. Thus, AT inflammation, excess VAT and an impaired AT expansion capacity are important determinants of the metabolic consequences of obesity [Barbarroja et al., 2010, Blüher, 2010, Karelis et al., 2004, Virtue and Vidal-Puig, 2010].

Obesity and adipose tissue inflammation

Expansion of AT is characterised by structural, functional and cellular changes that lead to local and systemic inflammation in obesity, which has been recognised as an underlying cause of the metabolic consequences of obesity [Calder et al., 2011A, Dalmas et al., 2011, Osborn and Olefsky, 2012]. In a chronic state of a positive energy balance (i.e. the onset of obesity), adipocytes increasingly accumulate intracellular TG and eventually become hypertrophic and start to secrete mediators that attract various immune cells that include in the early stage predominantly neutrophils, T cells and mast cells [Dalmas et al., 2011, Nishimura et al., 2009, Osborn and Olefsky, 2012]. As obesity develops into a chronic state, the hypertrophic adipocytes become inflamed and necrotic attracting macrophages which

increasingly infiltrate the tissue arranging themselves around the necrotic adipocytes forming crown-like structures (CLS) [Slim and Minihane, 2014]. The recruited AT macrophages (ATM) will predominantly acquire a pro-inflammatory (M1) phenotype associated with the expression of many pro-inflammatory genes (e.g. *TNF-α*, *IL-1β*, *IL-6*, *NOS2*) and start to outnumber the resident ATM that have predominantly an anti-inflammatory (M2) phenotype [Lumeng et al., 2007A, Weisberg et al., 2003, Wentworth et al., 2010]. Macrophages can adopt a range of phenotypes between M1 and M2, but the simplified M1/M2 model of macrophage polarisation is most frequently used [Morris et al., 2011]. The cellular alterations in obese AT are associated with an increased production of pro-inflammatory mediators and a decreased production of anti-inflammatory mediators by SVF cells (which includes macrophages) and hypertrophic adipocytes, which results in local and systemic inflammation [Fain et al., 2004, Maury and Brichard, 2010, Slim and Minihane, 2014]. It has been well established that specifically ATM infiltration is associated with systemic chronic low-grade inflammation, which in turn contributes to increased risk for systemic IR, metabolic disease and CVD [Barbarroja et al., 2010, Hotamisligil, 2006, Osborn and Olefsky, 2012, Shoelson et al., 2006, Van Gaal et al., 2006]. For example, Cancello *et al*. showed that weight loss was associated with a reduction in ATM and reduction in systemic low-grade inflammation in obese subjects [Cancello et al., 2005]. ATM infiltration in VAT is likely to be most detrimental to metabolic health, as excess VAT and the inflammatory response in VAT have not only been linked to the MUO phenotype, but VAT depots also have a greater ATM content than subcutaneous AT (SAT) [Barbarroja et al., 2010, Bruun et al., 2005, Cancello et al., 2006, Karelis et al., 2004]. Finally, AT from lean and metabolically healthy humans and mice also contains immune cells, including a considerable number of ATM with an anti-inflammatory M2 phenotype, which are involved in maintaining homeostasis [Lumeng et al., 2007A, Osborn and Olefsky, 2012]. These findings support that not all ATM have a detrimental effect on metabolic function and M2 ATM appear to be important for AT function [Dalmas et al., 2011, Morris et al., 2011].

In summary, excess AT in obesity is associated with AT inflammation and various metabolic complications resulting in an increased risk for T2DM and CVD. However, not all obese individuals show AT inflammation and a metabolically unhealthy phenotype, suggesting genetic, nutritional and other environmental factors may be involved in determining AT inflammation and metabolic complications in obesity.

Apolipoprotein E (*APOE*) genotype, adiposity and inflammation

Apolipoprotein E – protein function

There is accumulating evidence supporting the involvement of apolipoprotein E (apoE) in the development of AT, obesity and obesity-related metabolic complications [Huang et al., 2015, Kypreos et al., 2009, Slim and Minihane, 2014]. ApoE is a 34.2 kDa arginine-rich protein

consisting of 299 amino acids with lipoprotein-binding elements located in the C-terminal domain and receptor-binding elements in the N-terminal domain that is encoded by the *APOE* gene. ApoE is expressed in various tissues, including liver (≥ 80 %), brain and AT, and macrophages which can account for up to 20 % of its production in tissues [Kockx et al., 2007, Mahley, 1988]. ApoE is known to play an important role in lipoprotein metabolism, where it serves as a cofactor in hepatic very-low-density lipoprotein (VLDL) synthesis, a cofactor in the hydrolysis of VLDL remnants into low-density lipoprotein (LDL) particles in circulation, and a high-affinity ligand for receptor-mediated cellular uptake of remnants of chylomicron (CM) and VLDL particles [Mahley, 1988]. But besides its well-known role in lipoprotein metabolism, apoE has immunomodulatory, anti-inflammatory and anti-oxidative properties [Jofre-Monseny et al., 2008A, Minihane et al., 2007]. These immunomodulatory and anti-inflammatory effects have predominantly been shown *in vitro* using models of an induced-inflammatory response, where apoE modulated the immunological function of lymphocytes [Curtiss, 2000, Kelly et al., 1994, Pepe and Curtiss, 1986] and the phenotype of macrophages [Baitsch et al., 2011], reduced the lipopolysaccharide (LPS)-induced inflammatory response in microglia [Jofre-Monseny et al., 2008A, Lynch et al., 2003] and inhibited the gene expression of cell adhesion molecules in endothelial cells [Stannard et al., 2001]. Some examples of the anti-oxidative properties reported for apoE are, inhibition of lipid oxidation [Miyata and Smith, 1996, Ramassamy et al., 1999], inhibition of membrane oxidation [Jofre-Monseny et al., 2007A] and modulation of nitric oxide production [Colton et al., 2004, Jofre-Monseny et al., 2007A].

Apolipoprotein E and adipose tissue function

Early studies revealed that the expression of apoE increased linearly with time upon differentiation in adipocytes with apoE being essential for lipid accumulation [Huang et al., 2006, Zechner et al., 1991]. ApoE deficiency has been associated with an impaired fatty acid uptake, decreased TG synthesis and increased TG hydrolysis in primary adipocytes and with an impaired plasma TG clearance, reduced body fat mass and smaller adipocytes in apoE$^{-/-}$ mice [Hofmann et al., 2008, Huang et al., 2006]. Furthermore, apoE is a component of VLDL-derived TG-rich lipoproteins, which are the main source of fatty acids for AT in both the fasting and fed state and play an important role in AT development [Voshol et al., 2009]. A link between apoE, lipoprotein metabolism and the development of obesity was demonstrated by Chiba *et al.* who reported that VLDL-induced adipogenesis was apoE-dependent and that apoE-deficient *ob*/*ob* mice were protected from diet-induced obesity despite their higher plasma VLDL levels [Chiba et al., 2003]. In summary, these findings clearly indicate a role for apoE in AT development and the development of obesity.

Apolipoprotein E – genetic variants

Two common single nucleotide polymorphisms (SNPs) in the *APOE* gene 388T/C (ε4 variant, rs429358) and 526C/T (ε2 variant, rs7412) give rise to the three common allelic variants ε2, ε3 and ε4 which are associated with amino acid changes in the apoE protein

and referred to as *Epsilon* gene variants (Table 1.1). The most common variant and often referred to as wild type is apoE3 that contains a cysteine (Cys) and an arginine (Arg) at position 112 and 158. The apoE2 isoform is characterized by an amino acid change at position 158 (Arg -> Cys), which causes a 50 – 100 fold weaker binding affinity for the LDL receptor compared to the apoE3 isoform. The apoE4 isoform is distinguished by an amino acid change at position 112 (Cys -> Arg), which results in an interaction between the N- and C-terminal domain that is associated with a change in lipoprotein binding preference from HDL towards VLDL and CM and a lower chemical and thermal protein stability compared to the apoE3 isoform [Hatters et al., 2006]. The three common allelic variants of the *APOE* gene (*ε2*, *ε3* and *ε4*) give rise to six possible *APOE Epsilon* genotypes, which are in order of prevalence from most common to least common *E3/E3*, *E3/E4*, *E2/E3*, *E4/E4*, *E2/E4* and *E2/E2* [Singh et al., 2006]. The *APOE3* (*E3/E3*) genotype is often referred to as the wild type. The distribution of the *APOE* genotypes differs between ethnicities, but in a Caucasian population most people have the *APOE3* genotype (*E3/E3*; 65 %), 25 % are *APOE4* carriers (*E2/E4*, *E3/E4*, *E4/E4*) and 10 % are *APOE2* carriers (*E2/E3*, *E2/E2*) [Eichner et al., 2002, Singh et al., 2006].

Table 1.1. Human apoE isoforms and their key differences.

ApoE isoform	Amino acid at 112/158	Domain interaction	Stability, folding behaviour	LDLR binding affinity	Lipo-protein preference	Associated blood lipid changes
apoE2	Cys/Cys	No	Most stable, no folding intermediates	Low	HDL	Type II hyper-lipoproteinaemia
apoE3	Cys/Arg	No	Mediate stable, folding intermediates	High	HDL	
apoE4	Arg/Arg	Yes	Least stable, folding intermediates	High	VLDL, CM	Moderately (~8%) increased LDL cholesterol

Table describing key differences between the three common apoE isoforms, i.e. amino acid at position 112 and 158 of the protein; protein domain interaction between the N- and C-terminus; protein conformational stability and folding behaviour; LDL receptor binding affinity; lipoprotein preference; and associated blood lipid changes [Hatters et al., 2006, Minihane et al., 2007, Jofre-Monseny et al., 2008A]. ApoE, apolipoprotein E; Arg, arginine; Cys, cysteine; CM, chylomicron; HDL, high-density lipoprotein; LDLR, low-density lipoprotein receptor; Type II hyper-lipoproteinaemia, high triglyceride with normal cholesterol levels; VLDL, very low-density lipoprotein.

APOE Epsilon genotype and disease risk

Almost two decades ago, the *APOE4* allele was first discovered as a major risk factor for Alzheimer's Disease (AD) [Corder et al., 1993, Strittmatter et al., 1993, Takeda et al., 2010], with *E3/E4* and *E4/E4* at relative risk of AD of 3-fold and 15-fold relative to the *E3/E3* genotype [Bertram et al., 2007]. Subsequent studies revealed the *APOE4* allele was also associated with the risk of coronary heart disease (CHD), with *APOE4* carriers having 6 – 42 % increased CHD risk compared to those with *APOE3* genotype (Table 1.2) [Bennet et al., 2007, Song et al., 2004, Wilson et al., 1996].

Table 1.2. Meta-analyses on *APOE Epsilon* genotype and CHD risk.

Study details	*APOE Epsilon* genotype*	CHD risk compared to subjects with *APOE3* genotype	Reference
Meta-analysis including 9 studies published between 1987 and 1996	*APOE2* carriers†	OR = 0.98, 95% CI 0.85 – 1.14	[Bennet et al., 2007]
	APOE4 carriers†	OR = 1.26, 95% CI 1.13 – 1.41	
Meta-analysis including 48 studies published between 1966 and 2004	*APOE2* carriers	OR = 0.98, 95% CI 0.66 – 1.46	[Song et al., 2004]
	APOE4 carriers	OR = 1.42, 95% CI 1.26 – 1.61	
Meta-analysis including 112 studies between 1970 and 2007 with each study including at least 500 cases	*APOE2* carriers	OR = 0.80, 95% CI 0.70 – 0.90	[Wilson et al., 1996]
	APOE4 carriers	OR = 1.06, 95% CI 0.99 – 1.13	

APOE Epsilon genotypes were defined as *APOE3* genotype (*E3/E3*); *APOE2* carriers (*E2/E2*, *E2/E3*); and *APOE4* carriers (*E3/E4*, *E4/E4*), unless stated otherwise. † *APOE2* carriers (*E2/E2*, *E2/E3*, *E2/E4*) and *APOE4* carriers (*E2/E4*, *E3/E4*, *E4/E4*). 95% CI, 95% confidence interval; APOE, apolipoprotein E; CHD, coronary heart disease; OR, odds ratio.

Plasma apoE concentrations exist in an *APOE Epsilon* genotype-dependent manner with the highest plasma levels in *APOE2* carriers and lowest plasma levels in *APOE4* carriers [Moreno et al., 2009]. The increased CHD risk has traditionally been attributed to the approximately 8 % higher plasma LDL-cholesterol (LDL-C) levels in *APOE4* carriers relative to those with *APOE3* genotype, however this cannot solely explain the increased risk and suggests other lipoprotein-independent mechanisms, such as inflammation and oxidative stress, are involved in the *APOE4* – CHD association [Law et al., 1994, Minihane et al., 2007].

APOE Epsilon genotype and obesity

Limited human cross-sectional studies provide accumulating evidence supporting a link between *APOE Epsilon* genotype, adiposity and the metabolic consequences of obesity [Elosua et al., 2003, Kolovou et al., 2009, Sima et al., 2007, Slim and Minihane, 2014] (Table 1.3). Various studies indicated that the *APOE4* allele was more prevalent in obese people though there was some inconsistency [Elosua et al., 2003, Kolovou et al., 2009, Oh and Barrett-Connor, 2001, Sima et al., 2007, Volcik et al., 2006] and *APOE Epsilon* genotype interacted with BMI to determine metabolic and/or inflammatory parameters [Kofler et al., 2012, Marques-Vidal et al., 2003, Pouliot et al., 1990]. For example, the *APOE4* allele was more prevalent in obese people compared to people with a normal weight (30 % vs. 15 %, $p < 0.05$) and in people with MetS compared to normal weight healthy controls (13.5 % vs. 8.2 %, $p < 0.05$) in 279 subjects from Romanian population [Sima et al., 2007]. Besides, Marques-Vidal *et al*. showed that *APOE Epsilon* genotype interacted with BMI to determine plasma total cholesterol (TC), LDL-C, total insulin and bioactive insulin in 266 healthy men

without hypolipidemic or antidiabetic drug treatment, with *APOE4* carriers having higher levels compared to other *APOE Epsilon* genotypes and the difference enlarging with increasing BMI [Marques-Vidal et al., 2003]. These findings suggest that the *APOE Epsilon* genotype may influence adiposity and interact with adiposity (BMI) to determine the metabolic consequences of increased adiposity, something that may already manifest itself in childhood [Ellis et al., 2011]. In summary, literature suggests an interplay between *APOE Epsilon* genotype and AT to determine risk factors for disease (e.g. IR and MetS), which has recently been corroborated in a mouse model humanised for the different apoE isoforms [Arbones-Mainar et al., 2008 and 2010]. Arbones-Mainar *et al*. showed that the *APOE4* allele was associated with an impaired expandability of VAT, an impaired adipocyte differentiation and a greater predisposition to develop impaired glucose tolerance upon high-fat diet feeding compared to *APOE3* allele in human *APOE* targeted replacement mice. Thus, *APOE Epsilon* genotype is a potential determinant of AT expansion, adiposity and predisposition to develop metabolic complication in obesity.

APOE Epsilon genotype and oxidative stress

The potential involvement of oxidative stress in the *APOE4* – disease association is supported by a greater penetrance of the *APOE4* allele on CHD risk in smokers, who have a habitual pro-oxidative and low-grade pro-inflammatory status, relative to non-smokers after correction for plasma LDL-C [Humphries et al., 2001, Talmud et al., 2005]. For example, CHD hazard ratios corrected for classical CHD risk factors were 3.81 and 1.68 for male *APOE4* and *APOE3* smokers compared to non-smoking *APOE3* in the Framingham cohort [Talmud et al., 2005] and 2.79 and 1.47 for male *APOE4* and *APOE3* smokers compared to combined group of non-smokers in the Northwick Park Heart Study II [Humphries et al., 2001]. This implies that oxidative and inflammatory mechanisms could be involved in the *APOE4* – CHD association. In fact, the presence of a pro-oxidative status in *APOE4* carriers was supported by Dietrich *et al*. who reported 29 % higher F2-isoprostanes levels (a biomarker of lipid oxidation) in mildly-hypercholesterolaemic *APOE4* carriers compared to non-carriers [Dietrich et al., 2005]. These observations have been confirmed *in vitro* using stable transfected cell lines expressing apoE3 and apoE4 revealing the apoE4 isoform was associated with approximately 40 % higher membrane lipid oxidation and 30 – 60 % higher superoxide levels [Jofre-Monseny et al., 2008B]. Using *APOE3* and *APOE4* targeted replacement (TR) mice the *APOE4* allele was associated with lower alpha tocopherol tissue levels in liver, lung, muscle and heart (reaching only significance for lung) and 50 % higher plasma α-TOH uptake [Jofre-Monseny et al., 2008B, Huebbe et al., 2010]. Thus, current evidence suggests that the *APOE4* genotype is associated with higher levels of oxidative stress. The differential antioxidant capacity of the apoE isoforms (E2 > E3 > E4) has been suggested to be underlying the poorer oxidative status associated with *APOE4* genotype [Jofre-Monseny et al., 2007B, Miyata and Smith, 1996]. These differential antioxidant capacities of the apoE isoforms have been related to the differential folding and stability of

the proteins affecting the radical scavenging activity of the positively charged amino acids located in the receptor binding domain of the apoE protein [Jofre-Monseny et al., 2007B, Pham et al., 2005].

APOE Epsilon genotype and inflammation in humans

A pro-inflammatory status in *APOE4* carriers could also be involved in the *APOE4* – disease association, especially since inflammation is a well-recognised component of CHD and AD pathology [Hansson and Hermansson, 2011, Latta et al., in press]. Although previous *in vitro* studies and *in vivo* studies in mice have demonstrated the *APOE4* allele is associated with a pro-inflammatory state in predominantly microglia and brain tissue [Jofre-Monseny et al., 2008A], these findings are of relevance since microglia (considered to be the macrophages of the brain) share many functional characteristics with macrophages that play a key role in AT inflammation in obesity. However, the impact of *APOE Epsilon* genotype on the inflammatory status in humans is still unknown. The scarce human studies available have primarily investigated the impact of *APOE Epsilon* genotype on plasma levels of C-reactive protein (CRP), an acute phase protein released by the liver upon stimulation with inflammatory cytokines and a widely-used but not exclusive marker of CHD risk [Pearson et al., 2003]. These studies have demonstrates that in contrary to their 6 – 42 % increased CHD risk *APOE4* carriers have lower plasma CRP levels and *APOE2* carriers have higher CRP levels despite their 0 – 20 % lower CHD risk compared to the *APOE3* genotype (Table 1.4). For example, in the Ludwigshafen Risk and Cardiovascular Health Study involving patients with and without coronary artery disease, the *APOE4* genotype (*E3/E4*, *E4/E4*) was associated with 19 % lower and the *APOE2* genotype (*E2/E3*, *E2/E2*) was associated with 21 % higher circulating CRP levels compared to *APOE3* genotype (*E3/E3*) [März et al., 2004]. Besides, Tziakas *et al*. reported in patients with acute coronary syndrome and chronic stable angina lower circulating IL-10 levels in *APOE4* carriers compared to non-carriers [Tziakas et al., 2006], which is in line with *in vitro* data indicating a reduced production of IL-10 upon LPS-stimulation in *APOE4* macrophages compared to *APOE3* macrophages [Jofre-Monseny et al., 2007B]. Finally, plasma VCAM-1 levels have been reported to be significantly higher in *APOE2* and *APOE4* carriers compared to the *APOE3* genotype, with the opposite effect for plasma P-selectin levels, in 312 healthy subjects enrolled in a fish oil intervention trial [Kofler et al., 2012]. In this study, they also reported an interaction between *APOE Epsilon* genotype and BMI to determine plasma inflammatory marker concentrations. *APOE Epsilon* genotype had the strongest effect in the normal weight subjects (BMI ≤ 24.9 kg/m^2) and *APOE2* carriers were most sensitive to the detrimental effects of an increased BMI [Kofler et al., 2012]. These findings suggest that *APOE Epsilon* genotype could interact with adiposity to determine inflammatory status in humans, something that warrants further investigation in particular in relation to AT inflammation in obesity.

Table 1.3. Summary of key human studies investigating the association between *APOE Epsilon* genotype, adiposity and metabolic consequences of obesity.

Study design and study population	Main findings	Reference
Prospective birth cohort study investigating the associations between *APOE Epsilon* genotype, plasma lipids, PA, CRF, and adiposity (n = 292 children, age 8 yr)	*APOE4* genotype was associated with lower BMI (WMD 0.87 kg/m^2, 95%CI 0.26 – 1.47), truncal fat mass and waist circumference compared to non-*APOE4* carriers. *APOE Epsilon* genotype interacted with CRF for child BMI: Children with low CRF: *E4* had lower BMI vs. *E3* (WMD -1.78 kg/m^2, 95%CI -2.74 – -0.83) Children with high CRF: *E4* had similar BMI to *E3* (WMD 0.07 kg/m^2, 95%CI -0.59 – 0.73)	[Ellis et al., 2011]
Cross sectional study investigating the associations between *APOE Epsilon* genotype, adiposity and fasting plasma insulin and glucose levels (n = 2,929)	Fasting glucose and insulin in obese men: *E4 > E3* Fasting glucose and insulin in normal weight men: *E4 = E3*	[Elosua et al., 2003]
Study investigating association between *APOE Epsilon* genotype and MetS (n = 147 subjects with severe obesity (BMI 48.7 kg/m^2), of which 116 MetS patients).	*APOE Epsilon* genotype frequencies in MetS patients: *E2 > E3 = E4* *E4* was higher in obese without MetS vs. MetS patients (23 % vs. 9 %) Prevalence of MetS: Overall, 79 % In *E2*, 84 % In *E4*, 56 %	[Ferreira et al., 2011]
Study investigating the association between *APOE Epsilon* genotypes and obesity in CHD patients (n = 359 CHD patients and 248 controls).	*APOE Epsilon* genotype frequencies: *E2* was higher in normal weight CHD patients vs. controls (3 % vs. 23 %) *E3* was higher in normal weight CHD patients vs. controls (83 % vs. 57 %) *E4* was similar in normal weight CHD patients and controls (11 % vs. 20 %) *E3* was lower in obese vs. normal weight CHD patients (69 % vs. 83 %) *E4* was higher in obese vs. normal weight CHD patients (13 % vs. 6 %)	[Kolovou et al., 2009]
Study investigating the associations between *APOE Epsilon* genotype, alcohol intake, BMI, smoking and plasma lipid levels (n = 266 healthy men)	Plasma TC and LDL-C, *E2 < E3 < E4*, enlarged with increasing BMI Plasma apoE and apoB:apoE, *E2 > E3 > E4* Plasma bioactive insulin, *E2 = E3 < E4*, enlarged with increasing BMI	[Marques-Vidal et al., 2003]
Cross sectional study investigating the association between *APOE Epsilon* genotype and plasma lipids (n = 191 subjects with and 1,062 without family history of diabetes)	Non-diabetic men with/without family history: BMI, waist circumference, waist-to-hip ratio *E2 = E3 = E4* Non-diabetic women with family history: BMI, waist-to-hip ratio *E2 = E3 = E4* Waist circumference, *E2 = E3 < E4* Non-diabetic women without family history: BMI, waist circumference, waist-to-hip ratio, *E2 = E3 = E4*	[Oh and Barrett-Connor, 2001]

Table 1.3. continued.

Study design and study population	Main findings	Reference
Cross-sectional study investigating the association between *APOE Epsilon* genotypes, dietary intake, and BMI with serum lipid levels (n = 996)	BMI: *E2 = E3 = E4*	[Petkeviciene et al., 2012]
Cross sectional study investigating the associations between *APOE Epsilon* genotype, body fatness and plasma lipoproteins (n = 63 healthy sedentary premenopausal women)	*APOE3* genotype: body fatness ↑ → plasma VLDL-C, LDL-C, TG, apoB ↑, HDL-C ↓ *APOE2* genotype: body fatness ↑ → plasma VLDL-C ↑, apoB, LDL-C, HDL-C ↓ *APOE4* genotype: no association	[Pouliot et al., 1990]
Population-based cohort study investigating the association between SNPs in transcriptional pathways of glucose and lipid metabolism and features of MetS (n = 3,575)	*APOE4* was associated with abdominal obesity (PR = 1.12, 95% CI 1.03 – 1.23) *APOE4* was associated with low plasma HDL-C (PR = 1.21, 95% CI 1.07 – 1.37). *APOE2* was not associated with MetS features.	[Povel et al., 2011]
Study investigating the association between *APOE Epsilon* genotype and the risk of MetS or/and CHD (n = 91 control subjects, 117 MetS patients, 71 obese CHD patients).	*APOE Epsilon* allele frequencies: *E4* was higher in very obese vs. normal weight subjects (35 % vs. 15 %) *E4* was higher in MetS patients vs. controls (13.5 % vs. 8.2 %) *E2* was higher in controls vs. MetS patients (14.5 % vs. 7.3 %) *E2* was higher in controls vs. CHD patients (14.5 % vs. 6.4 %)	[Sima et al., 2007]
Prospective study investigating the impact of *APOE Epsilon* genotype on LDL-C, IMT and CHD risk (n = 3,187 African Americans, 9,304 Whites)	African Americans: BMI, *E2 > E3 > E4* Whites: BMI, *E2 = E3 = E4*	[Volcik et al., 2006]
Cross-sectional study investigating the association between abdominal obesity and *APOE Epsilon* genotype (n = 853)	*APOE Epsilon* allele frequencies were similar in subjects with and without abdominal obesity. Subjects with abdominal obesity: *E3* was associated with higher plasma TG (*E3 > E2 > E4*) Subjects without abdominal obesity: *E4* was associated with lower plasma HDL-C (*E2 > E3 > E4*)	[Zarkesh et al., 2012]

This table is a summary of the main findings from studies investigating the association between *APOE Epsilon* genotype, obesity and its complications. *APOE Epsilon* genotypes were defined as *APOE3* genotype (*E3/E3*); *APOE2* carriers (*E2/E2*, *E2/E3*); and *APOE4* carriers (*E3/E4*, *E4/E4*), unless stated otherwise. 95% CI, 95% confidence interval; APOE, apolipoprotein E; BMI, body mass index; CHD, coronary heart disease; CRF, cardiorespiratory fitness; HDL-C, high-density lipoprotein cholesterol; IMT, carotid artery intima-media thickness; LDL-C, low- density lipoprotein cholesterol; MetS, metabolic syndrome; PA, physical activity; PR, prevalence ratio; SNP, single nucleotide polymorphism; VLDL-C, very-low density lipoprotein cholesterol; WMD, weighted mean difference.

Table 1.4. Human studies investigating the association between *APOE Epsilon* genotype and circulating CRP levels.

Study design and study population	Results	Reference
Cross-sectional study (n = 562 Japanese Americans from 68 extended kindreds)	CRP, E2 > E3 = E4 (1.20 mg/L vs. 0.72 mg/L vs. 0.74 mg/L)	[Austin et al., 2004]
Study involving six-month aerobic exercise (n = 117 healthy sedentary subjects)	CRP, *E2 = E3 > E4 (*2.84 mg/L (*E2/E3)* vs. 2.59 mg/L (*E3/E3)* vs. 1.90 ± 2.13 (*E3/E4*)) Exercise did not change CRP levels.	[Angelopoulos et al., 2008]
Three prospective cohort studies (n = 2,010 subjects without CVD history)	PRINCE study: CRP, *E2 = E3 > E4* (1.6 mg/L (E2/E3) vs. 1.6 mg/L (E3/E3) vs. 1.2 mg/L (E3/E4)) Physicians' Health Study: CRP, *E2 = E3 > E4* (1.1 mg/L (E2/E3) vs. 1.6 mg/L (E3/E3) vs. 0.9 mg/L (E3/E4))	[Chasman et al., 2006]
Cross-sectional study (n = 211 subjects with low HDL-C† and 157 normolipidemic subjects)	Subjects with low HDL-C: CRP, E3 > E4 (1.72 mg/L (*E2/E3*, *E3/E3*) vs. 1.14 mg/L *(E2/E4*, *E3/E4*, *E4/E4*)) VCAM-1, ICAM-1 and E-sel, *E2 = E3 = E4* Normolipidemic subjects: CRP, E3 > E4 (1.29 mg/L (*E2/E3*, *E3/E3*) vs. 0.63 mg/L *(E2/E4*, *E3/E4*, *E4/E4*)) VCAM-1, ICAM-1 and E-sel, *E2 = E3 = E4*	[Kahri et al., 2006]
Placebo-controlled crossover fish oil intervention trail (n = 312 healthy subjects)	CRP, *E2 > E3 > E4* (82.8. ng/mL vs. 59.5 ng/mL vs. 56.1 ng/mL) TNF-α, IL-6, IL-10, E-sel, P-sel, ICAM-1 and VCAM-1 (E2 = E3 = E4) *APOE Epsilon* genotype interacted with BMI for CRP. Normal weight subjects (25 – 30 kg/m^2): CRP, E2 > E3 = E4	[Kofler et al., 2012]
Cross sectional study (n = 739 CAD patients and 570 controls)	CRP, *E2 > E3 > E4* (*E2* had 21% higher and *E4* had 19% lower levels than *E3*)	[März et al., 2004]
Cross sectional study (n = 166 ACS and 70 CSA patients)	ACS patients: CRP, *E2 = E3 > E4* (8.7 mg/L vs. 9.1 mg/L vs. 4.5 mg/L) IL-10, *E2 = E3 = E4* (6.9 pg/mL vs. 6.7 pg/mL vs. 5.6 pg/mL) CSA patients: *CRP, E3 > E2 > E4* (7.1 mg/L vs. 6.3 mg/L vs. 2.0 mg/L) IL-10, *E2 = E3 = E4* (28.6 pg/mL vs. 17.8 pg/mL vs. 14.3 pg/mL)	[Tziakas et al., 2006]

This table is not an exhaustive list of studies investigating the association between *APOE Epsilon* genotype and circulating CRP levels. Unless stated otherwise, results are summarised for *APOE2* carriers (*E2/E3*, *E2/E2*), *APOE3* genotype (*E3/E3*) and *APOE4* carriers (*E3/E4, E4/E4*). †Low HDL was defined as HDL-C levels below the 10th percentile cut-off level. 95% CI, 95% confidence interval; ACS, acute coronary syndrome; APOE, apolipoprotein E; BMI, body mass index; CAD, coronary artery disease; CSA, chronic stable angina; CVD, cardiovascular disease; E-sel, E-selectin; HDL-C, high-density lipoprotein-cholesterol; CRP, C-reactive protein; ICAM-1, intercellular cell adhesion molecule-1; IL, interleukin; LDL-C, low-density lipoprotein-cholesterol; P-sel, P-selectin; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

Apolipoprotein E and SNPs in *APOE* promoter region

Like the majority of other human genes *APOE* is highly polymorphic with 333 SNPs to date described (www.ncbi.nlm.nih.gov/snp/?term=apoE). In addition to the *APOE Epsilon* genotypes, SNPs in the *APOE* promoter region can alter the transcriptional activity of *APOE* thereby potentially impacting on disease risk. In fact, SNPs in the *APOE* promoter region have been shown to influence AD risk [Bizzarro et al., 2009, Lambert et al., 2002, Lescai et al., 2011, Wang et al., 2000]. Almost a decade ago five common genetic variations in the *APOE* promoter region were identified; -491A/T (rs449647), -427T/C (rs769446), -219G/T (rs405509), –186G/T and +113G/C (rs440446) [Artiga et al., 1998, Lambert et al., 1998]. The -491A/T (rs449647) and -219C/T (rs405509) SNPs, but not -427T/C (rs769446), have been shown to modulate the *APOE* transcriptional activity *in vitro*, where the -491T and -219G allelic variants were associated with respectively decreased and increased transcriptional activity of *APOE* compared to -491A and -219T variants [Artiga et al., 1998, Geng et al., 2011]. For example, Artiga *et al*. showed that the 491T variant was associated with 37 % decreased and the -219G variant with 69 % increased transcriptional activity of *APOE* compared to wild type haplotypes (-491A and -219T) in a human hepatoma cell line (HepG2) [Artiga et al., 1998]. In line with this, human studies demonstrated that the -219G/T polymorphism and *APOE Epsilon* genotype determine plasma apoE concentrations [Lambert et al., 2000, Moreno et al., 2003, Moreno et al., 2009]. The plasma apoE concentrations exist in a *APOE* genotype-dependent manner (E2 > E3 > E4) [Moreno et al., 2009]. The -219T allele was associated with decreased plasma apoE concentrations in dose-dependent manner ($p < 0.01$) in the ECTIM study adjusting for *APOE Epsilon* genotype (plasma apoE concentrations, 4.9 ± 1.8 (SD) for -219GG; 4.7 ± 1.7 for -219GT; and 4.1 ± 1.5 mmol/L for -219TT) [Lambert et al., 2000]. The -219T allele was also associated with an increased AD risk (OR = 1.30, 95% CI 1.06 – 1.60, for -219TT) in 2390 AD patients and controls, but this effect disappeared after correcting for presence of the *APOE4* allele with further investigation in *APOE4* carriers revealing no effect of the -219T allele on AD risk (OR = 4.77, 95% CI 3.18 – 7.17, for -219T * *APOE4* vs. OR = 5.31, 95% CI 2.93 – 9.63, for -219G * *APOE4*) [Lescai et al., 2011]. However, a potential involvement of SNPs in *APOE* promoter region in other disease pathologies associated with *APOE Epsilon* genotype, such as CHD, still needs to be investigated though there have been some reports concerning atherosclerosis, IR and T2DM [Banares et al., 2012, Geng et al., 2011, Komurcu-Bayrak et al., 2011]. In summary, the -219G/T (rs405509) and -491A/T (rs449647) polymorphism in the *APOE* promoter region have been shown to affect *APOE* gene expression levels and impact on AD risk, however the impact on other risk factors for disease including IR and inflammation remain to be elucidated.

APOE Epsilon genotype – adiposity, inflammation and disease risk

In summary, the pro-inflammatory effects of the *APOE4* allele compared to *APOE3* allele that have been well-established *in vitro* and *in vivo* in animal models, suggest that a

differential inflammatory status associated with *APOE Epsilon* genotype may be involved in the *APOE Epsilon* genotype – disease association, especially given the fact that both CHD and AD (on which *APOE Epsilon* genotype impacts) have a well-established inflammatory component. However, the impact of the *APOE4* genotype on inflammatory status in humans is still unknown and remains to be established. Furthermore, recent reports in humans and human *APOE* TR mice suggest that AT may be involved in mediating the effect of *APOE Epsilon* genotype on metabolic diseases associated with obesity [Kofler et al., 2012, Arbones-Mainar et al., 2008 and 2010]. For example, Arbones-Mainar *et al*. showed that *APOE Epsilon* genotype impacted on diet-induced obesity, VAT expansion and AT dysfunction in human *APOE* TR mice. In humans, obesity is associated with local and systemic low-grade inflammation and AT dysfunction [Hotamisligil, 2006, Calder et al., 2011A] that in turn leads to conditions such as MetS, IR and CVD [Maury and Brichard, 2010, Van Gaal et al., 2006]. Therefore, we hypothesize that *APOE Epsilon* genotype and SNPs in the *APOE* promoter region may impact on disease risk through modulating AT inflammation and function in particular in obesity, which in turn determines systemic inflammatory and metabolic parameters and subsequent disease risk. Finally, *APOE Epsilon* genotype has been shown to impact on long chain (LC) *n-3* polyunsaturated fatty acid (PUFA) status [Chouinard-Watkins et al., 2013, Conway et al., 2014, Plourde et al., 2009, Vandal et al., 2014], with LC *n-3* PUFA having important beneficial effects on inflammatory and metabolic processes which will be discussed in more detail in the next paragraphs. These findings suggest a potential involvement of LC *n-3* PUFA status in the relationship between *APOE Epsilon* genotype, adiposity and inflammation.

Omega 3 (*n-3*) polyunsaturated fatty acids

Long chain *n-3* PUFA – structure

Fatty acids are hydrocarbon chains consisting of 4 to 28 carbons (usually unbranched and even-numbered) that contain a methyl group (-CH_3) at one end at a carboxylic acid (-COOH) at the other end. Fatty acids can be saturated, i.e. without a double bond (C-C), or contain one (monounsaturated) or more (polyunsaturated) double bonds (C=C). Most unsaturated fatty acids have a *cis* configuration, i.e. the hydrogen atoms on either side of the double bond are oriented in the same direction, resulting in a bend in the molecular structure of the molecule. The omega (n-*x*) nomenclature classifies fatty acids based on the number of carbon atoms, the number of double bonds and the location of their first double bond from the methyl end. Fatty acids are not only an important source of energy providing 37 kJ per gram, but they are also important constituents of cell membrane and serve as signalling molecules regulating gene expression [Calder 2011B, Ruston and Drevon, 2005]. Fatty acids can be present as free NEFA or as part of more complex lipids TG and phospholipids (PL).

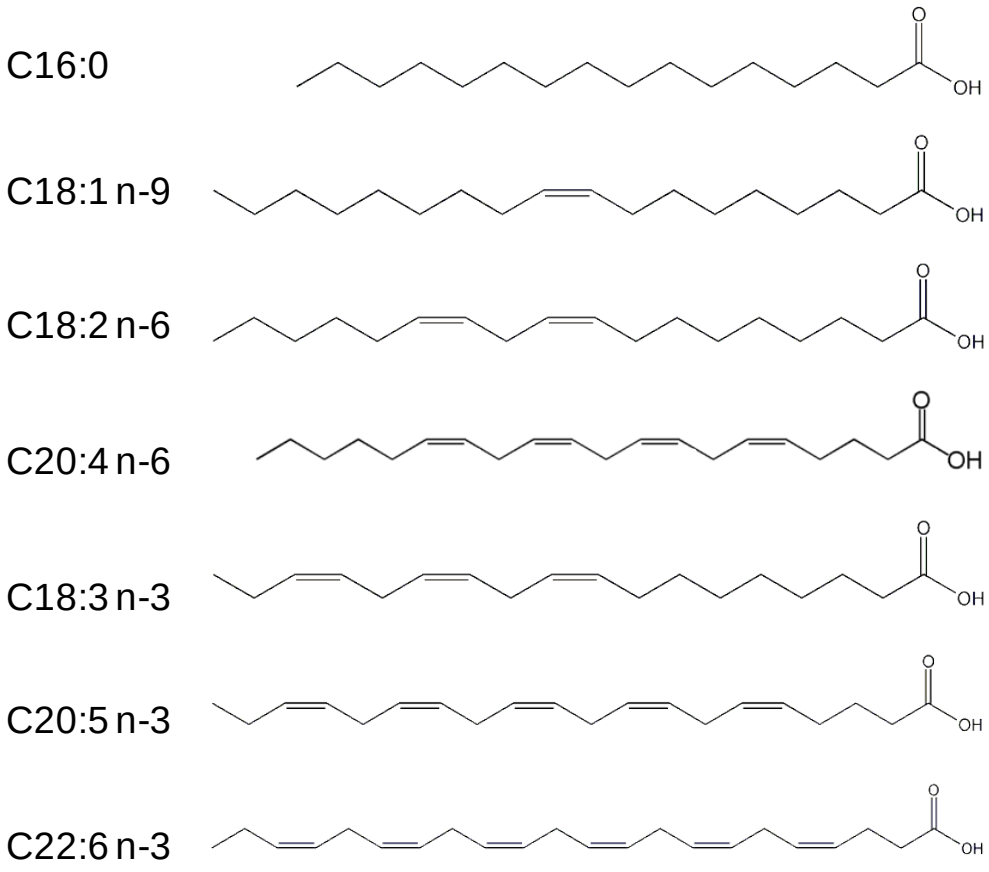

Figure 1.1. Structures of various common dietary fatty acids, illustrating palmitic acid (C16:0), oleic acid (C18:1 *n-9*), linoleic acid (C18:2 *n-6*), arachidonic acid (C20:4 *n-6*), α-linolenic acid (C18:3 *n-3*), eicosapentaenoic acid (C20:5 *n-3*) and docosahexaenoic acid (C22:6 *n-3*).

Long chain *n-3* PUFA – recommended intakes and dietary resources

PUFA can only be produced by plants and phytoplankton, and since mammals cannot interconvert *n-6* and *n-3* PUFA they have to acquire through their diet both linoleic acid (LA, C18:2 n-6) and α-linolenic acid (ALA, C18:3 *n-3*), which they can further metabolise through series of desaturation and elongation steps. The most important dietary *n-3* PUFAs are ALA and LC *n-3* PUFA eicosapentaenoic acid (EPA, C20:5 *n-3*) and docosahexaenoic acid (DHA, C22:6 *n-3*) (Figure 1.1). Only ALA is considered to be an essential fatty acid, since EPA and DHA can be synthesised from ALA, although the bioconversion is very low in humans (0.2 – 6% for EPA, 0 – 0.5% for DHA) [Arterburn et al., 2006, Minihane et al., 2000]. EPA and DHA are also referred to as marine *n-3* PUFA, since they are present in considerable amounts in oily fish (± 2 g EPA+DHA / 100g [SACN, 2004]) such as mackerel and salmon. Many organizations and expert committees have acknowledged the important role of LC *n-3* PUFA in human health, but so far no dietary reference intakes (DRI) have been set for EPA and DHA [Aranceta and Perez-Rodrigo, 2012, Flock et al., 2013, Harris et al., 2009, Kris-Etherton et al., 2009]. Various national and international organisations provide guidance on desirable intakes of EPA and DHA from fish. The recommendations in adults

are based mainly on the primary and secondary prevention of CVD and range from one to two portions of oily fish per week or 250 – 500 mg EPA and DHA per day [Aranceta and Perez-Rodrigo, 2012, Mozaffarian and Rimm, 2006]. For the maintenance of normal plasma TG and blood pressure in adult men and women intakes of 2 – 4 g/d and 3 g/d have been recommended [Aranceta and Perez-Rodrigo, 2012, Flock et al., 2013, Lorente-Cebrian et al., 2013]. Flock *et al*. reviewed the extensive data on the effect of LC *n-3* PUFA on CVD outcomes and recommended an intake of 566 mg/d for EPA and DHA, which is associated with the greatest risk reduction (37 %) in CHD mortality [Flock et al., 2013, Harris et al., 2008]. In the UK the current recommendation for optimal health is 450 mg/d, which would be available if people would consume at least two portions of fish a week of which one is oily [SACN, 2004]. This consumption would confer into significant public health benefits to the UK population in terms of reducing the risk of CVD, as the UK population has low fish intake and is considerd a high risk population for CVD. The tolerable upper intake level for EPA and DHA is still unknown, but long term intakes up to 5 g/d are considered safe for adults [EFSA, 2012]. Intakes of 2 – 6 g/d induce an increase in LDL-C concentration of about 3 %, however since it is associated with a decrease in TG and no change in total cholesterol it may not have an adverse effect on the risk of CVD [EFSA, 2012]. In summary, more studies are needed to determine the optimal DRI for different populations based on age, gender, body weight, race, health status and genotype [Flock et al., 2013].

Long chain *n-3* PUFA – function

LC *n-3* PUFA have various physiological roles in many different processes including the regulation of platelet function, vascular function, cognitive and visual development, bone turnover, immune function, insulin sensitivity and plasma TG concentrations [For full list see Calder, 2012], of which many processes are linked to cardiovascular and inflammatory diseases [Calder, 2012]. In fact, LC *n-3* PUFA intake has been associated with a reduced risk of CVD events that is primarily attributed to the prevention of cardiovascular mortality, though findings are somewhat inconsistent [Calder, 2004, Flock et al., 2013, Harris et al., 2008, Mozaffarian and Wu, 2011, Von Schacky, 2015]. For example, a meta-analysis of six epidemiologic studies conducted in the United States showed that an intake of about 500 mg/d of EPA and DHA achievable by the consumption of two portions of oily fish per week was associated with a 37 % reduced risk of CHD mortality [Harris et al., 2008]. Meta-analyses of randomized, double-blind, placebo controlled trials (RCTs) demonstrated that LC *n-3* PUFA reduce sudden death and fatal myocardial infarction, with no effect on all-cause mortality, in patients with existing or previous CVD (Table 1.5) [Bucher et al., 2002, Casula et al., 2013, Kwak et al., 2012]. A meta-analysis of 16 prospective cohort studies indicated that a moderate dietary EPA and DHA intake of 250 mg/d was sufficient to reduce the risk for cardiac death by about 30% in generally healthy individuals without known heart disease [Harris et al., 2009]. The cardioprotective effects of LC *n-3* PUFA have been ascribed to their anti-arrhythmic actions [Von Schacky, 2008, Harris et al., 2009] and

beneficial effect on various cardiovascular risk factors, including blood pressure [Gelijnse et al., 2002], platelet reactivity and thrombosis [Hornstra, 2001], plasma TG concentrations [Harris, 1996], vascular function [Nestel et al., 2002] and inflammation [Adkins and Kelly 2010, Calder, 2006, Calder, 2012]. Low doses of EPA and DHA (0.5 – 1 g EPA+DHA / d) are unlikely to reduce CVD risk and incidence, but they may reduce mortality by their anti-arrhythmic effects improving post-myocardial infarct survival [Von Schacky, 2008]. Besides, there is evidence indicating LC *n-3* PUFA could elevate the disease burden of conditions with an inflammatory component, which have been best-established for rheumatoid arthritis and inflammatory bowel disease [Calder, 2006, Calder et al., 2011A, Rangel-Huerta et al., 2012]. For example, recent meta-analyses showed that LC *n-3* PUFA reduce nonsteroidal anti-inflammatory drug use and tend to improve tender and swollen joint count, morning stiffness and physical function in patients with rheumatoid arthritis [Lee et al., 2012] and reduce the risk of relapse in patients with Crohn's Disease [Lev-Tzion et al., 2014]. These findings indicate that LC *n-3* PUFA can reduce the burden of inflammatory diseases. Finally, a comprehensive review on diet and low-grade inflammation with special focus on inflammation in overweight and obesity showed there is overwhelming evidence indicating LC *n-3* PUFA have anti-inflammatory effects in humans when consumed at sufficient doses (≥ 2 g/d) [Calder et al., 2011A]. Whether there may be effects at lower doses remains to be elucidated. Most studies have investigated the effects of LC *n-3* PUFA on plasma inflammatory marker concentrations which may not fully reflect tissue inflammatory status and may be rather an insensitive biomarker of inflammatory status in humans. Gene expression profiling of peripheral blood mononuclear cells, which can be obtained from whole blood, may provide a more sensititve biomarker of the effects of LC *n-3* PUFA on inflammation. Finally, LC *n-3* PUFA appear to have anti-obesogenic effects that could be important in the prevention of obesity [Buckley and Howe, 2010, Flachs et al., 2014, Mori et al., 1999]. The anti-obesogenic and anti-inflammatory effects of LC *n-3* PUFA will be discussed in more detail in the next paragraphs.

Long chain *n-3* PUFA in obesity and metabolic disease

LC *n-3* PUFA have been shown to exert potent anti-obesogenic effects in a considerable number of animal studies and disease models, but their effects in humans are inconsistent and human studies with appropriate design, size and duration are lacking [Buckley and Howe, 2010, Flachs et al., 2006, Lorente-Cebrián et al., 2013, Puglisi et al., 2011]. Prospective cohort studies found conflicting associations between fish intake and body weight in humans. For example, in the prospective Nurse's Health Study a high fish intake (≥ 5 portions / wk) was associated with a higher prevalence of obesity compared to low intake (< 1 portion / month) in women [Iso et al., 2001], whereas in the Health Professional Follow-Up Study a high fish intake (≥ 5 portions / wk) was associated with lower prevalence of overweight compared to low intake (< 1 portion / month) in men [He et al., 2002]. Most RCTs have investigated the effect of LC *n-3* PUFA either from fish or fish oil supplements on body

weight and body composition in combination with caloric restriction or exercise in overweight and obese individuals and reported either a beneficial or neutral effect of LC *n-3* PUFA (0.3 to 3.0 g/day) on body weight and/or body fat in combination with caloric restriction or exercise [Buckley and Howe, 2010, Lorente-Cebrián et al., 2013]. The first meta-analysis investigating the association between LC *n-3* PUFA intake either from fish or fish oil supplements and body composition indicated that LC *n-3* PUFA might influence body composition (Table 1.6) [Bender et al., 2014]. In more detail, individuals consuming fish or fish oil supplements showed a greater reduction in body weight (weighted mean difference (WMD) -0.59 kg, 95% CI -0.96 – -0.21), BMI (WMD 0.24 kg/m^2, 95% CI -0.40 – -0.08), body fat (WMD -0.49%, 95% CI -0.97 – -0.01) and waist circumference (WMD -0.81 cm, 95% CI -1.34 – -0.28) compared to control [Bender et al., 2014]. Only few studies (n = 3 RCT) investigated the association between LC *n-3* PUFA intake either from fish or fish oil supplements and lean body mass, which likely explains why Bender *et al.* observed no difference in fat mass (WMD -0.36 kg, 95% CI -0.96 – 0.24) or lean body mass (WMD -0.19 kg, 95% CI -0.72 – 0.33) [Bender et al., 2014]. In line with the above mentioned gender-difference in the association between fish intake and body weight in the prospective cohort studies they also observed a trend for a stronger effect on waist circumference in men. The effect of LC *n-3* PUFA either from fish consumption or fish oil supplements have shown inconsistent effects on body composition in RCTs and prospective cohort studies. One potential explanantion is that LC *n-3* PUFA intake achieved by fish consumption may have a differential effect on body composition than LC *n-3* PUFA intake achieved by fish oil supplements even if they provide a similar dose of LC *n-3* PUFA. Fish namely contains several important nutrients, such as vitamin D, iodine, selenium, and antioxidants, which are missing from supplements. Moreover, a portion of fish often replaces meat whereas supplements are generally taken on top of the normal diet not accounting for the additional calories available in these supplements. Thus, LC *n-3* PUFA intake achieved by fish consumption likely has addititional benificial effects on the overall dietary pattern and the dietary intake of other nutrients such as vitamin D. In summary, the effect of LC *n-3* PUFA on body weight and body composition in healthy individuals remains nevertheless controversial and more RCTs are needed [Lorente-Cebrián et al., 2013].

The potential mechanisms through which LC *n-3* PUFA may reduce body weight and improve body composition in overweight and obese individuals include an enhanced post-prandial satiety and regulation of genes involved in β-oxidation and adipogenesis [Buckley and Howe, 2010]. For example, a small cross-over study by Couet *et al*. showed that dietary fat replacement with fish oil (6 g/day containing 1.1 g EPA and 0.7 g DHA) resulted in a reduction in fat mass (-0.88 ± 0.16 (SD) vs. -0.30 ± 0.34 kg, $p < 0.05$) but no change in body weight (-0.70 ± 0.62 vs. -0.54 ± 0.52 kg) that was associated with an increased β-oxidation (1.06 ± 0.17 vs 0.87 ± 0.13 mg kg^{-1} min^{-1}, $p<0.05$) in six healthy young adults (n = 5 men; mean age 23 ± 2 (SD) year; BMI 21.9 ± 1.6 kg/m^2) [Couet et al., 1998]. More studies are needed to confirm the effects of LC *n-3* PUFA on body weight, fat mass and β-oxidation in

healthy individuals. The effect of LC *n-3* PUFA in animal studies are more convincing and include reduced AT expansion, reduced adipocyte proliferation and improved fatty acid oxidation that contribute to the prevention of diet-induced obesity in mice [Flachs et al., 2009, Puglisi et al., 2011].

Regarding metabolic parameters, LC *n-3* PUFA have been shown to improve insulin sensitivity and glucose homeostasis in various animal models of obesity and metabolic disease, but their effects in humans remain controversial [Akinkuolie et al., 2011, Flachs et al., 2014, Jafari et al., 2013]. In mice, LC *n-3* PUFA decrease AT mass and improve various associated metabolic parameters including systemic IR, dyslipidemia, hepatic steatosis and AT inflammation, with the changes in metabolic parameters being independent of changes in AT mass [Puglisi et al., 2011]. Epidemiological studies in humans revealed a lower prevalence of impaired glucose tolerance (IGT) and T2DM in populations with a high fish intake, but prospective cohort studies have found contradictive effects of fish intake on risk of T2DM [Jafari et al., 2013]. Various meta-analyses reported no effect of LC *n-3* PUFA intake on risk of T2DM in humans, but there is accumulating evidence that the effects are dependent on ethnicity or geographical location (Table 1.7). For example, a recent meta-analysis of 24 prospective cohort studies showed that LC *n-3* PUFA only exert beneficial on T2DM risk in Asian populations but not in Western populations, with a relative risk ratio for T2DM of 0.87 (95% CI 0.79 – 0.96) in Asian countries and 1.16 (95% CI 1.04 – 1.28) in Western countries comparing the highest with lowest LC *n-3* PUFA intake [Zheng et al., 2012]. These findings implicate the potential involvement of gene-diet interactions, although other factors such as type of fish (oily, lean, shellfish), other nutrients in fish (e.g. selenium, vitamin D) and preparation method (e.g. deep frying, steamed, raw), or other non-fish related differences in habitual diet or overall lifestyle may also be involved in the ethnic and geographical differences in the association between fish intake and risk of T2DM [Wallin et al., 2012, Zheng et al., 2012]. There is no effect of LC *n-3* PUFA on glycemic control and insulin sensitivity - a measure of both insulin responsiveness and insulin resistance - in T2DM patients and people without T2DM (Table 1.8). These finding are in line with a recent review on the effects of LC *n-3* PUFA on glucose homeostasis and insulin sensitivity in various categories of human subjects (healthy individuals, subject with MetS, T2DM patients, and CVD patients) reporting no change or a marginal improvement in IGT and IR in T2DM patients, a potential marginal improvement in IGT and IR in MetS patients, and no change or an impairment in glycemic control in healthy individuals [Flachs et al., 2014].

The fatty acid composition of plasma or serum lipids (cholesterol esters (CE) and phospholipids (PL)) is known to mirror dietary intake over the last 6 – 8 weeks and also reflects endogenous fatty acid metabolism [Warensjo et al. 2006], whereas the fatty acid composition of AT reflects long-term intake [Hodson et al. 2008]. Thus serum and AT fatty acid composition can be used as a biomarker of short and long term fat quality [Warensjo et al., 2006]. An altered fatty acid profile has been shown to predict the development of IR [Iggman et al., 2010, Warensjo et al, 2009], T2DM [Vessby et al., 1994], MetS [Warensjo et

al, 2005, Warensjo et al, 2006], CVD [Ohrvall et al., 1996, Sundstrom et al., 2001] and mortality [Warensjo et al, 2008] in healthy 50-year-old men over period of 10 – 20 years of follow-up. The unfavourable fatty acid profile is characterised by high levels of palmitic acid (16:0) and low levels of linoleic acid (18:2 n-6) in plasma lipid esters and a proportionally higher level of palmitoleic acid (16:1 n-7) and and dihomo-gamma-linolenic (20:3 n-6), and is induced by diets rich in saturated fat and probably lower in polyunsaturated fatty acids [Vessby 2003, Warensjo et al, 2006]. The concentrations of EPA and DHA in serum lipids and AT did not relate to IR [Iggman et al., 2010] or predict the development of MetS Warensjo et al., 2006]. Moreover, a moderate LC *n-3* PUFA supplementation (3.6 g/d for 12 weeks) failed to modulate insulin sensititvity and secretion in healthy individuals on either a high saturated or high monounsaturated fat diet [Giacco et al., 2007]. These findings suggest dietary LC *n-3* PUFA have no direct effect on insulin sensitivity and do not predict development of IR and MetS in healthy individuals. However, the fatty acid profile of saturated fatty acids and its desaturation products, i.e. palmitic acid (16:0) and palmitoleic acid (16:1 n-7), in serum lipids and AT predict the development of IR and MetS.

The beneficial effects of LC *n-3* PUFA on features of MetS observed in mice are likely mediated through AT, as LC *n-3* PUFA not only reduce AT inflammation and IR but also improve the storage and secretory functions of AT [Puglisi et al., 2011, Slim and Minihane, 2014]. For example, LC *n-3* PUFA increase mitochondrial biosynthesis and β-oxidation in AT that at least partially account for their anti-obesogenic effects [Flachs et al., 2009]. In summary, there is a neutral or modest beneficial effect of LC *n-3* PUFA on body weight and adiposity in humans and rodents. The effect of LC *n-3* PUFA on features of MetS in mice is established and include improvement of dyslipidemia, glucose tolerance and insulin sensitivity, but their effects in humans remain controversial. There appears to be no beneficial effect of LC *n-3* PUFA on plasma insulin or glucose homeostasis in healthy individuals, however its 20 – 30 % plasma TG lowering effect is well-established [Flachs et al., 2014].

Table 1.5. Overview of meta-analyses investigating the relationship between fish and long chain *n-3* PUFA intake and risk of cardiovascular disease events.

Study details	Outcome	Effect of *n-3* PUFA on CVD risk compared to control (unless specified otherwise)		Reference
Meta-analysis including 11 RCT (n = 15,806)	All-cause mortality and MI in MI and CHD patients	LC *n-3* PUFA supplementation (0.5 to 9.7 g/day, ≥ 6 months):		[Bucher et al., 2002]
		All-cause mortality	RR = 0.8, 95% CI 0.7 – 0.9 (9 studies, n = 15,495)	
		Sudden cardiac death	RR = 0.7, 95% CI 0.6 – 0.9 (5 studies, n = 13,060)	
		Fatal MI	RR = 0.7, 95% CI 0.6 – 0.8 (8 studies, n = 15,375)	
		Non-fatal MI	RR = 0.8, 95% CI 0.5 – 1.2 (9 studies, n = 15,015)	
Meta-analysis including 11 RCT (n = 15,348)	Secondary prevention in CVD patients	LC *n-3* PUFA supplementation (≥ 1 g/day, ≥ 1 yr):		[Casula et al., 2013]
		All-cause mortality	RR = 0.89; 95% CI 0.78 – 1.02	
		Sudden death	RR = 0.67; 95% CI 0.52 – 0.87	
		Cardiac death	RR = 0.68; 95% CI 0.56 – 0.83	
		MI	RR = 0.75; 95% CI 0.63 – 0.88	
		Stroke	RR = 1.31; 95% CI 0.90 – 1.90	
Meta-analysis including 29 RCT (n = 35,144)	Mortality and restenosis in high risk CV patients	*n-3* PUFA supplementation (0.9 to 6.9 g/d):		[Fillion et al., 2010]
		All-cause mortality	RR = 0.88, 95% CI 0.64 – 1.03 (25 studies, n = 34,501)	
		Restenosis	RR = 0.89, 95% CI 0.72 – 1.06 (14 studies, n = 3,553)	
Meta-analysis including 48 RCT (n = 36,913) and 41 prospective cohort studies	All-cause mortality, cardiovascular events, cancer	High vs. low LC *n-3* PUFA intake, prospective cohort studies (≥ 6 months):		[Hooper et al., 2004]
		All-cause mortality	RR = 0.65, 95% CI 0.48 – 0.88 (3 studies, n = 3,801)	
		Combined CV events	RR = 0.91, 95% CI 0.73 – 1.13 (7 studies, n = 69,702)	
		Cancer	RR = 1.02, 95% CI 0.87 – 1.19 (10 studies, n = 112,460)	
		Sudden death	RR = 0.44, 95% CI 0.21 – 0.91 (1 study, n = 5,734)	
		Cardiovascular death	RR = 0.79, 95% CI 0.63 – 0.99 (11 studies, n = 107,303)	
		Fatal MI	RR = 0.42, 95% CI 0.21 – 0.82 (2 studies, n = 6,534)	
		Non-fatal MI	RR = 0.93, 95% CI 0.69 – 1.26 (4 studies, n = 59,475)	
		Angina	N/A	
		Stroke	RR = 0.87, 95% CI 0.72 – 1.04 (4 studies, n = 52,026)	
		Heart failure	N/A	
		Peripheral vascular event	RR = 0.94, 95% CI 0.84 – 1.04 (1 study, n = 12,512)	
		Revascularisation intervention	RR = 1.07, 95% CI 0.76 – 1.50 (2 studies, n = 18,075)	
		Diagnosis of diabetes	RR = 1.20, 95% CI 1.05 – 1.37 (2 studies, n = 14,398)	

Table 1.5. continued.

Study details	Outcome	Effect of *n-3* PUFA on CVD risk compared to control (unless specified otherwise)		Reference
Meta-analysis including 48 RCT (n = 36,913) and 41 prospective cohort studies	All-cause mortality, cardiovascular events, cancer	High vs. low LC *n-3* PUFA supplementation, RCTs (0.4 to 7.0 g/d , ≥ 11 months):		[Hooper et al., 2004]
		All-cause mortality	RR = 0.87, 95% CI 0.73 – 1.03 (44 studies, n = 36,195)	
		Combined CV events	RR = 0.95, 95% CI 0.82 – 1.12 (31 studies, n = 35,140)	
		Cancer	RR = 1.07, 95% CI 0.88 – 1.30 (10 studies, n = 17,433)	
		Sudden death	RR = 0.85, 95% CI 0.49 – 1.48 (37 studies, n = 19,387)	
		Cardiovascular death	RR = 0.85, 95% CI 0.68 – 1.06 (44 studies, n = 36,195)	
		Fatal MI	RR = 0.86, 95% CI 0.60 – 1.25 (38 studies, n = 9,849)	
		Non-fatal MI	RR = 1.03, 95% CI 0.70 – 1.50 (25 studies, n = 14,145)	
		Angina	RR = 0.77, 95% CI 0.59 – 1.02 (25 studies, n = 17,198)	
		Stroke	RR = 1.17, 95% CI 0.91 – 1.51 (26 studies, n = 33,305)	
		Heart failure	RR = 0.51, 95% CI 0.31 – 0.85 (20 studies, n = 7,684)	
		Peripheral vascular event	RR = 0.26, 95% CI 0.07 – 1.06 (17 studies, n = 20,430)	
		Revascularisation intervention	RR = 1.05, 95% CI 0.97 – 1.12 (23 studies, n = 14,887)	
		Diagnosis of diabetes	RR = 0.87, 95% CI 0.15 – 5.08 (2 studies, n = 16,520)	
		Body weight	WMD = -0.59 kg, 95% CI -1.91 – 0.73 (7 studies, n = 1,970)	
		Serum TC	WMD = 0.03 mmol/L, 95% CI -0.06 – 0.12 (17 studies, n = 3,918)	
		Serum LDL-C	WMD = 0.13 mmol/L, 95% CI 0.03 – 0.22 (12 studies, n = 1,673)	
		Serum HDL-C	WMD = 0.01 mmol/L, 95% CI -0.03 – 0.05 (17 studies, n = 3,912)	
		Serum TG	WMD = -0.40 mmol/L, 95% CI -0.56 – -0.23 (14 studies, n = 2,096)	
		Blood pressure		
		systolic	WMD = -1.03 mmHg, 95% CI -3.30 – 1.25 (7 studies, n = 2,743)	
		diastolic	WMD = -0.23 mmHg, 95% CI -1.10 – 0.64 (7 studies, n = 2,743)	
Meta-analysis including 14 RCT (n = 20,485)	Secondary prevention in CVD patients	LC *n-3* PUFA supplementation (0.4 to 4.8 g/d , ≥ 1 yr):		[Kwak et al., 2012]
		All-cause mortality	RR = 0.96, 95% CI 0.90 – 1.02 (13 studies, n = 20,292)	
		Sudden cardiac death	RR = 0.93, 95% CI 0.66 – 1.30 (5 studies, n = 11,668)	
		Cardiovascular death	RR = 0.91, 95% CI 0.84 – 0.99 (11 studies, n = 13,974)	
		All CV events	RR = 0.99, 95% CI 0.89 – 1.09 (14 studies, n = 20,485)	
		MI	RR = 0.81, 95% CI 0.65 – 1.01 (11 studies, n = 16,207)	
		Angina	RR = 0.77, 95% CI 0.50 – 1.18 (7 studies, n = 1,671)	
		Congestive heart failure	RR = 0.92, 95% CI 0.73 – 1.17 (6 studies, n = 8,422)	
		TIA and stroke	RR = 1.13, 95% CI 0.77 – 1.66 (7 studies, n = 10,284)	

Table 1.5. continued.

Study details	Outcome	Effect of *n-3* PUFA on CVD risk compared to control (unless specified otherwise)		Reference
Meta-analysis including 12 RCT (n = 32,779)	Arrhythmias, sudden cardiac death, cardio-vascular death, all-cause mortality	LC *n-3* PUFA supplementation (0.5 to 2.8 g/day, ≥ 1 months):		[Leon et al., 2008]
		All-cause mortality	OR = 0.92, 95% CI 0.82 – 1.03 (11 studies, n = 32,439)	
		Sudden cardiac death	OR = 0.81, 95% CI 0.52 – 1.25 (6 studies, n = 31,111)	
		Cardiac death	OR = 0.80, 95% CI 0.69 – 0.92 (11 studies, n = 32,519)	
		Risk of appropriate implantable cardiac defibrillator intervention	OR = 0.90, 95% CI 0.55 – 1.46 (3 studies, n = 1148)	
Meta-analysis including 11 prospective RCT (n = 39,044)	Cardiovascular death	LC *n-3* PUFA supplementation (0.27 to 4.8 g/d, ≥ 1 yr):		[Marik and Varon et al., 2009]
		All-cause mortality	OR = 0.92, 95% CI 0.85 – 0.99 (11 studies, n = 39,044)	
		Sudden cardiac death	OR = 0.87, 95% CI 0.76 – 0.99 (6 studies, n = 37,796)	
		Cardiovascular death	OR = 0.87, 95% CI 0.79 – 0.95 (11 studies, n = 39,044)	
		Nonfatal cardiovascular event	OR = 0.92, 95% CI 0.85 – 0.99 (9 studies, n = 38,096)	
Meta-analysis including 20 RCT (n = 68,680)	All-cause mortality, sudden death, cardiac death, MI, stroke.	LC *n-3* PUFA supplementation (mean 1.5 g/d, ≥ 1 yr):		[Rizos et al., 2012]
		All-cause mortality	RR = 0.96; 95% CI 0.91 – 1.02 (17 studies, n = 63,279)	
		Sudden death	RR = 0.87; 95% CI 0.75 – 1.01 (7 studies, n = 41,751)	
		Cardiac death	RR = 0.91; 95% CI 0.85 – 0.98 (13 studies, n = 56,407)	
		MI	RR = 0.89; 95% CI 0.76 – 1.04 (13 studies, n = 53,875)	
		Stroke	RR = 1.05; 95% CI 0.93 – 1.18 (9 studies, n = 52,589)	
Meta-analysis including 18 RCT and 11 prospective studies (n = 51,264)	Cardiac, cardiovascular, all-cause mortality.	LC *n-3* PUFA supplementation, RCTs (0.27 to 6 g/d, ≥ 1 month):		[Trikalinos et al., 2012]
		All-cause mortality	RR = 0.95, 95% CI 0.89 – 1.01 (17 studies, n = 51,264)	
		Cardiac mortality	RR = 0.89, 95% CI 0.83 – 0.96 (14 studies, n = 48,500)	
		Dose-response relationships increasing intake from < 0.20 to 0.20 g/d LC *n-3* PUFA intake, prospective cohort studies (n ≥ 1000, ≥ 3 yr follow-up):		
		All-cause mortality	RR = 0.98, 95% CI 0.89 – 1.08 (8 studies, n = 194,037)	
		Cardiac, cardiovascular, or sudden cardiac death	OR = 0.64, 95% CI 0.46 – 0.89 (7 studies, n = 123,122)	

This table is a non-exhaustive list of recent meta-analyses investigating the association between LC *n-3* PUFA and risk of CVD. 95% CI, 95% confidence interval; CHD, coronary heart disease; CV, cardiovascular; CVD, cardiovascular disease; LC *n-3* PUFA, long chain n-3 polyunsaturated fatty acid; MI, myocardial infarction; N/A, not applicable; OR, odds ratio; RCT, randomized controlled trial; RR, relative risk; T2DM, Type 2 Diabetes Mellitus; TIA, transient ischemic attack.

Table 1.6. Review and meta-analyses investigating the effect of long chain *n-3* PUFA on body weight and body composition.

Study details	Outcome	Effect of LC *n-3* PUFA on body weight and body composition compared to control	Reference
Meta-analysis including 15 RCTs (n = 934)	Body composition measures	Total fish or fish oil intake (0.2 to 3.4 g/d for 3 wks up to 3 yr): BW WMD -0.59 kg, 95% CI -0.96 – -0.21 (12 studies) BMI WMD -0.24 kg/m^2, 95% CI -0.40 – -0.08 (13 studies) WC WMD -0.81 cm, 95% CI -1.34 – -0.28 (7 studies) BF WMD -0.49%, 95% CI -0.97 – -0.01 (7 studies) FM WMD -0.36 kg, 95% CI -0.96 – 0.24 (3 studies) FFM WMD -0.19 kg, 95% CI -0.72 – 0.33 (3 studies)	[Bender et al., 2014]
Meta-analysis including 7 RCTs (n = 1,970)	All-cause mortality, cardiovascular events, cancer	BW WMD = -0.59 kg, 95% CI -1.91 – 0.73	[Hooper et al., 2004]
Review of 10 studies	Body weight, fat mass	Prospective studies: Potential inverse relation between LC *n-3* PUFA and body weight and fat mass, but lacking consistency. RCTs: Conflicting results of the effect of LC *n-3* PUFA in combination with CR and/or exercise on body weight and fat mass in overweight and obese subjects.	[Lorente-Cebrián et al., 2013]

LC *n-3* PUFA, long chain n-3 polyunsaturated fatty acid; BF, body fat; BMI, body mass index; BW, body weight; CR, caloric restriction; FM, fat mass, FFM, lean body mass (fat free mass); RCT, randomized controlled trial; WC, waist circumference; WMD, weighted mean difference, i.e. difference in means.

Table 1.7. Recent meta-analyses investigating the relationship between fish and long chain *n-3* PUFA intake and risk of Type 2 Diabetes Mellitus.

Study details	T2DM risk compared to control		Reference
Meta-analysis including 16 prospective studies (n = 24,082 T2DM patients and 527,441 participants)	For each 1 serving / week increase in total fish intake:		[Wallin et al., 2012]
	Asia/Australia	RR = 0.98, 95% CI 0.97 – 1.00	
	Europe	RR = 1.03, 95% CI 0.96 – 1.11	
	U.S.	RR = 1.05, 95% CI 1.02 – 1.09	
	For each 0.3 g/day increase in LC *n-3* PUFA intake:		
	Asia/Australia	RR = 0.90, 95% CI 0.82 – 0.98	
	Europe	RR = 0.98, 95% CI 0.70 – 1.37	
	U.S.	RR = 1.17, 95% CI 1.09 – 1.26	
Meta-analysis including 16 prospective studies (n = 25,670 T2DM patients and 540,184 participants)	For each 100 g/day increase in fish/seafood intake (13 studies, n = 481,489):		[Wu et al., 2012]
	Overall	RR = 1.12, 95% CI 0.94 – 1.34	
	Asia	RR = 0.89, 95% CI 0·81 – 0.98	
	North America/ Europe	RR = 1.38, 95% CI 1.13 – 1.70	
	For each 250 mg/day increase in LC *n-3* PUFA intake (16 studies, n = 440,873):		
	Overall	RR = 1.04, 95% CI 0.97 – 1.10	
	Asia	RR = 0.95, 95% CI 0.91 – 0.99	
	North America/ Europe	RR = 1.12, 95% CI 1.05 – 1.20	
	For each 3% of total fatty acids increase in circulating LC *n-3* PUFA (5 studies, n = 10,382):		
	Overall	RR = 0.94, 95% CI 0.75 – 1.17	
Meta-analysis including 24 prospective cohort studies (n = 24,509 T2DM patients and 545,275 participants)	Total fish intake (highest vs. lowest):		[Zheng et al., 2012]
	Overall	RR = 1.07, 95% CI 0.91 – 1.25	
	Asian countries	RR = 0.89, 95% CI 0.81 – 0.98	
	Western countries	RR = 1.20, 95% CI 1.01 – 1.44	
	LC *n-3* PUFA intake (highest vs. lowest):		
	Overall	RR = 1.07, 95% CI 0.95 – 1.20	
	Asian countries	RR = 0.87, 95% CI 0.79 – 0.96	
	Western countries	RR = 1.16, 95% CI 1.04 – 1.28	

This table is a non-exhaustive list of recent meta-analyses investigating the association between LC *n-3* PUFA intake and risk of T2DM. LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid; RR, relative risk; T2DM, Type 2 Diabetes Mellitus.

Table 1.8. Recent meta-analyses investigating the effect of long chain *n-3* PUFA on glycemic control and insulin sensitivity.

Study details	Outcome	Effect of LC *n-3* PUFA compared to control	Reference
Meta-analysis including 11 RCTs (n = 618)	IS	By study population: Overall SMD = 0.08, 95% CI -0.11 – 0.28 T2DM SMD = -0.02, 95% CI -0.81 – 0.77 Without T2DM SMD = 0.09, 95% CI -0.09 – 0.28 By measure of IS: HOMA SMD = 0.30, 95% CI 0.03 – 0.58 QUICKI SMD = 0.08, 95% CI -0.51 – 0.68 Other test SMD = -0.02, 95% CI -0.33 – 0.29	[Akinkuolie et al., 2011]
Meta-analysis including 16 studies in T1DM and T2DM patients (n = 425)	Fasting glucose, HbA1c	Fasting glucose (mmol/L): Overall WMD = -0.06, 95% CI -0.71 – 0.59 T1DM WMD = -1.86, 95% CI -3.1 – -0.61 T2DM WMD = 0.43, 95% CI 0.00 – 0.87 HbA1c (%): Overall WMD = 0.16, 95% CI -0.10 – 0.41 T1DM WMD = 0.17, 95% CI -0.09 – 0.43 T2DM WMD = 0.14, 95% CI -0.41 – 0.68	[Friedberg et al., 1998]
Meta-analysis including 23 RCTs in T2DM patients (n = 1,075)	Fasting glucose, fasting insulin, HbA1c	Fasting glucose (mmol/L) (16 studies, n = 930): WMD = 0.16, 95% CI -0.13 – 0.46 Fasting insulin (pmol/L) (8 studies, n = 529): WMD = -4.19, 95% CI -13.09 – 4.71 HbA1c (%) (15 studies, n = 848): WMD = 0.01, 95% CI -0.03 – 0.01	[Hartweg et al., 2008]
Meta-analysis including 18 RCTs in T2DM patients (n = 823)	Fasting glucose, HbA1c	Fasting glucose (mmol/L) (12 studies, n = 649): WMD = 0.26, 95% CI -0.08 – 0.60 HbA1c (%) (15 studies, n = 660): WMD = 0.15, 95% CI -0.08 – 0.37	[Montori et al., 2000]

This table is a non-exhaustive list of recent meta-analyses investigating the association between LC *n-3* PUFA intake and insulin sensitivity and glycemic control. HbA1c, glycated haemoglobin; HOMA, homeostasis model assessment; IR, insulin resistance; IS, insulin sensitivity; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid; QUICKI, quantitative insulin sensitivity check index; RCT, randomized controlled trial; RR, relative risk; SMD, standard mean difference; T1DM, Type 1 Diabetes Mellitus; T2DM, Type 2 Diabetes Mellitus; WMD, weighted mean difference, i.e. difference in means.

Long chain *n-3* PUFA in inflammatory disease

LC *n-3* PUFA are anti-inflammatory and have been associated with a decreased inflammatory response in *in vitro* models and in humans, which is of relevance to the low-grade inflammation in obesity [Calder, 2006, Calder et al., 2011A, Puglisi et al., 2011, Rangel-Huerta et al., 2012]. For example, a meta-analysis of sixty-eight RCTs showed that LC *n-3* PUFA lower circulating CRP (weighted mean difference (WMD) -0.20, 95% CI -0.28 – -0.12, $p < 0.001$) and IL-6 (WMD -0.22, 95% CI -0.38 – -0.06, $p < 0.01$), but not TNF-α (WMD -0.11, 95% CI -0.24 – 0.03, ns.) in subjects with chronic non-autoimmune disease [Li et al., 2014]. In addition, LC *n-3* PUFA lower circulating CRP (WMD -0.18, 95% CI -0.28 – -0.08, $p < 0.01$), TNF-α (WMD -0.12, 95% CI -0.16 – -0.07, $p < 0.001$) and possibly IL-6 (WMD -0.09, 95% CI - 0.18 – 0.01, $p = 0.07$) in healthy subjects. In another study LC *n-3* PUFA supplementation (1.8 g/d of EPA and DHA for 2 months) decreased the plasma plasminogen activator inhibitor-1 levels and inflammatory gene expression in AT (i.e. chemoattractant plasminogen activator urokinase receptor, macrophage surface markers CD11b and CD18 and macrophage phagocytic activity marker CD68) which was associated with a lower fat mass and adipocyte diameter, in overweight women (BMI 30 kg/m^2) with T2DM [Kabir et al., 2007]. However in this study there was no effect of LC *n-3* supplementation on plasma TNF-α and IL-6 levels. The dose of LC *n-3* PUFA, EPA and DHA seems to be important for the effects on inflammatory outcomes [Calder et al., 2011A, Li et al., 2014]. For example, in the meta-analysis described above a higher dose of EPA (≥ median) has been associated with a greater CRP-lowering effect whereas a lower dose of DHA (< median) was associated with a greater IL-6 lowering effect in healthy subjects [Li et al., 2014].

In vitro studies have confirmed the anti-inflammatory effects of EPA and DHA in macrophages, which are known to play an important role in AT inflammation in obesity [Mullen et al., 2010, Oliver et al., 2012]. For example, EPA and DHA (25 mM) decreased the LPS-induced cytokine production of IL-1β, IL-6 and TNF-α and nuclear factor-kappa B (NF-κB) transcription activity in human THP-1-derived macrophages [Mullen et al., 2010]. In line with this, DHA (50 μM) significantly attenuated the LPS-induced inflammatory response (lower NF-κB activation, lower TNF-α production and greater IL-10 production) in J774.2 macrophages, which in turn decreased adipocyte inflammation (lower IL-6 and TNF-α production, and greater IL-10 production) and maintained adipocyte insulin-sensitivity in an *in vitro* co-culture model with 3T3-L1 adipocytes [Oliver et al., 2012]. Besides LC *n-3* PUFA reduced ATM infiltration and/or AT inflammation markers in various mouse models of diet-induced obesity [Muurling et al., 2003, Oh et al., 2010, Saraswathi et al., 2007, Todoric et al., 2006]. A recent fish oil intervention trial investigating the effect of LC *n-3* PUFA (3.36 g EPA+DHA / d) on AT inflammation in 55 severely obese non-diabetic patients undergoing elective bariatric surgery, showed LC *n-3* PUFA decreased inflammatory gene expression in subcutaneous, but not visceral, AT [Itariu et al., 2012]. In summary, LC *n-3* PUFA are anti-inflammatory molecules with the potential to reduce inflammation in obesity.

Long chain *n-3* PUFA and mechanism of action

The effects of LC *n-3* PUFA on cell and tissue function have been suggested to be mediated through three general mechanisms; 1) transcription factor function and cell signalling; 2) cell membrane and lipid raft fatty acid composition influencing membrane fluidity, protein composition and receptor function; 3) cell membrane fatty acid composition influencing production of fatty acid-derived bioactive mediators [Calder, 2012, Kalupahana et al., 2011].

Effect on receptors and sensors

The first mechanism through which LC *n-3* PUFA exert their anti-inflammatory effect is via surface and/or intracellular receptors and sensors, including transcription factors NF-κB, peroxisome proliferator-activated receptors (PPARs) and nuclear factor erythroid 2 related factor 2 (Nrf2), which are discussed separately in more detail in the following paragraphs. Recently it has also been suggested that LC *n-3* PUFA can exert beneficial effects though G-protein coupled receptor (GPR)120 and GPR40 which are able to bind LC *n-3* PUFA and are involved in signal transduction [Calder 2011B, Kalupahana et al., 2011, Tomita et al., 2014]. Interestingly, it was shown that an agonist of the GPR120 receptor that is highly expressed in inflammatory macrophages was able to diminish the LPS-induced inflammatory response (TNF-α and IL-6 production, IκB phosphorylation) in macrophages, whereas the anti-inflammatory effect of DHA on macrophage response to LPS was abolished in GPR120 knock-down cells [Calder, 2011B, Oh et al., 2010]. On the other hand, activation of GPR40 that is highly expressed in intestinal and pancreatic cells by LC n-3 PUFA is involved in the regulation of glucose metabolism through the augmentation of glucose-dependent insulin secretion [Tomita et al., 2014].

Effect on nuclear factor kappa-B (NF-κB) target genes

NF-κB is the key transcription factor involved in the upregulation of inflammatory cytokine, adhesion molecule and COX-2 genes [May and Ghosh, 1998] and has been linked to inflammation-associated metabolic diseases [Baker et al., 2011]. Some examples of extracellular stimuli resulting in NF-κB activation are binding of TNF-α to its receptor and binding of LPS (also known as endotoxin) to the Toll-like receptor 4 receptor. LC *n-3* PUFA have been shown to reduce the expression of inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8, IL-12) and proteins (COX-2, iNOS) upon LPS stimulation in various cell types [Calder, 2011B]. For example, Lee *et al*. showed that DHA (5 - 50 μM) decreased the LPS-induced protein expression of COX-2, iNOS, and IL-1α in RAW 264.7 macrophages which involved the inhibition of NF-κB activation and decreased IκB degradation [Lee et al., 2004]. In addition, they showed that lauric acid (C12:0), which is a potential ligand for Toll-like receptors, augmented the NF-κB activation in macrophages, but this could be reversed by EPA and DHA. Furthermore, recent data indicate that LC *n-3* PUFA, in particular DHA, can also influence NF-κB activation far more upstream at cell membrane level via modulation of lipid raft formation [Calder, 2011B], with two recent studies showing DHA exerts its inhibitory

effects on NF-κB activation at the Toll-like receptor 4 receptor level [Lee et al., 2004, Wong et al., 2009].

Effect on peroxisome proliferator-activated receptors (PPARs)

The PPAR family includes PPAR-α, PPAR-γ and PPAR-δ, which are regulators of fatty acid metabolism, glucose homeostasis, cellular proliferation, differentiation and apoptosis and inflammation [Chinetti et al., 2000, Delerive et al., 2001]. PPAR-α and PPAR-γ have been well described and, besides their function in metabolic control, are also involved in the control of the inflammatory response. They have anti-inflammatory effects which encompass the direct regulation of anti-inflammatory gene expression and interference with NF-κB activation [Calder, 2011B, Chinetti et al., 2000, Delerive et al., 2001]. PPAR-α is predominantly expressed in the liver and an important regulator of fatty acid β-oxidation and energy homeostasis, with its target genes being involved in peroxisome proliferation, fatty acid oxidation and uptake of fatty acid from circulation (hypolidemia) [Chinetti et al., 2000, Delerive et al., 2001]. PPAR-γ is mainly expressed in adipocytes where it is essential for adipocyte differentiation and stimulates lipid storage and insulin sensitivity [Chinetti et al., 2000, Delerive et al., 2001]. However, macrophages also express PPAR-α and PPAR-γ, in particular upon differentiation, where they are important in controlling the inflammatory mediator production [Chinetti et al., 2000]. LC *n-3* PUFA and their metabolites are endogenous ligands for PPARs [Calder, 2011B, Chinetti et al., 2000]. The activation of PPAR by LC *n-3* PUFA involves binding to PPAR followed by translocation from the cytosol to the nucleus where it either forms heterodimer with retinoic acid receptor or binds to transcription factor NF-κB. The dimerisation with retinoic acid receptor results in binding to peroxisome proliferator response element and induction of the expression of its target genes (including anti-inflammatory mediators). The binding to NF-κB prevents binding of NF-κB to its response element inhibiting the expression of NF-κB target genes. Thus, PPAR activation by LC *n-3* PUFA has both metabolic and anti-inflammatory down-stream effects.

Effect on nuclear factor erythroid 2 related factor 2 (Nrf2)

Nrf2 is a key transcription factor involved in the oxidative stress response, which plays an important role in health resilience (i.e. the ability to cope with stress, produce a protective response and return to homeostasis after a stress challenge) [De Roos and Duthie, 2015, Stefanson and Backovic, 2014]. Nrf2 regulates the transcription of many important antioxidant and detoxification genes, including glutathione peroxidise (GPx), superoxide dismutase (SOD), heme oxygenase-1 (HO-1), catalase, glutathione-S-transferases (GST) and NAD(P)H:quinone oxidoreductase-1 (NQO1), and activates proteasomal and chaperone proteins that are involved in repairing and degradating damaged proteins. Reactive oxygen species (ROS) are produced by normal energy metabolism and function as signalling molecules. At low and moderate concentrations ROS activate Nrf2 resulting in an antioxidant response, whereas high levels of ROS activate NF-κB resulting in a proinflammatory response [Stefanson and Backovic, 2014]. Dietary fatty acids, in particular EPA and DHA,

are prone to oxidation and are believed to increase ROS levels just enough to activate Nrf2 [De Roos and Duthie, 2015]. For example, oxidized EPA and DHA have been shown to activate Nrf2 and induce Nrf2 target gene expression in endothelial cells [Ishikado et al., 2013, Majkova et al., 2011]. Furthermore, the induction of the Nrf2 target gene HO-1 has been shown to modulate polarisation towards the anti-inflammatory M2 phenotype and increase the production of IL-10 in macrophages [Naito et al., 2014], which provides a potential link between the Nrf2 pathway and the pro- and anti-inflammatory macrophage phenotype. Thus dietary LC *n-3* PUFA are able to prime the Nrf2 pathway resulting in upregulation of the above described enzymes prior to oxidative stress, which increases the cellular fitness to respond more robustly to an oxidative assault without activating the more intense inflammatory NF-κB pathway (i.e. improved resilience) [Stefanson and Backovic, 2014].

Effect on bioactive mediator production

The second and third mechanism through which LC *n-3* PUFA exert anti-inflammatory effects is via incorporation into cell membrane phospholipids where they are involved in membrane protein function [Murphy, 1990], membrane fluidity [Stubbs and Smith, 1984], lipid raft formulation [Yaqoob et al., 2009], and serve as substrates for enzymes to produce bioactive lipid mediators including eicosanoids, resolvins, protectins and maresins [Buckley et al., 2014, Calder, 2011B, Spite et al., 2014]. Eicosanoids are a family of active mediators consisting of prostaglandins, prostacyclins, thromboxanes and leukotrienes, that serve as regulators of inflammation and are produced from arachidonic acid (AA, C20:4 n-6) and 20-carbon PUFA EPA (C20:5, n-3). Phospholipid membranes of immune cells are in general rich in AA, which serves as major substrate for enzymes cyclooxygenase (COX) and lipoxygenase (LOX) to produce the predominantly pro-inflammatory eicosanoids 2-series prostaglandins (PG) and 4-series leukotrienes (LT) [Bagga et al., 2003, Ratnayake and Galli, 2009]. On the other hand, EPA serves as a substrate for the same enzymes to produce less inflammatory 4-series PG and 5-series LT [Bagga et al., 2003, Ratnayake and Galli, 2009]. Besides, EPA and DHA are incorporated into phospholipid membranes in a dose-response manner partly at the expense of AA, resulting in less substrate available for production of its more pro-inflammatory eicosanoids [Calder, 2006]. Thus, LC *n-3* PUFA cause a shift in eicosanoid production towards more anti-inflammatory mediators. Finally, EPA and DHA give rise to lipid mediators with potent pro-resolving properties, including resolvins, protectins and maresins [Buckley et al., 2014, Spite et al., 2014]. The E-series (EPA-derived) and D-series (DHA-derived) resolvins and DHA-derived protectins have potent anti-inflammatory effects in macrophages and microglia [Calder, 2009]. The lipid mediators appear to be potent mediators involved in the resolution of inflammation and metabolic diseases [Buckley et al., 2014, Spite et al., 2014].

Research gaps

Obesity has become a worldwide problem with major health, social and financial implications by increasing the risk for metabolic complications, various diseases and premature death [World Obesity Federation, 2014]. However, a proportion of obese people are not at an increased risk for these obesity-related comorbidities and display a MHO phenotype [Karelis et al., 2004, Wildman et al., 2008]. Investigations into the determinants of MHO phenotype is of great importance to increase our understanding of what factors determine metabolic health in obesity, which can be possibly used to reduce the disease burden associated with obesity. So far, a low cardiorespiratory fitness, low AT expansion capacity, excess VAT and AT inflammation have been shown to be determinants of MHO phenotype [Barbarroja et al., 2010, Karelis et al., 2004, Virtue and Vidal-Puig, 2010]. However, our knowledge of how common genetic variants interact with nutrition and other environmental factors to cause metabolic disease in obesity is still very poor, and the identification of protective and susceptibility genes is essential to help us understand why not all people with obesity develop metabolic disease [Osborn and Olefsky, 2012]. The immune system and metabolism are highly integrated and obesity-associated inflammation is an important contributing factor to T2DM and CVD, therefore unravelling what genetic variants interact with nutrition and other environmental factors to determine AT inflammation is of great importance [Osborn and Olefsky, 2012].

In the past decades, the *APOE Epsilon* genotype has been identified as an important determinant of AD and CHD risk [Bennet et al., 2007, Corder et al., 1993, Song et al., 2004, Strittmatter et al., 1993, Takeda et al., 2010, Wilson et al., 1996], with recent studies suggesting involvement in obesity and its associated metabolic complication as well [Elosua et al., 2003, Kolovou et al., 2009, Marques-Vidal et al., 2003, Pouliot et al., 1990, Sima et al., 2007]. Research from our group and other research groups has demonstrated that the *APOE4* genotype is associated with a pro-inflammatory state [Jofre-Monseny et al., 2008], an impaired AT expandability and greater predisposition to the metabolic complications of obesity [Arbones-Mainar et al., 2008 and 2010], an altered LC *n-3* PUFA status [Chouinard-Watkins et al., 2013, Conway et al., 2014, Plourde et al., 2009, Vandal et al., 2014] and *APOE Epsilon* genotype interacts with adiposity to determine plasma inflammatory marker concentrations [Kofler et al., 2012]. Furthermore, LC *n-3* PUFAs exert various beneficial effects on inflammatory and metabolic parameters altered in obesity [Flachs et al., 2009, Puglisi et al., 2011, Kalupahana et al., 2011], and these effects may be influenced by factors determining their plasma and tissue concentrations [Walker et al., 2013]. The impact of *APOE Epsilon* genotype on obesity and inflammation in humans is still relatively unknown and its impact on AT inflammation and ATM phenotype (polarisation) has never been investigated. Moreover, the potential involvement of LC *n-3* PUFA status in the relationships between *APOE Epsilon* genotype, adiposity and inflammation has not been established.

Research question and objectives

The overall aim of this PhD project is to investigate the relationships between *APOE Epsilon* genotype, adiposity, LC *n-3* PUFA status and inflammation in order to advance the knowledge in the area, with the following specific objectives;

1.) To investigate the relationships between *APOE Epsilon* genotype, adiposity, LC *n-3* PUFA status and inflammation, and the potential involvement of SNPs in *APOE* promoter region, in participants involved in a fish oil intervention trial (FINGEN) [Caslake et al., 2008, Kofler et al., 2012].

2.) To investigate the independent and interactive impact of *APOE Epsilon* genotype and fish oil on the development of diet-induced obesity (DIO) and whole-body glucose tolerance in human *APOE3* and *APOE4* TR mice.

3.) To investigate the independent and interactive impact of *APOE Epsilon* genotype and fish oil on LC *n-3* PUFA status and AT inflammation (ATM infiltration rate and phenotype) in human *APOE3* and *APOE4* TR mice.

4.) To conduct a pilot study investigating the impact of *APOE Epsilon* genotype on the 'basal' phenotype of macrophages in primary murine bone marrow-derived macrophages (BMM) to corroborate our findings from *in vivo* studies in human *APOE* TR mice.

Hypotheses

We hypothesise that the *APOE Epsilon* genotype is a determinant of inflammatory status, adiposity (i.e. AT expansion), and metabolic consequences of obesity, as well as a determinant of LC *n-3* PUFA metabolism and the responsiveness to LC *n-3* PUFA supplementation. In more detail, we hypothesize that compared to *APOE3* allele the *APOE4* allele will be associated with;

1.) a pro-inflammatory status,

2.) a reduced AT expandability,

3.) a greater predisposition to metabolic consequences of obesity,

4.) an altered LC *n-3* PUFA metabolism,

5.) an impaired responsiveness to LC *n-3* PUFA supplementation.

Chapter 2. *APOE* genotype, adiposity, long chain *n-3* polyunsaturated fatty acids and inflammation: Relationships between -219G/T and *E2/E3/E4 APOE* genotypes, adiposity, long chain *n-3* polyunsaturated fatty acids and plasma inflammatory markers in the FINGEN cohort.

Retrospective analysis of baseline and response to intervention data from a double-blind placebo controlled cross-over fish oil intervention trial

Introduction

Overview of obesity, inflammation and long chain *n-3* PUFA status

Worldwide approximately 1 billion people are overweight (BMI 25 – 29.9 kg/m²) and a further 475 million people are obese (BMI ≥ 30 kg/m^2) [World Obesity Federation, 2014]. Obesity is associated with a state of systemic chronic low-grade inflammation and metabolic complications, such as metabolic syndrome (MetS) and insulin resistance (IR), which in turn increase the risk of developing Type 2 Diabetes Mellitus (T2DM) and cardiovascular disease (CVD) [Maury and Brichard, 2010, Van Gaal et al., 2006]. However, not all obese individuals develop the metabolic complications which lead to the increased risk of T2DM or CVD. Approximately one-third of obese people, termed metabolically healthy obese (MHO), have a normal metabolic profile characterised by a low visceral fat (VAT) content, high-density lipoprotein-cholesterol (HDL-C) levels, low triglyceride (TG) levels, high insulin sensitivity and low inflammation, despite their high fat mass [Barbarroja et al., 2010, Karelis et al., 2004, Perrault et al., 2014, Wildman et al., 2008]. The MHO individuals have a lower risk of T2DM and CVD compared to metabolically unhealthy obese (MUO) and a similar risk of CVD compared to normal weight and metabolically healthy individuals [Primeau et al., 2011]. Tissue inflammatory status has emerged as an important determinant of the metabolic consequences of obesity, as the immune system and metabolic pathways involved in macronutrient metabolism and utilisation, including ATP production, are highly integrated [Osborn and Olefsky, 2012]. For example, a nutrient surplus and saturated fatty acids (SFA) can trigger intracellular stress signals potentiating pro-inflammatory signalling, whereas long chain (LC) *n-3* polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) exert anti-inflammatory effects [Osborn and Olefsky, 2012].

Furthermore, lower plasma EPA and DHA levels have been reported in MUO, but not MHO, compared to healthy lean individuals suggesting a lower whole-body availability of these LC *n-3* PUFA that might also persist at tissue levels in MUO [Fernandez-Real et al., 2003, Perrault et al., 2014]. In summary, metabolic disease in obesity is associated with low-grade chronic inflammation and a poor LC *n-3* PUFA status.

Long chain *n-3* PUFA and inflammation

LC *n-3* PUFA intake and status have been shown to be associated with a decreased inflammatory response in *in vitro* and animal models and in humans [Calder, 2006, Calder et al., 2011A, Puglisi et al., 2011]. For example, LC *n-3* PUFA reduced ATM infiltration and AT inflammatory marker expression in various mouse models of diet-induced obesity [Muurling et al., 2003, Oh et al., 2010, Saraswathi et al., 2007, Todoric et al., 2006]. Besides, EPA and DHA supplementation (3.36 g/d) for 8 weeks decreased inflammatory gene expression in subcutaneous AT in 55 severely obese non-diabetic patients undergoing elective bariatric surgery [Itariu et al., 2012]. In healthy elderly subjects, EPA and DHA supplementation (1.8 g/d) for 26 weeks decreased the expression of genes involved in inflammatory and atherogenic-related pathways, such as nuclear factor kappa-B (NF-κB) pathway, in peripheral blood mononuclear cells compared to a high-oleic acid sunflower control oil (4.0 g/d) [Bouwens et al., 2009]. Therefore, LC *n-3* PUFA may provide a potential strategy to resolve the chronic low-grade inflammation associated with obesity.

APOE Epsilon genotype, inflammation and long chain *n-3* PUFA status

In addition to dietary fatty acids and associated tissue fatty acid status, common gene variants in inflammatory genes are likely to be independently or interactively important determinants of inflammatory status [Slim and Minihane, 2014]. Genetic variants in the *apolipoprotein E* (*APOE)* gene have emerged as an important determinant of inflammation, with the *APOE Epsilon 4* (*APOE4)* genotype being associated with a pro-inflammatory state [Jofre-Monseny et al., 2008A, Lynch et al., 2003, Maezawa et al., 2006, Ophir et al., 2005, Tsoi et al., 2007]. ApoE is produced by hepatocytes, astrocytes in the brain and by adipocytes and macrophages in adipose tissue (AT). There is increasing evidence that apoE plays a role in AT development, the development of obesity and its related metabolic complications [Kypreos et al., 2009]. In the FINGEN study we demonstrated that *APOE Epsilon* genotype interacts with adiposity to determine plasma inflammatory marker concentrations [Kofler et al., 2012] suggesting that *APOE Epsilon* genotype may exert it effects on inflammation in part through modulation of AT metabolism. Limited studies investigating the association between *APOE Epsilon* genotype and adiposity in humans have provided accumulating evidence supporting a link between *APOE Epsilon* genotype, adiposity and the metabolic consequences of obesity [Elosua et al., 2003, Kolovou et al., 2009, Sima et al., 2007]. Although there is some inconsistency, the *APOE4* allele appears to be more prevalent in obese people [Elosua et al., 2003, Kolovou et al., 2009, Oh and Barrett-Connor, 2001, Sima et al., 2007, Volcik et al., 2006]. A few studies have reported an

interaction between *APOE Epsilon* genotype and BMI to determine metabolic and/or inflammatory parameters [Kofler et al., 2012, Marques-Vidal et al., 2003, Pouliot et al., 1990]. These findings suggest that the *APOE Epsilon* genotype may influence adiposity and inflammatory status and interact with adiposity to determine the metabolic consequences of increased adiposity.

Besides, *APOE Epsilon* genotype has been shown to impact on LC *n-3* PUFA status, with *APOE4* carriers having an altered DHA metabolism and suboptimal LC *n-3* PUFA bioavailability [Plourde et al., 2009, Chouinard-Watkins et al., 2013, Vandal et al., 2014]. For instance, DHA concentrations in plasma triglycerides (TG) were increased by only 75 % in *APOE4* carriers after a 6 week supplementation with 3 g/d EPA and DHA compared to a 240 % increase in non-carriers [Plourde et al., 2009]. A kinetics study using isotopically labelled DHA revealed 31 % lower DHA levels in plasma total lipids and a more rapid β-oxidation of DHA 1 – 28 days post-dose (40 mg [^{13}C]DHA) in *APOE4* carriers compared to non-carriers [Chouinard-Watkins et al., 2013]. These findings suggest that *APOE Epsilon* genotype modulates the incorporation of EPA and DHA into plasma lipids, which in turn may affect their bioavailability and ability to exert beneficial effects [Carvalho-Wells et al., 2012, Minihane et al., 2000, Olano-Martin et al., 2010]. Currently little is known regarding the impact of *APOE Epsilon* genotype on tissue fatty acid status, which will be investigated in this thesis.

In addition, the impact of *APOE Epsilon* genotype on inflammatory status and the responsiveness to EPA and DHA supplementation may be modulated by single nucleotide polymorphisms (SNPs) in the *APOE* promoter region, which are known to affect gene expression. As discussed in Chapter 1, the -219G/T (rs405509) and -491A/T (rs449647) polymorphisms in the *APOE* promoter region affect *APOE* expression levels and impact on Alzheimer's Disease (AD) risk, which is independent of *APOE Epsilon* genotype for -491A/T polymorphism but not for -219G/T polymorphism [Lambert et al., 2002, Wang et al., 2000, Lescai et al., 2011, Artiga et al., 1998]. In more detail, the -491T allele and -219G allele were associated with respectively a 63 % decreased and 169 % increased transcriptional activity of *APOE* in a human hepatic cell line compared to wild type haplotypes (-491A and -219T) [Artiga et al., 1998]. In line with this, the -219T allele was associated with decreased plasma apoE concentrations in dose-dependent manner in the ECTIM study, adjusting for *APOE Epsilon* genotype (plasma apoE concentrations, 4.9 ± 1.8 (SD) for -219GG; 4.7 ± 1.7 for -219GT; and 4.1 ± 1.5 mmol/L for -219TT, $p < 0.01$) [Lambert et al., 2000]. Besides, the -219T allele was associated with an increased AD risk (OR = 1.30, 95 % CI 1.06 – 1.60 for -219TT) in 2,390 AD patients and controls, but this effect disappeared after correcting for presence of the *APOE4* allele and further investigation in *APOE4* carriers revealed no significant effect of -219T allele on AD risk (OR = 4.77, 95% CI 3.18 – 7.17 for -219T/*APOE4* vs. OR = 5.31, 95% CI 2.93 – 9.63 for -219G/*APOE4*) [Lescai et al., 2011]. These findings suggest that *APOE Epsilon* genotype interacts with SNP in the *APOE* promoter region to determine its expression levels and likely its impact on disease risk. However, the

independent and interactive (with *APOE Epsilon E2* / *E3* / *E4* genotypes) impact of SNPs in the *APOE* promoter region on inflammatory status and the responsiveness to EPA and DHA supplementation remains to be elucidated.

In conclusion, inflammation is likely to be an important modulator of the phenotype associated with obesity which may be influenced by *APOE Epsilon* genotype and LC *n-3* PUFA status. However our understanding of how these factors interact (Figure 2.1) is incomplete.

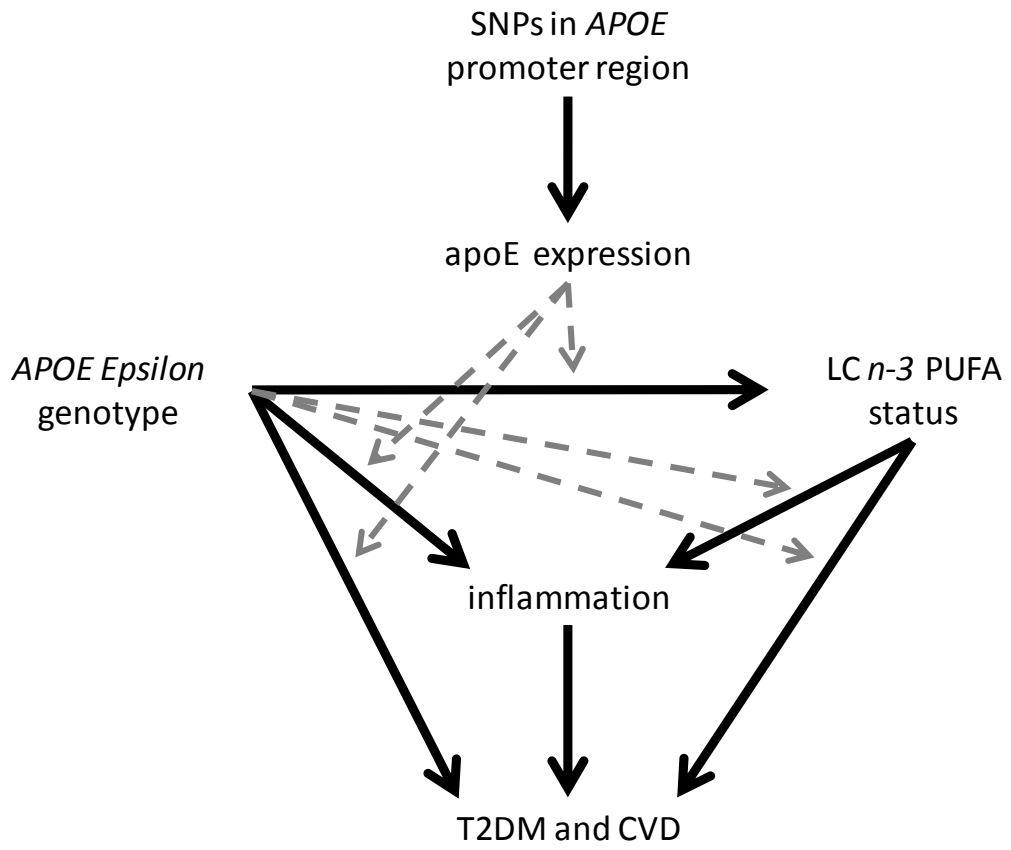

Figure 2.1. Schematic overview of the associations and interactions between *APOE Epsilon* genotype, SNPs in *APOE* promoter region, LC *n-3* PUFA status, inflammation and disease. Black solid arrows indicate a direct effect of one factor on another factor. Grey dotted arrows indicate how one factor can modulate the relationship between two other factors. APOE, apolipoprotein E; CVD, cardiovascular disease; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acids; T2DM, type 2 Diabetes Mellitus.

Research gaps

Our understanding of how common genetic variants interact with dietary and other environmental factors to cause metabolic disease is still very poor [Osborn and Olefsky 2012]. The identification of protective and susceptibility genes is crucial to help us, among others, explain why not all people with obesity develop metabolic diseases. In addition, more studies are needed to obtain a full understanding of the determinants of LC *n-3* PUFA

status, which is particularly important for predicting the likely health benefits associated with dietary intakes [Walker 2013]. We previously discussed how *APOE Epsilon* genotype and LC *n-3* PUFA status may influence inflammation, which is an important modulator of the phenotype associated with obesity. The interactive impact of *APOE Epsilon* genotype and SNP in the *APOE* promoter region to determine LC *n-3* PUFA status and inflammatory marker concentrations is currently unknown.

Research question

The overall aim of this study was to further investigate the relationships between *APOE* genotype, adiposity, fatty acid status and inflammation by using the existing data set from the FINGEN study [Caslake 2008, Kofler 2012] and perform novel genotyping for the -219G/T polymorphism (rs405509) in *APOE* promoter region. The data was used to, 1.) perform an analysis of the determinants of LC *n-3* PUFA status; 2.) investigate the impact of *APOE Epsilon* genotype, -219G/T genotype and -219G/T * *APOE Epsilon* haplotype on the plasma inflammatory marker concentrations; and 3.) to investigate the impact of the *APOE* genotypes on the plasma inflammatory marker responsiveness to LC *n-3* PUFA supplementation.

Hypotheses

1) Age, gender, adiposity, *APOE Epsilon* genotype and the -219G/T polymorphism (rs405509) in *APOE* promoter region influence plasma LC *n-3* PUFA status, with a poorer LC *n-3* PUFA status in young versus old; men versus women; obese versus lean individuals; and *APOE4* carriers versus those with *APOE3* genotype, which may interact with the -219G/T genotype.
2) *APOE Epsilon* genotype will interact with -219G/T polymorphism to determine plasma apoE and possibly plasma inflammatory marker concentrations. The highest plasma apoE concentrations are expected in those with *APOE2* and -219GG genotype. The highest plasma inflammatory marker concentrations are expected in those with -219GG * *APOE4* haplotype.
3) *APOE Epsilon* genotype interacts with the -219G/T polymorphism to determine the plasma inflammatory marker responsiveness to LC *n-3* PUFA supplementation. A poorer response is expected in *APOE4* carriers compared to *APOE3* genotype, which may interact with the -219G/T allele.

Materials & Methods

This study used an existing data set from the FINGEN study [Caslake et al., 2008, Kofler et al., 2012, Rudkowska et al., 2014]. The primary aim of the original FINGEN study was to investigate the independent and interactive impact of *APOE Epsilon* genotype, age and gender on the responsiveness of over 40 biochemical markers of CHD to a modest dose fish oil intervention [Caslake et al., 2008, Miles et al., 2010, Kofler et al., 2012, Rudkowska et al., 2014]. All data regarding *APOE Epsilon* genotype, habitual diet, plasma biochemistry, plasma fatty acid status in the phosphatidylcholine (PC) fraction, and plasma inflammatory marker concentrations was available from the existing FINGEN study data set. Only genotyping for the -219 G/T polymorphism (rs405509) in the *APOE* promoter region had to be performed as part of my PhD analysis. For completeness the current largely original analysis includes a small amount of published FINGEN data; where this occurs it is clearly indicated and referenced.

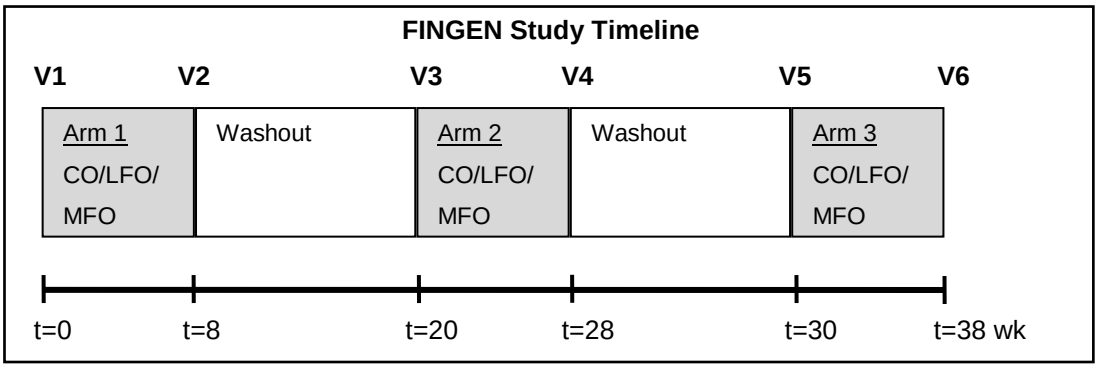

Figure 2.2. Timeline of the FINGEN study; a double-blind placebo controlled cross-over trial with three dietary intervention arms; 0.7 g EPA+DHA / day (LFO), 1.8 g EPA+DHA / day (MFO) and control oil (CO). The volunteers received each dietary intervention (CO / LFO / MFO) in random order separated by a 12 week washout period. At each visit (V1 – V6) a 12-h fasting blood sample was collected. A food frequency questionnaire (FFQ) was completed twice during the study period. t, time point; V, visit; wk, week.

FINGEN study design and volunteers

The FINGEN study was a double-blind placebo controlled cross-over trial with three intervention arms (0.7 g EPA+DHA / d and 1.8 g EPA+DHA / d and control oil) conducted at the Universities of Glasgow, Newcastle, Reading and Southampton between 2003 and 2006 (Figure 2.2). Volunteers in the FINGEN study were generally fit and healthy and were prospectively recruited according to *APOE Epsilon* genotype (*E2/E4* carriers were excluded), age (20 – 70 years) and gender, creating a study population with fairly equal distribution of males and females, all ages and all *APOE* genotypes. The dietary intervention consisted of modest doses of EPA and DHA (0.7 g/d and 1.8 g/d), which can be achieved through increased consumption of 2 – 4 portions of oily fish per week. The study design, fish oil capsule composition and volunteer exclusion criteria have been described in detail before [Caslake et al., 2008]. Briefly, randomization of the subjects to treatment order was achieved

by using a computer-generated random number table. *APOE Epsilon* genotypes were evenly randomised over the six treatment arm options. -219 G/T genotypes were not randomised over the treatment arm options, as the genotyping for the -219G/T alleles was performed retrospectively. An 8 week intervention period is long enough to observe an effect on plasma lipid profile, but is unlikely long enough to establish a new homeostasis in AT fatty acid composition or establish an effect on AT biology since the adipocyte turnover in humans is approximately 10 % per year independently of age or BMI [Spalding et al., 2008]. The FINGEN study was approved by the local research ethics committees (LRES) and all individuals provided informed written consent prior to participation.

Study data description

The *APOE Epsilon* genotyping, biochemical analysis of CHD risk markers and analysis of the fatty acid composition in the plasma PC lipid fraction has been described elsewhere [Caslake et al., 2008, Kofler et al., 2012]. The measures of interest regarding plasma LC *n-3* PUFA status were EPA, docosapentaenoic acid (DPA), DHA and total LC *n-3* PUFA (EPA+DPA+DHA) measured as a percentage (%) of total fatty acids, and the ratio of EPA to arachidonic acid (AA) and SFA to PUFA in the plasma PC lipid fraction. The plasma inflammatory markers investigated were C-reactive protein (CRP), tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, intercellular adhesion molecule (ICAM)-1, vascular cellular adhesion molecule (VCAM)-1, E-selectin (E-sel) and P-selectin (P-sel). A food frequency questionnaire (FFQ) was completed twice during the study period to characterise habitual diet. The FFQ was modified from the European Prospective Investigation into Cancer and Nutrition FFQ that contained 154 items. An additional eight questions regarding fish consumption were included and fish groups were classified according to Welch *et al*. [Welch et al., 2002]. The data from the FFQ was used to calculate intakes of energy and nutrients using portion size information and nutrient composition values from the 6th edition of McCance and Widdowson food tables and Windiets2005 (developed by Dr. Alan Wise, Robert Gordon University, UK). Data was available for total energy intake, percentage of total energy derived from fat, percentage of total energy derived from alcohol, fatty acid intake split into SFA, monounsaturated fatty acids (MUFA) and PUFA, ratio of PUFA:SFA, portions of oily fish per week, fruit and vegetable intake (number of portions per week, absolute daily intake (g/d) and percentage of total energy).

Analysis of single nucleotide polymorphisms

The selection of the additional SNP in the *APOE* promoter region rs405509 was based on the literature review summarised in the introduction of this chapter and in the main introduction. Briefly, the selected non-synonymous SNPs in the *APOE* promoter region have been shown to impact on 1.) *APOE* gene expression, 2.) apoE protein levels, and 3.) interact with *APOE Epsilon* genotype to determine disease risk. The -219G/T (rs405509) and -491A/T polymorphism (rs449647) in the *APOE* promoter region were selected for further analysis. We added novel data to the existing data set from the FINGEN study by

conducting additional genotyping for the -219G/T (rs405509) polymorphism using existing DNA samples. Unfortunately, we were unable to genotype the FINGEN participants for the -491A/T polymorphism (rs449647) due to a lack of validity of the Custom TaqMan SNP Genotyping Assay (Assay ID: AHFAYSD; Life Technologies, Paisly, UK). Briefly, genotyping of the FINGEN participants using this SNP Genotyping Assay identified a single population homozygous for -491A allele suggesting all FINGEN participants (n = 312) are homozygous for the -491A allele. We think this is very unlikely a true result given a minor allele frequency (MAF) of T = 0.18 in Caucasian populations (www.ncbi.nlm.nih.gov/SNP) that predicts approximate 50 carriers of the -491T allele in the FINGEN study. Therefore the data was not used and this SNP is not further discussed in this piece of work. The genotyping of -219G/T (rs405509) was performed using real-time polymerase chain reaction (RT-PCR) and Pre-designed TaqMan SNP Genotyping Assay (Assay ID: C____905013_10; Life Technologies) on the Applied Biosystems 7500 Real-Time PCR system (Life Technologies). Each reaction (10 µl) contained 5 µl TaqMan Genotyping Master Mix (Life Technologies), 0.5 µl primer/probe mix and 4.5 µl DNA sample (containing 1 to 20 ng purified genomic DNA). The following reaction cycles were performed: pre-PCR read (1x 1 min 60 °C), holding stage (1x 10 min 95 °C), 40 apmplification cycles (40x 15 sec 95 °C, 1 min 60°C), followed by a post-PCR read (1 min 60 °C). A negative control without genomic DNA template (NTC) was taken along with every assay. The -219G/T alleles were automatically assigned by the Applied Biosystems software and presented in the Allelic Discrimination Plot. The amplification data and Allelic Discrimination Plot were visually inspected to verify the allocation of the -219G/T alleles. The DNA had been previously isolated from the buffy coat layer of 10 mL of blood drawn into tubes containing EDTA with the use of Qiagen DNA Blood Mini Kit (Qiagen Ltd, Crawley, United Kingdom).

Statistical analysis

Data is shown for the participants who completed the study (n = 312) excluding participants with significant outlying values. Outliers were identified prior to analysis by visual inspection of the frequency distribution plot with the fitted normal distribution for the individual outcome measures. Values outside of the fitted normal distribution and outside of the physiological range were considered outliers and excluded from further analysis. Age, gender, BMI, smoking and habitual oily fish intake were chosen as covariates because of their potential impact on plasma fatty acid status and/or plasma inflammatory marker concentrations. Study centre (Glasgow, Newcastle, Reading and Southampton) was also added as covariate.

Analysis of Variance (ANOVA) was performed on baseline data (visit 1 values) to determine the effect of *APOE Espilon* genotype, -219G/T genotype, age, gender and BMI on fatty acid composition in the plasma PC lipid fraction and plasma inflammatory marker concentrations adding the factors as single explanatory variable, followed by adjusted ANOVA analysis (ANCOVA) including additional covariates age, gender, BMI, centre, smoking and habitual

oily fish intake in the model (Figure 2.3). Subsequently, interaction terms were added to the ANCOVA model to determine the interactive effect of *APOE Espilon* genotype, -219G/T genotype, age, gender and BMI on the outcome measures. The effects (main and interaction effect) were considered statistically significant when $p < 0.05$.

Figure 2.3. Primary analysis model using mixed effect model.

$$y_{ij} = \mu + \tau_{\sigma ij} + \pi_j + \xi_i + \varepsilon_{ij}$$

y_{ij} is trait value for subject i at end of time period j
$\tau_{\sigma ij}$ is effect of treatment on subject i during time period j
π_j is effect of time period j
ξ_i is person specific effect (modelled as random effect)
ε_{ij} is normally distributed random error

ANCOVA analysis on outcome data (end of treatment values) with baseline (value at beginning of respective treatment period) and sequence (order of treatments) as covariates was performed to determine the effect of treatment, followed by adjusted ANCOVA analysis including additional covariates age, gender, BMI, centre, smoking and habitual oily fish intake in the model. Subsequently, interaction terms were added to the adjusted ANCOVA model to determine potential interaction effects between treatment and BMI, *APOE* genotype, -219G/T genotype or -219G/T * APOE haplotype. The effects (main and interaction effect) were considered statistically significant when $p < 0.05$. Upon finding a significant main effect of the factors, differences between groups were investigated using the student's *t*-test with Bonferroni correction.

A repeated–measures ANOVA analysis on visit (V)1, V3 and V5 baseline data (beginning of respective treatment period) had been undertaken to test for period, sequence and carry-over effects. The V1, V3 and V5 baseline values (value at beginning of intervention) for all outcome measures did not change over time ($p > 0.05$). In addition, there was no effect of sequence (treatment order) on response to treatment for plasma fatty acid composition outcome measures and for most of the plasma inflammatory markers ($p > 0.05$), with the exception of TNF-α and ICAM-1 for which the effect of treatment was investigated per treatment arm. Although subsequent investigation of treatment effect according to treatment arm for TNF-α and ICAM-1 revealed a significant effect of treatment in arm 1 for plasma TNF-α and ICAM-1 concentrations ($p < 0.05$), comparison of plasma TNF-α and ICAM-1 concentrations according to treatment revealed no difference between end-of-treatment and baseline (beginning-of-treatment) values for the different treatments in intervention arm 1 ($p > 0.05$).

Select multiple linear regression analysis had been undertaken to determine 1.) what factors, i.e. *APOE Epsilon* genotype, -219G/T genotype, age, gender, BMI and habitual oily fish intake, were the strongest determinants of baseline plasma LC *n-3* PUFA, EPA and DHA status and plasma inflammatory marker concentrations and 2.) how much of the variation in these outcomes was explained by our model. Smoking was added as a covariate to the regression model because of its potential impact on plasma fatty acid status and plasma inflammatory marker concentrations. The regression model was based on the univariate ANOVA analysis and contained for plasma LC *n-3* PUFA, EPA and DHA status, *APOE Epsilon* genotype (reference is *APOE3*), age (continuous), gender (reference is male), BMI (continuous), smoking (reference is not-smoker) and habitual oily fish intake (continuous) as explanatory variables. The regression model for plasma inflammatory marker concentrations contained *APOE Epsilon* genotype (reference is *APOE3*), -219G/T genotype (reference is -219GG), age (continuous), gender (reference is male), BMI (continuous), smoking (reference is not-smoker) and habitual oily fish intake (continuous) as explanatory variables.

All statistical analysis was performed using STATA 11.0 software.

Results

Key findings from previous publications

The primary aim of the FINGEN study was to investigate the independent and interactive impact of *APOE Epsilon* genotype, age and gender on the responsiveness of over 40 biochemical markers of CHD to a modest dose of fish oil intervention, with its primary findings published [Caslake et al., 2008, Miles et al., 2010, Kofler et al., 2012] and summarised in Table 2.2.

Table 2.1. Baseline characteristics according to gender.

	Women	Men	All
Characteristics			
APOE Epsilon genotype (n)			
E2	49	38	87
E3	55	56	111
E4	59	55	114
Age (yr)	45 ± 1.0	45 ± 1.0	45 ± 0.7
BMI (kg/m^2)	24.3 ± 0.3	26.1 ± 0.2*	25.1 ± 0.2
Weight (kg)	64.8 ± 0.8	81.9 ± 0.9*	73.0 ± 0.8
Waist (cm)	61.6 ± 0.7	66.7 ± 0.6*	64.1 ± 0.5
MetS (%, n)	11% (18)	26% (39)*	18% (57)
Habitual diet			
Energy intake (kJ/d)	9.7 ± 0.3	10.6 ± 0.3*	10.1 ± 0.2
Fat intake (% of total energy)	30.0 ± 0.5	29.7 ± 0.3	29.9 ± 0.3
SFA intake (% of total energy)	11.6 ± 0.3	11.6 ± 0.2	11.6 ± 0.2
MUFA intake (% of total energy)	8.5 ± 0.2	8.9 ± 0.1	8.7 ± 0.1
PUFA intake (% of total energy)	4.5 ± 0.1	4.5 ± 0.1	4.5 ± 0.1
Oily fish intake (portions / week)	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
Plasma biochemistry			
TG (mmol/L)	1.04 ± 0.03	1.34 ± 0.05*	1.18 ± 0.03
TC (mmol/L)	5.15 ± 0.08	5.15 ± 0.08	5.15 ± 0.06
LDL-C (mmol/L)	3.17 ± 0.07	3.33 ± 0.90	3.25 ± 0.05
HDL-C (mmol/L)	1.60 ± 0.03	1.25 ± 0.02*	1.44 ± 0.02
glucose (mmol/L)	4.96 ± 0.04	5.33 ± 0.04*	5.14 ± 0.03

All values are mean ± SEM. Women (n = 163), men (n = 149) and all participants (n = 312). Metabolic syndrome was defined as having at least three of the following criteria: increased waist circumference (≥ 102 cm in men and ≥ 88 cm in women; ≥ 90 cm in Asian men and ≥ 80 cm in Asian women); elevated triglycerides (≥ 1.7 mmol/L); decreased high-density lipoprotein cholesterol (< 1.03 mmol/L for men, < 1.29 mmol/L for women); blood pressure > 130/85 mmHg or active treatment for hypertension; fasting plasma glucose level > 5.6 mmol/L or active treatment for hyperglycaemia. Student's *t*-test was used to test for differences in baseline characteristics for women and men, *p < 0.05. Pearson's chi-square test was used to test for differences in frequency distribution of *APOE Epsilon* genotype and MetS (%) in women and men, *p < 0.05. APOE, apolipoprotein E; BMI, body mass index; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; MetS, metabolic syndrome; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TG, triglycerides; TC, total cholesterol.

The study population description, plasma PC fatty acid composition, plasma lipid, apolipoprotein and oxidative stress marker response to treatment have been previously described by Caslake *et al*. [Caslake et al., 2008]. Briefly, the study population consisted of 312 individuals with roughly equal number of men and women, *APOE2* carriers (*E2/E2* and *E2/E3*), wild-type *APOE3* (*E3/E3*) and *APOE4* carriers (*E3/E4* and *E4/E4*), and roughly

equal numbers across the decades of life 20 – 70 years (Table 2.1). The men were somewhat overweight (BMI 26.1 ± 0.2 kg/m^2) and pre-hypertensive (systolic blood pressure 129 ± 1.3 and diastolic blood pressure 76 ± 0.09 mmHg). The blood lipids and plasma glucose and insulin levels were within the normal ranges as recommended by the British Cardiac Society and others [British Cardiac Society, 1998]. The habitual energy intake was higher in men than women (10.6 ± 0.26 vs. 9.69 ± 0.24 kJ/d, $p < 0.05$) with similar habitual fat intake (total fat, SFA, MUFA and PUFA) (Table 2.1) [Caslake et al., 2008], which were comparable to averages in the United Kingdom (UK) [Swan, 2004]. The consumption of oily fish in both men (0.9 portions / day) and women (1.1 portions / day) was far above the UK average of 0.33 portions / wk [Swan, 2004]. Fish oil supplementation increased the plasma LC *n-3* PUFA concentration in the PC lipid fraction by 3 – 4 absolute percentage from 7.3 % to 10.3 % in response to 0.7 g EPA+DHA / day and from 7.1 % to 11.9 % in response to 1.8 g EPA+DHA / day, with a greater response in women than men ($p < 0.05$) [Caslake et al., 2008]. There was no impact of *APOE Epsilon* genotype or age on the fatty acid response to treatment.

Caslake *et al*. showed that *APOE Epsilon* genotype interacted with gender to determine plasma TG response to treatment (Table 2.2). The findings in Kofler *et al*. suggested that *APOE Epsilon* genotype influences the inflammatory status in human subjects, but its impact on cardiovascular and neurodegenerative disease risk is likely to be modulated a by number of factors, such as gender and BMI (Table 2.2).

Table 2.2. Key FINGEN findings from previous publications.

Study	Key findings
Caslake *et al*. (2008)	Plasma LC *n-3* PUFA concentration in response to treatment Plasma LC *n-3* PUFA concentration in PC lipid fraction increased from 7.3 % to 10.3 % in response to 0.7 g EPA+DHA / day and from 7.1 % to 11.9 % in response to 1.8 g EPA+DHA / day. The response was greater in women than men. There was no impact of *APOE Epsilon* genotype or age on the response to treatment. Plasma lipid response to treatment The plasma TG-lowering effect of EPA and DHA was 8 % in response to 0.7 g EPA+DHA / day and 11 % in response to 1.8 g EPA+DHA / day. The greatest plasma TG-lowering response was observed in *APOE4* men (15 – 23 %). The plasma LDL-C-raising effect of EPA and DHA was 2 – 3 % in response to 0.7 g and 1.8 g EPA+DHA / day. There was no effect of *APOE Epsilon* genotype on the plasma LDL-C response.
Kofler *et al*. (2012)	Plasma lipids and CVD risk score There was a significant impact of the *APOE Epsilon* genotype on the plasma TC:HDL-C ratio, TG, %HDL3 and the Framingham 10-year CVD risk score, with an overall trend for a lower and higher risk in *APOE2* and *APOE4* carriers compared to the *APOE3* genotype. The impact of *APOE Epsilon* genotype interacted with gender for TG and %HDL3, with *APOE Epsilon* genotype explaining respectively 7 % and 4 % of the variation in these outcomes. The impact of *APOE Epsilon* genotype on TC:HDL-C was greater in females compared to males, which explained 16 % and 6 % of the variability in this outcome in females and males. Plasma inflammatory marker concentrations There was an effect of *APOE Epsilon* genotype on plasma CRP, VCAM-1 and P-sel, of which the effect interacted with BMI for CRP and P-sel. The effect of genotype was most pronounced in the normal weight BMI group (≤ 24.9 kg/m^2). In this subgroup, *APOE2* carriers had lower concentrations compared to the other *APOE Epsilon* genotypes, but these benefits were entirely negated at a higher BMI. Plasma CRP levels were in the order of *E2* > *E3* > *E4*, with 23% higher levels in *APOE2* carriers and 21% lower levels in *APOE4* carriers compared to *APOE3* genotype. Plasma VCAM-1 levels were higher in *APOE2* and *APOE4* carriers compared to *APOE3* genotype. Plasma P-sel were lower in *APOE2* and *APOE4* carriers compared to *APOE3* genotype

APOE, apolipoprotein E; CRP, C-reactive protein; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL3, the most dense HDL subfraction; HDL-C, high-density lipoprotein cholesterol; LC n-3 PUFA, long chain n-3 polyunsaturated fatty acids; LDL-C, low-density lipoprotein cholesterol; P-sel, P-selectin; PC, phosphatidylcholine; TC, total cholesterol; TG, triglycerides; VCAM-1, vascular cell adhesion molecule-1.

Findings from newly analysed outcomes

The following paragraphs describe the results of the newly analysed outcomes that were part of the original analysis conducted as part of this PhD project. The -219G/T genotyping data will be described first, followed by the determinants of plasma long chain *n-3* PUFA status and the determinant of plasma inflammatory marker concentrations.

Single nucleotide polymorphism in the *APOE* gene region

The FINGEN participants were genotyped for the -219G/T (rs405509) polymorphism in the *APOE* promoter region to investigate its independent impact and its interaction with the *APOE Epsilon* genotype on plasma inflammatory marker concentrations and their responsiveness to treatment.

-219G/T alleles across *APOE Epsilon* genotypes

Overall, half of the participants were heterozygous carrying one copy of the -219G and -219T allele, a quarter carried two copies of the -219G allele and a quarter carried two copies of the -219T allele. The MAF for -219G/T (rs405509) was T = 0.50, which is in line with the reported MAF of T = 0.49 in European populations [Ensemble Genome Browser]. The -219G allele was associated with the *APOE2* genotype (Pearson $\chi^2_{(4)} = 52.2$, $p < 0.001$), whereas the -219T allele was more frequent in those with *APOE3* and *APOE4* genotype (Table 2.3).

Table 2.3. Frequency distribution of the -219G/T alleles across *APOE Epsilon* genotypes.

***APOE Epsilon* genotype**	**-219G/T allele frequency (%)**		
	GG	**GT**	**TT**
APOE2	40 (46%)	45 (52%)	2 (2%)
APOE3	24 (22%)	49 (44%)	37 (34%)
APOE4	12 (11%)	63 (55%)	39 (34%)
All	76 (24%)	157 (51%)	78 (25%)

-219G/T alleles were associated with *APOE Epsilon* genotype, Pearson $\chi^2_{(4)} = 52.2$, $p < 0.001$. APOE2 (*E2/E2*, *E2/E3*, n = 87), APOE3 (*E3/E3*, n = 111), APOE4 (*E3/E4*, *E3/E4*, n = 114). APOE, apolipoprotein E.

Plasma apoE concentrations according to -219G/T * *APOE Epsilon* haplotype

Baseline characteristics of the -219G/T genotype subgroups are presented in Table 2.4. The -219G/T polymorphism in the *APOE* promoter region has been shown to affect *APOE* expression levels [Artiga et al., 1998, Geng et al., 2011] and potentially modulate disease risk [Lambert et al., 2002, Wang et al., 2000, Lescai et al., 2011]. Therefore, the impact of -219G/T genotype on the baseline plasma apoE concentrations was investigated independently and according to *APOE Epsilon* genotype. The average plasma apoE concentration at baseline was 35.8 ± 0.5 (SEM) µg/ml. Plasma apoE concentrations were determined by *APOE Epsilon* genotype ($p < 0.001$), but not -219G/T genotype ($p = 0.87$), adjusting for age, gender, BMI, centre, smoking and habitual oily fish intake (Table 2.5).

APOE2 carriers had approximately 28 % higher plasma concentrations compared to those with *APOE3* and *APOE4* genotype ($p < 0.05$). However, the impact of *APOE Epsilon* genotype on plasma apoE concentrations interacted with -219G/T genotype ($p < 0.05$) with the greatest effect in the -219GG group. In this subgroup, *APOE2* carriers and *APOE4* carriers had respectively 18 % higher and 25 % lower plasma apoE concentrations compared to those with *APOE3* genotype ($p < 0.05$). In addition, we observed a trend for lower plasma apoE concentrations with an increasing number of -219T alleles in those with the *APOE2* and *APOE3* genotype, but not in those with *APOE4* genotype for which the opposite was evident. In *APOE4* carriers the presence of the minor -219T allele restored plasma apoE concentrations to levels comparable to the *APOE3* genotype.

Table 2.4. Baseline characteristics according to -219G/T genotypes.

	-219GG	-219GT	-219TT	All
Characteristics				
Women (%, n)	61% (46)	48% (76)	51% (40)	53% (163)
Age (yr)	46 ± 2	44 ± 1	45 ± 1	45 ± 1
Weight (kg)	72.5 ± 1.5	73.1 ± 1.1	73.2 ± 1.5	73.0 ± 0.8
BMI (kg/m^2)	25.5 ± 0.4	25.0 ± 0.3	25.2 ± 0.4	25.2 ± 0.2
Waist (cm)	64.7 ± 1.0	63.6 ± 0.7	64.1 ± 0.9	64.0 ± 0.5
MetS (%, n)	20% (15)	18% (29)	17% (13)	18% (57)
Smokers (%, n)	5% (4)	17% (26)	10% (8)*	12% (38)
Plasma inflammatory markers				
CRP (mg/l)	1.75 ± 0.24	1.38 ± 0.12	1.33 ± 0.13	1.45 ± 0.09
TNF-α (pg/ml)	1.61 ± 0.11	1.48 ± 0.06	1.63 ± 0.09	1.55 ± 0.05
IL-6 (pg/ml)	1.20 ± 0.15	1.28 ± 0.11	1.38 ± 0.14	1.28 ± 0.07
IL-10 (pg/ml)	0.92 ± 0.11	1.08 ± 0.08	1.02 ± 0.13	1.03 ± 0.06
ICAM-1 (ng/ml)	319 ± 16	287 ± 9	317 ± 15	302 ± 7
VCAM-1 (ng/ml)	1974 ± 107	1804 ± 72	1997 ± 98	1892 ± 51
E-sel (ng/ml)	70.9 ± 4.3	76.1 ± 3.0	70.0 ± 3.6	73.2 ± 2.1
P-sel (ng/ml)	68.6 ± 7.3	63.7 ± 4.9	60.8 ± 6.5	64.1 ± 3.4

All values are mean ± SEM. -219G/T genotype groups, -219GG (n = 76), -219GT (n = 157), -219TT (n = 78). One participant had missing -219G/T data. Metabolic syndrome was defined as having at least three of the following criteria: increased waist circumference (≥ 102 cm in men and ≥ 88 cm in women; ≥ 90 cm in Asian men and ≥ 80 cm in Asian women); elevated triglycerides (≥ 1.7 mmol/L); decreased high-density lipoprotein cholesterol (< 1.03 mmol/L for men, < 1.29 mmol/L for women); blood pressure > 130/85 mmHg or active treatment for hypertension; fasting plasma glucose level > 5.6 mmol/L or active treatment for hyperglycaemia. Student's *t*-test using Bonferroni correction was used to test for differences in baseline characteristics for the -219G/T genotype groups, there were no significant differences. Pearson's chi-square test was used to test for differences in frequency distribution of women (%), MetS (%) and Smokers (%) in the -219G/T genotype groups, *p < 0.05. APOE, apolipoprotein E; BMI, body mass index; CRP, C-reactive protein; E-sel, E-selectin; ICAM, intercellular adhesion molecule; IL, interleukin; MetS, metabolic syndrome; P-sel, P-selectin; TNF, tumor necrosis factor; VCAM, vascular cellular adhesion molecule.

Table 2.5. Baseline plasma apoE concentrations (μg/mL) according to -219G/T * *APOE Epsilon* haplotype.

	-219GG	-219GT	-219TT	All	ANOVA	Model 1	Model 2
APOE2	43.9 ± 1.3[a]	41.7 ± 1.2[a]	41.5 ± 1.6[a]	42.7 ± 0.9[a]	APOE Epsilon	p<0.001	p<0.001
APOE3	37.3 ± 1.9[b]	33.1 ± 1.0[b]	31.7 ± 0.9[a]	33.5 ± 0.7[b]	-219G/T	p=0.89	p=0.87
APOE4	28.1 ± 1.7[c]	32.8 ± 0.8[b]	34.6 ± 1.5[a]	32.9 ± 0.7[b]	-219G/T*APOE Epsilon	p<0.01	p<0.05
All	39.3 ± 1.1[a]	35.4 ± 0.6[b]	33.4 ± 0.9[b]				

All values are mean ± SEM. -219G/T * *APOE Epsilon* haplotypes, -219GG * E2 (n = 40), -219GT * E2 (n = 45), -219TT * E2 (n = 2), -219GG * E3 (n = 24), -219GT * E3 (n = 49), -219TT * E3 (n = 37), -219GG * E4 (n = 12), -219GT * E4 (n = 63), -219TT * E4. (n = 39). Model 1, ANOVA main-effects model (unadjusted) including *APOE Epsilon* genotype, -219G/T genotype and -219G/T * *APOE Epsilon* as explanatory variables. Model 2, ANOVA mixed-effects model (adjusted) including covariates age, gender, BMI, centre, smoking, habitual oily fish intake. Group differences were determined with Student's *t*-test with Bonferroni correction, different letters in the columns indicate different means $p < 0.05$ and different letters in the row 'all by -219G/T genotype' indicate different means $p < 0.05$. There were no mean differences between the -219G/T genotypes in *APOE2* and *APOE3* carriers. In *APOE4* carriers, the plasma apoE concentrations were significantly lower in those with -219GG genotype compared to -219TT genotype, $p < 0.05$. APOE, apolipoprotein E.

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Determinants of plasma long chain *n-3* PUFA status

Plasma LC *n-3* PUFA status has been linked to metabolic health in obesity and low-grade inflammation [Fernandez-Real et al., 2003, Perrault et al., 2014, van Bussel et al., 2011]. Therefore, we investigated potential determinants of LC *n-3* PUFA concentrations in the plasma PC lipid fraction. In the phospholipid membrane, *n-3* PUFA EPA and *n-6* PUFA AA serve as ligands for enzymes to produce respectively more anti-inflammatory and proinflammatory lipid mediators which play an important role in the inflammatory response [Buckley et al., 2014]. Hence, we investigated the ratio of EPA to AA in the plasma PC lipid fraction. Overall, there was a significant effect of habitual oily fish intake, age and BMI on baseline plasma LC *n-3* PUFA concentrations ($p < 0.05$), whereas *APOE Epsilon* genotype, -219G/T genotype, -219G/T * *APOE Epsilon* haplotype, gender, smoking, alcohol intake and habitual diet other than oily fish intake had no significant impact on the plasma LC *n-3* PUFA concentrations at baseline (Appendix 2-1). The factors with a significant effect and a considerable effect size are discussed in more detail below.

Habitual oily fish intake impacts on plasma long chain *n-3* PUFA status

As expected, habitual oily fish intake (portions / wk) had a significant effect on the LC *n-3* PUFA, EPA and DHA concentrations (% of total fatty acids) and the EPA to AA ratio ($p < 0.001$), but not on DPA concentrations and SFA to PUFA ratio in plasma PC lipid fraction at baseline (Table 2.6). The LC *n-3* PUFA, EPA and DHA concentrations and the EPA to AA ratio increased across quintiles of oily fish intake, with higher values in the highest three quintiles (≥ 0.7 portions / wk) compared to lowest two quintiles (< 0.5 portions / wk) of oily fish intake ($p < 0.05$).

Age influences plasma long chain *n-3* PUFA status

A significant effect of age on the baseline LC *n-3* PUFA, EPA, DPA and DHA concentrations (% of total fatty acids) and EPA to AA ratio ($p < 0.01$), but not SFA to PUFA ratio ($p = 0.36$) in plasma PC lipid fraction was evident in the study population adjusted for gender, BMI, centre, smoking and habitual oily fish intake. Since gender has been shown to affect LC *n-3* PUFA status in blood lipid fractions [Caslake et al., 2008, Crowe et al., 2008], the impact of age on plasma fatty acid concentrations was further investigated in men and women separately. This revealed a similar effect of age in both men and women with an overall trend of increasing levels and ratios with increasing age (Table 2.7), which reached only statistical significance in women ($p < 0.01$). Plasma EPA concentrations were higher in women aged 50 – 70 yr compared to those aged < 50 yrs ($p < 0.05$) and plasma LC *n-3* PUFA and DHA concentrations were higher in women aged 60 – 70 yr compared to women aged 20 – 29 yr and 40 – 49 yr ($p < 0.05$). Moreover, a trend was observed for increasing plasma DPA concentrations with age from 0.9 % to 1.2 % in women, but not men. The EPA to AA ratio in the plasma PC lipid fraction was higher in women aged 50 – 70 yr compared to those aged 40 – 49 yr ($p < 0.05$).

Table 2.6. Baseline plasma LC *n-3* PUFA status across quintiles of oily fish intake.

	Q1	Q2	Q3	Q4	Q5		
Oily fish intake (portions / wk)	0	0.2 – 0.5	0.7 – 0.9	1.0 – 1.6	1.7 – 7.7	**Model 1**	**Model 2**
EPA	1.35 ± 0.08[a]	1.43 ± 0.06[a]	1.68 ± 0.10[ab]	1.68 ± 0.10[ab]	2.02 ± 0.14[b]	p<0.001	p<0.001
DPA	1.11 ± 0.04	1.08 ± 0.03	1.13 ± 0.04	1.10 ± 0.01	1.13 ± 0.03	p=0.85	p=0.69
DHA	3.66 ± 0.14[a]	3.78 ± 0.12[a]	4.47 ± 0.14[ab]	4.47 ± 0.15[b]	4.89 ± 0.16[b]	p<0.001	p<0.001
LC *n-3* PUFA	5.93 ± 0.19[a]	6.35 ± 0.16[a]	7.35 ± 0.23[b]	7.43 ± 0.24[bc]	8.44 ± 0.29[c]	p<0.001	p<0.001
EPA:AA ratio	0.14 ± 0.01[a]	0.15 ± 0.01[ab]	0.19 ± 0.01[c]	0.18 ± 0.01[bc]	0.22 ± 0.01[c]	p<0.001	p<0.001
SFA:PUFA ratio	1.02 ± 0.01	1.04 ± 0.01	1.03 ± 0.01	1.04 ± 0.01	1.01 ± 0.01	p=0.83	p=0.71

All values are mean ± SEM. Model 1, ANOVA main-effects model (unadjusted) including only habitual oily fish intake (portions / wk) as explanatory variable. Model 2, ANOVA mixed-effects model (adjusted) including covariates age, gender, BMI, centre, smoking. Quintile differences were determined with Student's *t*-test using Bonferroni correction, different letters indicate different means $p < 0.05$. EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid; EPA:AA ratio, ratio of EPA to arachidonic acid; SFA:PUFA ratio, ratio of saturated fatty acids to PUFA.

Table 2.7. Baseline plasma LC *n-3* PUFA status across age in men and women.

Men	**20 - 29 yr**	**30 - 39 yr**	**40 - 49 yr**	**50 - 59 yr**	**60 - 70 yr**	**Model 1**	**Model 2**
EPA	1.56 ± 0.15	1.50 ± 0.09	1.55 ± 0.09	1.63 ± 0.10	1.78 ± 0.15	p=0.50	p=0.52
DPA	1.14 ± 0.04	1.11 ± 0.04	1.18 ± 0.04	1.16 ± 0.04	1.21 ± 0.03	p=0.48	p=0.62
DHA	3.87 ± 0.24	3.88 ± 0.20	4.14 ± 0.18	4.15 ± 0.18	4.58 ± 0.23	p=0.14	p=0.61
LC *n-3* PUFA	6.44 ± 0.34	6.55 ± 0.28	6.80 ± 0.25	7.09 ± 0.25	7.71 ± 0.33	p<0.05	p=0.18
EPA:AA ratio	0.16 ± 0.02	0.16 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.20 ± 0.02	p=0.09	p<0.05
SFA:PUFA ratio	1.00 ± 0.02	1.02 ± 0.01	1.03 ± 0.02	1.05 ± 0.01	1.04 ± 0.02	p=0.31	p=0.40
Women	**20 - 29 yr**	**30 - 39 yr**	**40 - 49 yr**	**50 - 59 yr**	**60 - 70 yr**	**Model 1**	**Model 2**
EPA	1.35 ± 0.17^{ab}	1.55 ± 0.14^{ab}	1.33 ± 0.08^{a}	1.96 ± 0.18^{b}	2.00 ± 0.19^{b}	p<0.01	p<0.01
DPA	0.84 ± 0.06^{a}	1.03 ± 0.05^{ab}	1.05 ± 0.04^{b}	1.16 ± 0.04^{b}	1.02 ± 0.05^{b}	p<0.001	p<0.001
DHA	3.86 ± 0.22^{a}	4.26 ± 0.21^{ab}	4.05 ± 0.19^{a}	4.50 ± 0.21^{ab}	5.04 ± 0.24^{b}	p<0.01	p<0.01
LC *n-3* PUFA	6.38 ± 0.43^{a}	7.07 ± 0.37$^{a\ b}$	6.54 ± 0.28^{a}	7.61 ± 0.37^{ab}	8.40 ± 0.41^{b}	p<0.01	p<0.01
EPA:AA ratio	0.15 ± 0.02^{ab}	0.17 ± 0.02^{ab}	0.14 ± 0.01^{a}	0.21 ± 0.02^{b}	0.22 ± 0.02^{b}	p<0.01	p<0.01
SFA:PUFA ratio	1.03 ± 0.01	1.02 ± 0.02	1.01 ± 0.01	1.02 ± 0.01	1.05 ± 0.02	p=0.46	p=0.52

All values are mean ± SEM. Age groups 20 – 29 yr (n = 21 men, n = 29 women), 30 – 39 yr (n = 34 men, n = 31 women), 40 – 49 yr (n = 34 men, n = 34 women), 50 – 59 yr (n = 36 men, n = 41 women), 60 – 70 yr (n = 24 men, n = 28 women). Model 1, ANOVA main-effects model (unadjusted) including only age as explanatory variable. Model 2, ANOVA mixed-effects model (adjusted) including covariates gender, BMI, centre, smoking, habitual oily fish intake. Group differences were determined with Student's *t*-test using Bonferroni correction, different letters indicate different means $p < 0.05$. EPA, eicosapentaenoic acid ; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid; EPA:AA ratio, ratio of EPA to arachidonic acid; SFA:PUFA ratio, ratio of saturated fatty acids to PUFA.

BMI is associated with plasma long chain *n-3* PUFA and DHA

There was a significant effect of BMI on plasma LC *n-3* PUFA and DHA concentrations in the PC lipid fraction at baseline ($p < 0.05$) adjusting for age, gender, centre, smoking, habitual oily fish intake (Table 2.8). To get a clearer picture of the impact of BMI on the plasma LC *n-3* PUFA concentrations adjusted predicted plasma concentrations are presented in Figure 2.4. These predictions reveal that plasma LC *n-3* PUFA and DHA concentrations decrease with increasing BMI, with LC *n-3* PUFA concentrations being significantly lower in obese subjects (BMI ≥ 30 kg/m^2) compared to normal weight subjects (BMI < 25 kg/m^2) ($p < 0.05$).

Table 2.8. Baseline plasma LC *n-3* PUFA status according to body mass index (BMI).

	< 25 kg/m^2	25 - 29.9 kg/m^2	≥ 30 kg/m^2	Model 1	Model 2
EPA	1.65 ± 0.08	1.58 ± 0.06	1.64 ± 0.10	p=0.76	p=0.23
DPA	1.08 ± 0.02	1.15 ± 0.02	1.08 ± 0.05	p<0.05	p=0.45
DHA	4.37 ± 0.10	4.03 ± 0.10	4.40 ± 0.21	p<0.05	p<0.01
LC *n-3* PUFA	7.20 ± 0.18	6.87 ± 0.15	7.16 ± 0.29	p=0.36	p<0.05
EPA:AA ratio	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	p=0.47	p=0.09
SFA:PUFA ratio	1.02 ± 0.01^a	1.04 ± 0.01^b	1.01 ± 0.01^{ab}	p=0.09	p<0.05

All values are mean ± SEM. BMI groups < 25 kg/m^2 (n = 155), 25 – 29.9 kg/m^2 (n = 128) and ≥ 30 kg/m^2 (n = 29). Model 1, ANOVA main-effects model (unadjusted) including only BMI as explanatory variable. Model 2, ANOVA mixed-effects model (adjusted) including covariates gender, centre, smoking, habitual oily fish intake. Group differences were determined with Student's *t*-test using Bonferroni correction, different letters indicate different means $p < 0.05$. EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid; EPA:AA ratio, ratio of EPA to arachidonic acid; SFA:PUFA ratio, ratio of saturated fatty acids to PUFA.

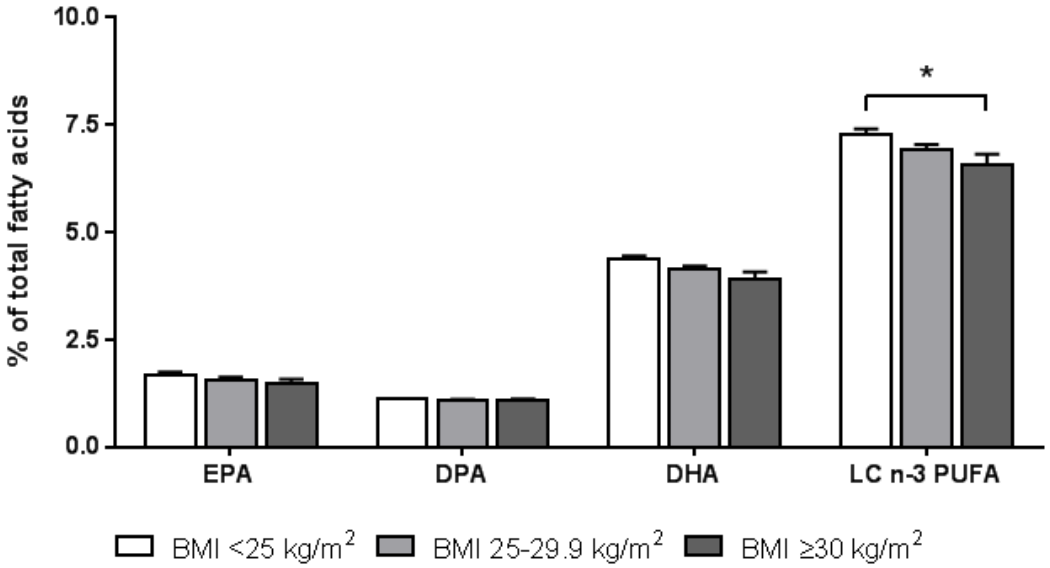


Figure 2.4. Predicted baseline eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA) and long chain (LC) *n-3* polyunsaturated fatty acid (PUFA) concentrations (% of total fatty acids) in plasma phosphatidylcholine lipid fraction tended to decrease with increasing body mass index (BMI). Data are predicted concentrations adjusted for age, gender, centre, smoking and habitual oily fish intake and are presented as mean ± SEM. BMI groups < 25 kg/m^2 (n = 155), 25 – 29.9 kg/m^2 (n = 128) and ≥ 30 kg/m^2 (n = 29). Group differences were determined with Student's *t*-test using Bonferroni correction, different letters indicate different means $p < 0.05$.

To investigate if the decreased LC *n-3* PUFA concentration in obese subjects could be attributed to an increased oxidation of these fatty acids, the ratio of plasma oxidized LDL-C (oxLDL-C) to plasma apolipoprotein B (apoB) concentrations was investigated as a marker of oxidative status. The oxLDL-C to apoB ratio reflects the proportion of oxidatively modified LDL-C relative to the total LDL particles and is a measure of lipid oxidation. There was no effect of BMI on the plasma oxLDL-C to apoB ratio at baseline adjusting for age, gender, centre, smoking and habitual oily fish intake ($p = 0.80$) (Appendix 2-2).

Plasma long chain *n-3* PUFA status in response to treatment

Caslake *et al*. have reported that gender, but not *APOE Epsilon* genotype or age, influenced the LC *n-3* PUFA response to treatment in the FINGEN study [Caslake et al., 2008]. A potential impact of BMI on the plasma fatty acid response to treatment was not investigated. Here, we report no effect of BMI ($p \geq 0.41$), -219G/T genotype ($p \geq 0.57$) or -219G/T * *APOE Epsilon* haplotype ($p \geq 0.57$) on the plasma LC *n-3* PUFA response to treatment (Table 2.9).

Table 2.9. The impact of *APOE* genotype and BMI on the plasma long chain *n-3* PUFA response to treatment.

	CO		0.7FO		1.8FO		Model 1	Model 2				
	0 wk	8 wk	0 wk	8 wk	0 wk	8 wk	T	T	T*BMI	T*G	T*E	T*G*E
EPA	1.60 ± 0.04	1.60 ± 0.04[a]	1.66 ± 0.05	2.88 ± 0.06[b]	1.63 ± 0.05	3.78 ± 0.07[c]	p<0.001	p<0.001	p=0.42	p=0.31	p=0.71	p=0.49
DPA	1.09 ± 0.01	1.08 ± 0.01[a]	1.10 ± 0.01	1.21 ± 0.01[b]	1.08 ± 0.01	1.29 ± 0.02[c]	p<0.001	p<0.01	p=0.18	p=0.48	p=0.49	p=0.69
DHA	4.41 ± 0.07	4.33 ± 0.07[a]	4.38 ± 0.07	6.22 ± 0.07[b]	4.29 ± 0.07	6.78 ± 0.08[c]	p<0.001	p<0.001	p=0.88	p=0.63	p=0.71	p=0.90
LC *n-3* PUFA	7.17 ± 0.11	7.06 ± 0.11[a]	7.21 ± 0.12	10.3 ± 0.12[b]	7.06 ± 0.11	11.9 ± 0.14[c]	p<0.001	p<0.001	p=0.35	p=0.50	p=0.85	p=0.57
EPA:AA ratio	0.18 ± 0.01	0.18 ± 0.01[a]	0.18 ± 0.01	0.34 ± 0.01[b]	0.18 ± 0.01	0.47 ± 0.01[c]	p<0.001	p<0.001	p=0.36	p=0.60	p=0.76	p=0.80
SFA:PUFA ratio	1.03 ± 0.01	1.03 ± 0.01	1.03 ± 0.01	1.03 ± 0.01	1.03 ± 0.01	1.03 ± 0.01	p=0.62	p=0.79	p=0.88	p=0.37	p=0.73	p=0.71

All values are percentage (%) of total fatty acids in the plasma phosphatidylcholine lipid fraction, mean ± SEM. Model 1, ANCOVA main-effects model containing treatment (T) with sequence and baseline value as covariates. Model 2, ANCOVA mixed-effects model containing treatment (T), treatment * BMI (T*BMI), treatment * -219G/T genotype (T*G), treatment * *APOE Epsilon* genotype (T*E), and treatment * -219G/T * *APOE Epsilon* haplotype (T*G*E) and covariates sequence, baseline value, age, gender, BMI, centre, smoking, habitual oily fish intake. Student's *t*-test using Bonferroni correction revealed no difference in the before treatment (0 wk) values between treatment groups, $p > 0.05$. Student's *t*-test using Bonferroni correction was used to determine differences in the after treatment (8 wk) values between treatment groups, different letters indicate different means $p < 0.05$. 0.7FO, 0.7 g EPA+DHA / day; 1.8FO, 1.8 g EPA+DHA / day; CO, control oil; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid; EPA:AA ratio, ratio of EPA to arachidonic acid; SFA:PUFA ratio, ratio of saturated fatty acids to PUFA.

Determinants of plasma inflammatory marker concentrations

Kofler *et al*. reported the effect of *APOE Epsilon* genotype and its interaction with age, gender and BMI on plasma inflammatory marker concentrations [Kofler et al., 2012], as summarised in Table 2.2 and detailed in Table 2.10 below with *APOE Epsilon* genotype influencing select inflammatory markers. Besides, we demonstrated that *APOE Epsilon* genotype interacted with -219G/T genotype to determine plasma apoE concentrations. Therefore, we investigated the impact of -219G/T genotype and -219G/T * *APOE Epsilon* haplotype on baseline plasma inflammatory marker concentrations. Furthermore, we investigated the impact of other potential determinants of plasma inflammatory marker concentrations, including age, gender, BMI and habitual oily fish intake. In line with the literature, we observed a significant effect of gender ($p < 0.05$), BMI ($p < 0.05$), but not of age ($p \geq 0.07$), on plasma inflammatory marker concentrations at baseline (Appendix 2-3). Men had higher baseline plasma TNF-α and E-sel concentrations than women ($p < 0.05$). Obese individuals (≥ 30 kg/m^2) had higher baseline plasma CRP and E-sel compared to lean individuals (< 25 kg/m^2) ($p < 0.05$).

Table 2.10. Impact of *APOE Epsilon* genotype on plasma inflammatory marker concentrations as published by Kofler *et al*. (2012).

	APOE2	APOE3	APOE4	G	G*BMI
CRP (mg/l)	1.69 ± 0.13^{a}	1.38 ± 0.10^{b}	1.09 ± 0.06^{c}	p<0.01	p<0.05
TNF-α (pg/ml)	1.53 ± 0.07	1.61 ± 0.12	1.76 ± 0.08	ns	ns
IL-6 (pg/ml)	1.54 ± 0.29	1.34 ± 0.16	1.63 ± 0.15	ns	ns
IL-10 (pg/ml)	1.09 ± 0.15	1.24 ± 0.16	1.20 ± 0.18	ns	ns
ICAM-1 (ng/ml)	298 ± 13	307 ± 13	304 ± 7	ns	ns
VCAM-1(ng/ml)	1975 ± 89^{a}	1721 ± 91^{ab}	2043 ± 55^{bc}	p<0.05	ns
E-sel (ng/ml)	70.6 ± 4.1	79.5 ± 3.7	68.5 ± 1.8	p=0.05	p<0.001
P-sel (ng/ml)	59.5 ± 8.2^{a}	82.8 ± 6.7^{b}	56.1 ± 2.8^{a}	p<0.01	p<0.05

These results have been published by Kofler *et al*. (2012) and are not part of our original data analysis. All values are mean ± SEM. *APOE Epsilon* genotypes, APOE2 (n = 87), APOE3 (n = 111), APOE4 (n = 114). General linear modelling was used to determine the independent and interactive impact of *APOE Epsilon* genotype (G), gender, age and BMI on the plasma inflammatory marker concentrations. There was no significant interaction between age and genotype and gender and genotype. Group differences were determined using post hoc analysis with Bonferroni correction, different letter indicate different means $p < 0.05$. APOE, apolipoprotein E; CRP, C-reactive protein; E-sel, E-selectin; ICAM, intercellular adhesion molecule; IL, interleukin; ns, non-significant; P-sel, P-selectin; TNF, tumor necrosis factor; VCAM, vascular cellular adhesion molecule.

Habitual oily fish intake determined plasma IL-10

There was a significant effect of habitual oily fish intake (portions / week) on plasma IL-10 concentrations adjusting for age, gender, BMI, centre and smoking ($p < 0.01$), but not on any of the other inflammatory markers (Appendix 2-4). Individuals in the highest quintile of oily fish intake (1.7 – 7.7 portions / week) had borderline significant higher plasma IL-10 concentrations compared to those who consume no oily fish (lowest quintile of oily fish intake) (1.35 ± 0.16 (SEM) vs. 0.84 ± 0.09 pg/ml, $p = 0.05$). The predicted plasma IL-10 concentrations adjusted for age, gender, BMI, centre and smoking illustrate how predicted

plasma IL-10 concentrations increase across quintiles of oily fish intake after accounting for covariates (Figure 2.5). Oily fish is rich in LC *n-3* PUFA and to explore if the beneficial effect of oily fish intake on plasma IL-10 was mediated through plasma LC *n-3* PUFA status, the association between quintiles of plasma LC *n-3* PUFA and plasma IL-10 concentrations was investigated. There was no association between the quintiles of plasma LC *n-3* PUFA and plasma IL-10 concentrations (p = 0.17; Appendix 2-5).

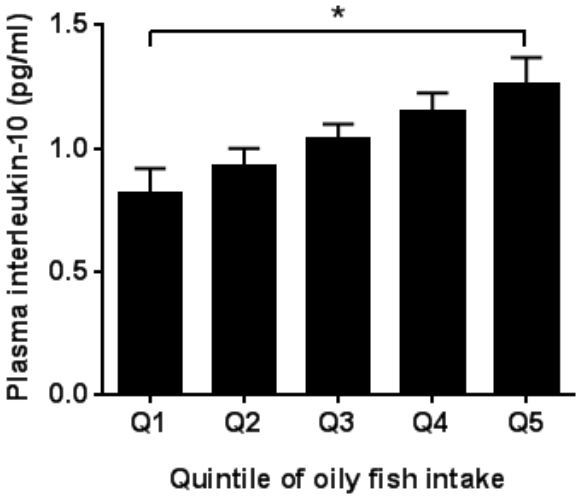


Figure 2.5. Predicted plasma interleukin (IL)-10 concentrations increase across quintiles (Q) of oily fish intake, with individuals in the highest quintile of oily fish intake (Q5, 1.7 – 7.7 portions / week) having significantly higher predicted IL-10 levels compared to individuals in the lowest quintile of oily fish intake (Q1, 0 portions / week) (p < 0.05). Habitual oily fish intake quintiles (Q) ranges were Q1, 0; Q2, 0.2 – 0.5; 0.7 – 0.9; Q4, 1.0 – 1.6; Q5, 1.7 – 7.7 portions / week. Data are predicted concentrations adjusted for age, gender, BMI, centre and smoking, mean ± SEM. Group differences were determined with Student's *t*-test using Bonferroni correction, *p < 0.05.

-219G/T * *APOE Epsilon* haplotype determined plasma P-sel and VCAM-1

Baseline characteristics of the -219G/T genotype subgroups are presented in Table 2.8. We found no effect of the -219G/T genotype on baseline plasma inflammatory marker concentrations (p ≥ 0.10). However, there was an effect of the -219G/T * *APOE Epsilon* haplotype on baseline plasma P-sel and VCAM-1 concentrations adjusting for age, gender, BMI, centre, smoking and habitual oily fish intake (p < 0.01) (Table 2.11). Individuals with -219GG * *APOE3* haplotype had higher plasma P-sel concentrations compared to those with -219TT * *APOE4* haplotype (p < 0.05).

Table 2.11. Baseline plasma inflammatory marker concentrations according to -219G/T * *APOE Epsilon* haplotype.

		-219GG	-219GT	-219TT	Model 1	Model 2
CRP (mg/l)	APOE2	1.67 ± 0.29	1.46 ± 0.17	2.09 ± 1.22	p=0.40	p=0.34
	APOE3	1.41 ± 0.19	1.55 ± 0.23	1.23 ± 0.16		
	APOE4	2.45 ± 1.03	1.20 ± 0.22	1.39 ± 0.20		
TNF-α (pg/ml)	APOE2	1.51 ± 0.08	1.53 ± 0.12	2.16 ± 1.20	p=0.60	p=0.24
	APOE3	1.62 ± 0.25	1.42 ± 0.11	1.58 ± 0.13		
	APOE4	1.92 ± 0.36	1.50 ± 0.09	1.66 ± 0.11		
IL-6 (pg/ml)	APOE2	1.24 ± 0.23	1.36 ± 0.23	0.46 ± 0.22	p=0.73	p=0.81
	APOE3	1.36 ± 0.25	1.11 ± 0.15	1.33 ± 0.22		
	APOE4	0.74 ± 0.16	1.36 ± 0.18	1.49 ± 0.20		
IL-10 (pg/ml)	APOE2	0.99 ± 0.12	0.97 ± 0.14	0.62 ± 0.11	p=0.86	p=0.90
	APOE3	0.97 ± 0.27	1.19 ± 0.15	1.10 ± 0.22		
	APOE4	0.61 ± 0.15	1.08 ± 0.15	0.97 ± 0.163		
ICAM-1 (ng/ml)	APOE2	319 ± 23	286 ± 15	345 ± 93	p=0.54	p=0.57
	APOE3	314 ± 27	271 ± 18	330 ± 22		
	APOE4	327 ± 42	300 ± 14	303 ± 22		
VCAM-1 (ng/ml)	APOE2	2135 ± 145	1806 ± 108	2450 ± 507	p<0.01	p<0.01
	APOE3	1607 ± 184	1487 ± 136	2042 ± 117		
	APOE4	2172 ± 264	2052 ± 115	1933 ± 159		
E-sel (ng/ml)	APOE2	73.6 ± 6.4	69.7 ± 5.4	52.8 ± 3.23	p=0.12	p=0.08
	APOE3	71.1 ± 7.5	88.6 ± 6.2	74.3 ± 5.0		
	APOE4	61.4 ± 8.1	71.0 ± 3.9	66.9 ± 5.3		
P-sel (ng/ml)	APOE2	56.7 ± 11^{ab}	45.7 ± 6.4^{ab}	68.8 ± 21^{ab}	p<0.01	p<0.01
	APOE3	97.9 ± 12^{a}	83.1 ± 11^{ab}	72.5 ± 11^{ab}		
	APOE4	48.5 ± 6.8^{ab}	61.0 ± 6.9^{ab}	49.4 ± 7.4^{b}		

All values are mean ± SEM. -219G/T * *APOE Epsilon* haplotypes, -219GG * APOE2 (n = 40), -219GT * APOE2 (n = 45), -219TT * APOE2 (n = 2), -219GG * APOE3 (n = 24), -219GT * APOE3 (n = 49), -219TT * APOE3 (n = 37), -219GG * APOE4 (n = 12), -219GT * APOE4 (n = 63), -219TT * APOE4 (n = 39). Model 1, ANOVA main-effects model (unadjusted) including only -219G/T * *APOE Epsilon* haplotype as explanatory variable. Model 2, ANOVA mixed-effects model (adjusted) including covariates age, gender, BMI, centre, smoking, habitual oily fish intake. Group differences were determined with Student's *t*-test with Bonferroni correction, different letters indicate different means $p < 0.05$. APOE, apolipoprotein E; CRP, C-reactive protein; E-sel, E-selectin; ICAM, intercellular adhesion molecule; IL, interleukin; P-sel, P-selectin; TNF, tumor necrosis factor; VCAM, vascular cellular adhesion molecule.

Plasma inflammatory markers response to treatment

Overall, there was no effect of treatment on any of the plasma inflammatory markers CRP, TNF-α, IL-6, IL-10, ICAM-1, VCAM-1, E-sel and P-sel evident in crude analysis and after adjusting for age, gender, BMI, centre, smoking and habitual oily fish intake ($p \geq 0.24$) (Table 2.12). Although a main effect of treatment was absent a significant interaction between treatment and *APOE Epsilon* genotype was evident for plasma VCAM-1 ($p = 0.02$), where stratification according to *APOE Epsilon* genotype did not reveal an effect of treatment ($p \geq 0.22$) indicating that *APOE Epsilon* genotype largely explains the variation in plasma VCAM-1 concentrations (Figure 2.6). No significant interactions between treatment and *APOE Epsilon* genotype were evident for the other inflammatory markers, although a borderline non-significant treatment * *APOE Epsilon* genotype interaction was observed for IL-6 ($p = 0.11$). There were no significant interactions between treatment and -219G/T genotype ($p \geq 0.19$), -219G/T * *APOE Epsilon* haplotype ($p \geq 0.06$) or BMI ($p \geq 0.30$).

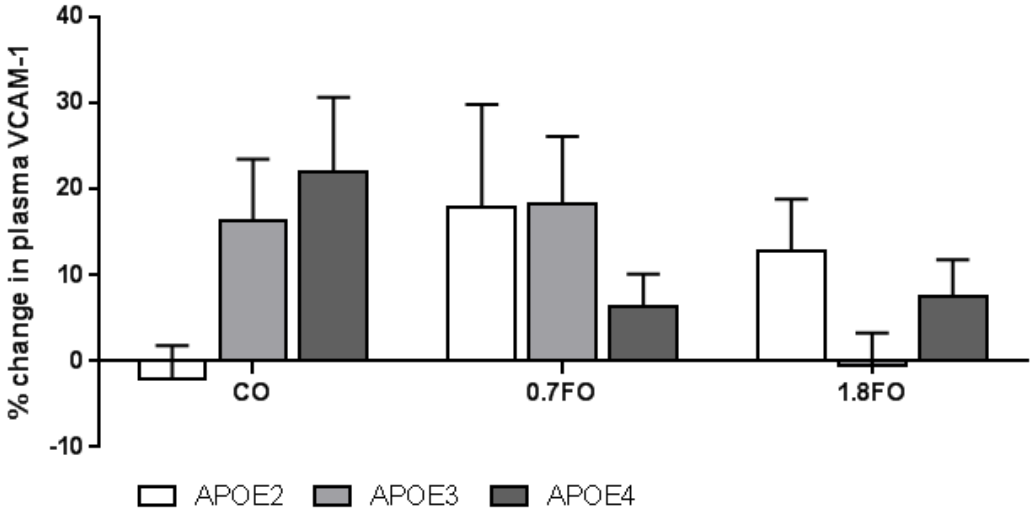


Figure 2.6. Change (%) in plasma vascular cell adhesion molecule 1 (VCAM-1) concentration according to *APOE Epsilon* genotype after treatment with control oil (CO) or fish oil (0.7g EPA+DHA / d (0.7FO) and 1.8g EPA+DHA / d (1.8FO)) for 8 weeks. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. APOE2 (*E2/E2*, *E2/E3*, n = 87), APOE3 (*E3/E3*, n = 111), APOE4 (*E3/E4*, *E3/E4*, n = 114). Groups did not differ, Student's *t*-test with Bonferroni correction p > 0.05. APOE, apolipoprotein E.

Table 2.12. Plasma inflammatory marker response to treatment.

	CO		0.7FO		1.8FO			
	0 wk	8 wk	0 wk	8 wk	0 wk	8 wk	Model 1	Model 2
CRP (mg/l)	1.68 ± 0.12	1.69 ± 0.11	1.53 ± 0.11	1.57 ± 0.11	1.46 ± 0.09	1.56 ± 0.10	p=0.60	p=0.56
TNF-α (pg/ml)	1.56 ± 0.04	1.51 ± 0.04	1.55 ± 0.04	1.47 ± 0.04	1.58 ± 0.05	1.50 ± 0.04	p=0.64	p=0.64
IL-6 (pg/ml)	1.25 ± 0.06	1.17 ± 0.06	1.23 ± 0.07	1.12 ± 0.06	1.25 ± 0.07	1.05 ± 0.06	p=0.31	p=0.30
IL-10 (pg/ml)	1.14 ± 0.07	1.06 ± 0.07	1.00 ± 0.05	1.03 ± 0.07	1.02 ± 0.07	0.96 ± 0.06	p=0.47	p=0.47
ICAM-1 (ng/ml)	299 ± 7	320 ± 7	301 ± 7	311 ± 7	311 ± 8	315 ± 7	p=0.32	p=0.32
VCAM-1(ng/ml)	1881 ± 51	1891 ± 51	1833 ± 52	1832 ± 52	1868 ± 49	1831 ± 50	p=0.72	p=0.71
E-sel (ng/ml)	74 ± 2	76 ± 2	73 ± 2	77 ± 2	75 ± 2	76 ± 2	p=0.41	p=0.42
P-sel (ng/ml)	64 ± 4	67 ± 4	59 ± 3	63 ± 3	62 ± 3	68 ± 4	p=0.82	p=0.83

All values are mean ± SEM. Model 1, ANOVA main-effects model (unadjusted) including treatment as explanatory variable with sequence and baseline value as covariates. Model 2, ANOVA mixed-effects model (adjusted) including covariates sequence, baseline value, age, gender, BMI, centre, smoking, habitual oily fish intake. 0.7FO, 0.7 g eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) / day; 1.8FO, 1.8 g EPA+DHA / day; CO, control oil; CRP, C-reactive protein; E-sel, E-selectin; ICAM, intercellular adhesion molecule; IL, interleukin; P-sel, P-selectin; TNF, tumor necrosis factor; VCAM, vascular cellular adhesion molecule.

Summary results

Table 2.13. Main findings from this study of the FINGEN trial.

Determinants of plasma LC *n-3* PUFA, EPA and DHA concentrations **1. Habitual plasma LC *n-3* PUFA, EPA and DHA concentrations** Oily fish intake moderate intake (≥ 0.7 portions / wk) > low intake (< 0.5 portions / wk) Age and gender 50 years and older > younger than 50 years (trend) age effect was stronger in women than men BMI obese (BMI ≥ 30 kg/m^2) < normal weight subjects (BMI < 25 kg/m^2) *APOE* genotype no effect of *APOE Epsilon* genotype, -219G/T genotype or -219G/T * *APOE Epsilon* haplotype **2. Response to supplementation** no effect of -219G/T genotype or -219G/T * *APOE Epsilon* haplotype
***APOE Epsilon* genotype interacts with -219G/T to determine plasma apoE** -219G/T genotype -219G allele is associated with the *APOE2* genotype plasma apoE concentrations *APOE2* > *APOE3* and *APOE4* *APOE Epsilon* genotype interacted with -219G/T genotype. In -219GG subgroup: *APOE2* > APOE3 > *APOE4* Plasma apoE increased with increasing number of -219T alleles in those with *APOE2* and *APOE3* genotype. The opposite was evident for *APOE4* genotype.
Plasma inflammatory marker concentrations **1. Habital plasma inflammatory marker concentrations** Habitual oily fish intake and plasma IL-10 highest quintile (1.7 – 7.7 portions / week) > lowest quintile (0 portions / week). -219G/T * *APOE Epsilon* haplotype and plasma P-sel and VCAM-1 P-sel: -219GG * *APOE3* > -219TT * *APOE4* haplotype VCAM-1: no difference between haplotypes **2. Response to supplementation** no effect of supplementation on plasma inflammatory markers

Plasma LC *n-3* PUFA, EPA and DHA concentrations in the plasma PC lipid fraction. APOE, apolipoprotein E; BMI, body mass index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acids; P-sel, P-selectin; PC, phosphatidylcholine; VCAM-1, vascular cell adhesion molecule-1.

The main findings from this study are summarised in Table 2.13. To determine what factors, i.e. *APOE Epsilon* genotype, age, gender, BMI and habitual oily fish intake, were the

strongest determinant of the baseline plasma LC *n-3* PUFA concentrations, we performed multiple linear regression analysis (Appendix 2-6). Briefly, this analysis revealed that age and habitual oily fish intake were significant determinants of plasma LC *n-3* PUFA (age β = 0.035, $p < 0.001$ and oily fish intake β = 0.632, $p < 0.001$), EPA (age β = 0.011, $p < 0.01$ and oily fish intake β = 0.151, $p < 0.001$) and DHA concentrations (age β = 0.019, $p < 0.001$ and oily fish intake β = 0.291, $p < 0.001$). BMI was only a significant determinant of the plasma LC *n-3* PUFA (β = -0.093, $p < 0.01$) and DHA concentrations (β = -0.057, $p < 0.01$). *APOE Epsilon* genotype and gender did not determine plasma LC *n-3* PUFA, EPA and DHA concentrations. Our regression model explained only 26 %, 11 % and 17 % of the variation in the plasma LC *n-3* PUFA, EPA and DHA concentrations respectively, indicating that additional factors not included in our model are important in determining plasma LC *n-3* PUFA concentrations.

To determine what factors, i.e. -219G/T genotype, *APOE Epsilon* genotype, age, gender, BMI and habitual oily fish intake, were the strongest determinant of the baseline plasma inflammatory marker concentrations, we performed similar multiple linear regression analysis (Appendix 2-6). In brief, *APOE Epsilon* genotype was a significant determinant of the plasma VCAM-1 (*APOE2* β = 307.609, $p < 0.05$ and *APOE4* β = 344.072, $p < 0.01$), E-sel (*APOE2* β = -9.609, ns. and *APOE4* β = -12.030, $p < 0.05$) and P-sel concentrations (*APOE2* β = -36.536, $p < 0.001$ and *APOE4* β = -26.772, $p < 0.01$). In contrast, -219G/T genotype was not a determinant of plasma inflammatory marker concentrations. BMI was a determinant of plasma CRP (β = 0.090, $p < 0.01$) and VCAM-1 concentrations (β = -35.856, $p < 0.05$). Gender was a determinant of plasma IL-6 (β = -0.257, $p < 0.01$) and E-sel concentrations (β = -8.645, $p < 0.01$) and habitual oily fish intake (portions / week) was determinant of plasma IL-10 concentrations (β = 0.181, $p < 0.001$). Age and smoking did not determine plasma inflammatory marker concentrations. Our regression model explained only between 3 % and 10 % of the variation indicating that our model was a poor predictor of plasma inflammatory marker concentrations and other additional factors are important in determining plasma inflammatory marker concentrations.

Discussion

This study used an existing data set from the FINGEN study [Caslake et al., 2008, Kofler et al., 2012] to further investigate the *APOE Epsilon* genotype * adiposity * LC *n-3* PUFA * inflammation relationships and included novel genotyping for the -219G/T polymorphism (rs405509) in the *APOE* promoter region. The aim of the study was to 1.) perform an analysis of the determinants of LC *n-3* PUFA status; 2.) investigate the impact of *APOE Epsilon* genotype, -219G/T genotype and -219G/T * *APOE Epsilon* haplotype on plasma inflammatory marker concentrations; and 3.) to investigate the impact of the *APOE* genotypes on the plasma inflammatory marker concentrations response to LC *n-3* PUFA supplementation. Our main findings were that habitual oily fish intake, age and BMI determined plasma LC *n-3* PUFA status; *APOE Epsilon* genotype interacted with -219G/T polymorphism to determine plasma apoE concentrations; -219G/T * *APOE Epsilon* haplotype determined plasma P-sel and VCAM-1 concentrations but not their response to treatment; and *APOE Epsilon* genotype determined plasma VCAM-1 response to treatment. Furthermore, there was no effect of treatment (i.e. fish oil supplementation) on plasma inflammatory marker concentrations, although habitual oily fish intake was a determinant of plasma IL-10 concentrations at baseline.

Determinants of plasma fatty acid status

In summary, we showed that habitual oily fish intake, age and BMI are determinants of plasma LC *n-3* PUFA status. As expected habitual oily fish intake increased plasma LC *n-3* PUFA concentrations. Plasma LC *n-3* PUFA concentrations also increased with age but only in women and not in men following full adjustment, with individuals aged ≥ 50 years having a higher plasma LC *n-3* PUFA status compared to individuals aged < 50 years. Obese subjects (BMI ≥ 30 kg/m^2) had lower plasma LC *n-3* PUFA status compared to subjects with a normal weight (BMI < 25 kg/m^2).

In published FINGEN findings, Caslake *et al*. reported a greater increase in LC *n-3* PUFA concentrations in the plasma PC lipid fraction upon fish oil supplementation in women compared to men, but observed no effect of age on the LC *n-3* PUFA responsiveness to supplementation [Caslake et al., 2008]. Our findings are supported by other studies reporting an effect of age and gender on the EPA and DHA concentration in plasma lipid fractions [Childs et al., 2008, Crowe et al., 2008, Tavendale et al., 1992, Dewailly et al., 2001A and 2001B, Babin et al., 1999, Vandal et al., 2008, Walker et al., 2013], red blood cells [Sands et al., 2011, Ogura et al., 2010, Walker et al., 2013] and AT [Bolton-Smith et al., 1997, Childs et al., 2008, Ogura et al., 2010, Walker et al., 2013]. The strength of the current FINGEN analysis is the prospective recruitment by gender and age approach, allowing a meaningful comparison across the decades of life (20 – 70 yr) in groups matched for gender. This provides considerable power to examine the impact of gender and age independently and interactively. Several studies have shown that women have higher DHA levels compared to men [Crowe et al., 2008, Bakewell et al., 2006, Garneau et al., 2012]. The impact of age on

the LC *n-3* PUFA response to supplementation appears to be dependent on the plasma lipid fraction, cells or tissues studied. A recent comprehensive study investigating the impact of age and gender on the incorporation of EPA and DHA in plasma PC, cholesterol esters (CE), NEFA, TG, mononuclear cells, red blood cells, platelets, buccal cells and subcutaneous AT in a double-blind randomised, controlled intervention trial providing fish oil supplements corresponding to 0, 1, 2, or 4 portions of oily fish per week showed that the effect of age and gender is dependent on the plasma lipid fraction, cell type or tissue studied [Walker et al., 2013]. For example, they reported increasing EPA and DHA concentrations in plasma TG and AT with increasing age, with significantly higher DHA levels in AT of old (60 – 79 yr) compared to middle aged (40 – 59 yr) and young (30 – 39 yr) individuals. Moreover, women had higher baseline DHA concentrations in AT compared to men, with no difference in EPA concentrations. Regarding the response to supplementation, the increase in EPA levels in plasma TG was higher with increasing age, whereas the opposite was true for the increase in DHA levels in AT. However, no age or gender differences were observed in the plasma PC fraction, although the increase in EPA concentrations in response to supplementation in the plasma PC fraction tended to be larger in old compared to young individuals. In conclusion, these and other results clearly indicate a role for age and gender in determining EPA and DHA concentrations and their response to supplementation in plasma lipids, cells and AT.

The gender related effects on LC *n-3* PUFA, in particular EPA and DHA, concentrations and their incorporation into plasma lipids, cells and AT have been suggested to be attributed to the sex hormones [Walker et al., 2013, Crowe et al., 2008]. Studies using stable isotopes have shown that women are more efficient than men in converting α-linolenic acid (ALA) into EPA and DHA [Pawlowsky et al., 2001, Burdge et al., 2002A and B] and the female sex hormones are responsible for this [Childs et al., 2008]. The increased conversion may account for the higher habitual LC *n-3* PUFA status in women. However, it should be mentioned that any difference in fatty acid metabolism, including their synthesis, interconversion and degradation may be responsible for differences in LC *n-3* PUFA status [Arterburn et al., 2006].

There was a large jump in plasma EPA concentrations in women between the age of 40 – 49 yr and 50 – 59 year in our study. This finding is the opposite of what one would expect given that the female sex hormones play an important role in the biosynthesis of EPA from ALA and postmenopausal women suffer from lower oestrogen levels. It suggests a difference in partitioning of fatty acids in postmenopausal women with less stored for potential pregnancy and more found in plasma lipid pools. The higher plasma LC *n-3* PUFA concentrations with increasing age in women in our study may be reflective of, as previously reported, a higher LC *n-3* PUFA concentration in AT with age, especially in women. This is reasonable given that the total body fat mass increases with age and ALA and EPA are preferably mobilised from AT relative to the other *n-3* PUFA [Puglisi et al., 2011, Childs et al., 2008]. ALA is the most abundant *n-3* PUFA in AT with a concentration of approximately

1 % of total fatty acids, whereas EPA and DHA are present in only very small amounts (< 0.3 % of total fatty acids) which increase upon dietary fish oil supplementation [Arterburn et al., 2006, Puglisi et al., 2011, Clifton et al., 2004, Andersen et al., 1999]. The age-related increase in plasma and tissue LC *n-3* PUFA status is relevant based on the current opinion that low-grade inflammation is underlying various age-related diseases, including MetS, T2DM and CVD [Osborn and Olefsky, 2012, Maury and Brichard, 2010, Van Gaal et al., 2006]. In addition, the lower plasma EPA and DHA levels in overweight subjects and MUO, but not MHO, compared to lean healthy subjects suggests a lower whole-body availability of these LC *n-3* PUFA that might also persist at tissue levels in overweight and MUO individuals [Fernandez-Real et al., 2003, Perrault et al., 2014]. In line with this we also observed a lower LC *n-3* PUFA concentration in the plasma PC lipid fraction in obese subjects (BMI ≥ 30 kg/m^2) compared to subjects with normal weight (BMI < 25 kg/m^2), suggesting depletion of circulating LC *n-3* PUFA in obesity. These findings suggest a potential clinical relevance of LC *n-3* PUFA in maintaining AT function and metabolic health [Puglisi et al., 2011]. Thus the increased plasma LC *n-3* PUFA concentration in older individuals could be a natural response to hold down the age-related inflammatory disease progression.

Plasma phospholipid (PL) fatty acid profile reflects dietary intake and endogenous fatty acid metabolism. Thus, the increasing levels of LC *n-3* PUFA in older subjects and decreased LC *n-3* PUFA levels in obese subjects could reflect a differential fat intake and/or altered fatty acid metabolism. A low fat diet has been shown to alter fatty acid pattern in plasma phospholipid fatty acids and cholesteryl esters in a manner similar to that observed with feeding of LC *n-3* PUFA [Raatz et al., 2001]. Besides, when the relative supply of *n-3* fatty acids is more abundant, *n-3* fatty acid are preferentially desaturated and elongated relative to *n-6* fatty acids [Holman, 1998]. Hence, the increased LC *n-3* PUFA levels in older subjects may reflect increased endogenous synthesis which may result from an relative abundance of *n-3* fatty acids and/or low fat intake. On the other hand, the lower LC *n-3* PUFA levels in obese subject may relate to a high absolute fat and/or n-6 fatty acid intake.

We found no significant effect of *APOE Epsilon* genotype on the fatty acid composition of the PC lipid fraction - the most abundant plasma PL fraction - compared to scarce other human studies which determined the fatty acid composition of plasma TG [Plourde et al., 2009] and plasma total lipids [Chouinard-Watkins et al., 2013]. Thus, it is possible that the plasma lipid fraction, i.e. TG, PL, CE and total lipids, will influence the impact of *APOE Epsilon* genotype on circulating fatty acid status in humans.

In this study we corrected for habitual oily fish intake hence our reported age-dependent effects on plasma EPA and DHA concentrations were independent of dietary intake. In line with this, several studies have reported higher EPA and DHA levels in older individuals independent of a differential dietary intake [Bolton-Smith et al., 1997, Crowe et al., 2008]. These findings all together clearly indicate a role for age and gender in determining EPA and DHA concentrations and their response to supplementation in plasma lipids, cells and

adipose tissue, which appears to be independent of dietary intake [Walker et al., 2013, Bolton-Smith et al., 1997, Crowe et al., 2008].

Determinants of plasma inflammatory marker concentrations

To our knowledge, this study was the first to investigate the impact of the -219G/T * *APOE Epsilon* haplotype on plasma inflammatory marker concentrations. We showed that the -219G/T * *APOE Epsilon* haplotype determined baseline plasma P-sel and VCAM-1 concentrations, with *APOE Epsilon* genotype generally being a stronger determinant than the -219G/T genotype. The plasma P-sel concentrations tended to be consistently higher in those with *APOE3* genotype compared to *APOE2* and *APOE4* genotype across the -219G/T genotypes, with a trend for decreasing plasma P-sel concentrations with an increasing number of -219T alleles in those with *APOE3* genotype. Plasma VCAM-1 levels tended to be higher in *APOE2* and *APOE4* carriers compared to those with *APOE3* genotype, with no differences across the -219G/T genotypes.

-219G/T * APOE Epsilon haplotype

In line with previous reports, we also observed an association between the -219G allele and the *APOE2* genotype in the FINGEN study [Lambert et al., 2002, Lescai et al., 2011, Wang et al., 2000]. The MAF for -219G/T (rs405509) in the FINGEN study was T = 0.50 and corresponded to the reported MAF of T = 0.494 [Ensemble Genome Browser, NCBI dbSNP database]. The -219G/T polymorphism in the *APOE* promoter region has been shown to modulate the *APOE* expression levels both *in vitro* and *in vivo*, where the -219T allele decreases *APOE* transcriptional activity and plasma apoE levels compared to the other allelic variant [Artiga et al., 1998, Lambert et al., 2000, Moreno et al., 2003]. In agreement with previous findings [Moreno et al., 2003, Lambert et al., 2000], we also observed a dose-dependent decrease in plasma apoE concentrations with increasing number of -219T alleles in *APOE2* carriers and those with the *APOE3* genotype, but not in *APOE4* carriers for which the opposite effect was evident. This *APOE Epsilon* genotype-dependent effect contradicts with a previous report from the ECTIM-study, in which there was a similar effect of -219G to T substitution on plasma apoE concentrations after stratification for *APOE Epsilon* genotype [Lambert et al., 2000]. At this moment, we do not have a mechanistic explanation for the *APOE Epsilon* genotype-dependent effect of the -219T allele on plasma apoE concentrations. A SNP located very close to the promoter of a gene can change the expression of that gene or a more distant gene. The most common mechanism through which the SNP modulates gene expression is by altering the binding of proteins in the regulatory gene region, although it could also alter CpG sites affecting methylation of the region or alter binding of non-coding RNA (ncRNA). The altered binding of proteins in the regulatory gene region can lead to a loss or gain of enhancer or repressor transcription factor binding, nucleosome repositioning and/or altered long-range chromatin interactions which will affect gene expression. The -219G/T polymorphism may affect the binding of proteins in the regulatory gene region of *APOE* which subsequently alters *APOE* gene

expression levels and potentially plasma apoE concentrations. The apoE isoforms differ in their stability, apoE2 is most stable, apoE3 intermediate stable and apoE4 least stable [Hatters et al., 2006], and in their protein turnover and recycling [Heeren et al., 2004, Huang et al., 2011, Rellin et al., 2008]. Hence the -219G/T polymorphism may interact with the apoE isoforms to determine its effects on plasma apoE concentrations.

Although findings are somewhat inconsistent, studies suggest an impact of the -219G/T polymorphism and a potential interaction with *APOE Epsilon* genotype to determine disease risk [Bizzaro et al., 2009, Lambert et al., 2000, Lambert et al., 2002 Lescai et al., 2011, Heijmans et al., 2002], something that warrants further investigation especially given that most studies have not specifically looked at -219G/T * *APOE Epsilon* haplotypes. For example, Lambert *et al*. showed that -419A/T (rs449647) and -219G/T (rs405509) polymorphisms in the *APOE* promoter region interact with *APOE Epsilon* genotype to determine risk of dementia in elderly, the 491A/−219T/*APOE4* haplotype was associated with increased risk of dementia (OR = 3.5, 95 % CI 2.5 – 5.0) whereas the −491A/−219G/*APOE3* haplotype was associated with a decreased risk (OR = 0.6, 95 % CI 0.5 – 0.9) [Lambert et al., 2004]. To our knowledge, we are the first to report an effect of -219G/T * *APOE Epsilon* haplotype on plasma inflammatory marker concentrations in a study population comprising of healthy individuals without disease, with a normal weight (BMI < 25 kg/m^2, 50 %) and mainly non-smokers (88 %). The -219G/T * *APOE Epsilon* haplotype determined plasma P-sel concentrations, which tended to be consistently higher in those with *APOE3* genotype compared to *APOE2* and *APOE4* carriers across the -219G/T genotypes. In addition, there was a trend for decreasing plasma P-sel concentrations with an increasing number of -219T alleles in those with *APOE3* genotype. P-sel is expressed by endothelium and platelets upon activation by inflammatory mediators (such as thrombin and TNF-α) [Hartwell et al., 1998] where it mediates leukocyte rolling and recruitment, and its plasma concentrations are increased in people with thrombotic disorders, ischemic heart disease, and obesity [Blann and Lip, 1997, Ferri et al., 1999]. Therefore, -219G/T * *APOE Epsilon* haplotype may impact on plasma P-sel levels through modulation of the inflammatory response and/or platelet activation.

Here, we also report a significant effect of -219G/T * *APOE Epsilon* haplotype on plasma VCAM-1 concentrations. However, only non-significant differences in the order of higher levels in *APOE2* and *APOE4* carriers compared to those with *APOE3* genotype and no differences across the -219G/T genotypes were observed. The differences between the *APOE Epsilon* genotypes were published by Kofler *et al*. [Kofler et al., 2012] and are in line with previous reports that apoE protein suppresses VCAM-1 expression in a genotype-dependent manner (E2 > E3 > E4) in endothelial cells [Stannard et al., 2001]. VCAM-1 is an important protein involved leukocyte rolling and transmigration that is upregulated during endothelial activation, although its soluble levels may not necessary represent cell-surface levels [Videm and Albrigtsen, 2008]. Thus, our findings suggests a potential differential

endothelial activation according to -219G/T * *APOE Epsilon* haplotypes, something that warrants further investigation.

In summary, we demonstrated for the first time that the *APOE Epsilon* genotype and -219G/T * *APOE Epsilon* haplotype, but not the -219G/T genotype, were a determinant of the plasma P-sel and VCAM-1 concentrations, indicating that *APOE Epsilon* genotype rather than -219G/T genotype is a strong determinant of plasma P-sel and VCAM-1 concentrations. These findings were unlikely to be mediated through plasma LC *n-3* PUFA status as there was no independent or interactive effect of the -219G/T and *APOE Epsilon* genotype on plasma LC *n-3* PUFA concentrations. However, plasma apoE concentrations were affected by the -219G/T * *APOE Epsilon* haplotype suggesting that *APOE* transcriptional activity may interact with *APOE Epsilon* genotype to determine its effects disease risk. All together, the current data suggests that the impact of *APOE Epsilon* genotype may depend on other factors, such as SNPs in *APOE* gene region, BMI and gender, and illustrates the complex interplay of factors in the development of physiological conditions, such as a pro-inflammatory state. Furthermore, this data highlights the importance of research in specific homogenous populations in order to be able to determine genotype-phenotype associations.

Plasma inflammatory marker responsiveness to treatment

A low and modest dose fish oil intervention (0.7 and 1.8 g EPA+DHA / d) had no effect on any of the plasma inflammatory marker concentrations in the FINGEN study. These findings are in line with the inconsistent and often neutral effects of LC *n-3* PUFA on plasma inflammatory markers in healthy individuals in marine *n-3* PUFA intervention trials [Calder, 2006, Calder et al., 2011A, Myhrstad et al., 2011A, Yang et al., 2012]. On the contrary, a large review on the dietary factors and low-grade inflammation in relation to overweight and obesity reviewing marine *n-3* PUFA intervention studies showed that LC *n-3* PUFA can lower circulating levels of CRP, IL-6, TNF-α, IL-18, ICAM-1, VCAM-1 and E-sel in various study populations [Calder et al., 2011A]. In addition, a meta-analysis analysing sixty-eight RCTs showed that LC *n-3* PUFA lower circulating CRP, IL-6 and TNF-α levels in subjects with chronic non-autoimmune disease and healthy subjects [Li et al., 2014]. There was a negative linear relationship between the daily dose of EPA and the effect size on circulating CRP levels in healthy subjects, suggesting that a higher dose of EPA results in a greater CRP lowering effect. The null-findings in this study are most likely resulting from the relatively low physiological doses of EPA and DHA used, since most studies reporting anti-inflammatory effect of EPA and DHA have used a high dose (≥ 3.0 g EPA+DHA / d) [Calder 2006, Myhrstad et al., 2011A, Yang et al., 2012, Calder et al., 2011A] which could only realistically be achieved through fish oil supplementation rather than diet. A meta-analysis analysing seven studies reported that EPA and DHA decrease plasma ICAM-1 levels (5 ng/ml, 95 % CI -10.07 – -0.27) regardless of dose in young (≤ 55 yr), but not older (> 55 yr) subjects [Yang et al., 2012]. The failure to observe an effect on plasma ICAM-1

concentrations in our study could therefore be in part due to the wide age of our study population. However, we did report a positive effect of habitual oily fish intake on plasma IL-10 concentrations, suggesting that there may be other compounds in oily fish that exert beneficial effects on certain plasma inflammatory marker concentrations.

A strength of the current FINGEN data analysis is the prospective recruitment according to *APOE Epsilon* genotype (*E2*, *E3* and *E4*) (*E2/E4* carriers were excluded), age and gender. This provided us significant power to evaluate the effects of *APOE Epsilon* genotype, age and gender on plasma LC *n-3* PUFA status and plasma inflammatory marker concentrations independently and interactively. The equal distribution of the -219G/T alleles (50 % / 50 %) in the FINGEN study population also allowed a meaningful comparison across the -219G/T genotypes. The FINGEN study was not specifically designed to evaluate the effect of *APOE Epsilon* genotype and its interaction with adiposity (BMI) to determine plasma LC *n-3* PUFA status and plasma inflammatory marker concentrations and their response to LC *n-3* PUFA supplementation. However, the study protocol had a broad BMI inclusion range of 18.5 to 32.0 kg/m^2, which resulted in a study population with a large range of BMI and fairly equal number of normal weight (< 25 kg/m^2, n = 155) and overweight individuals (25 – 29.9 kg/m^2, n = 128) with some obese individuals (≥ 30 kg/m^2, n = 29) also included. This provided us significant power to evaluate a potential interaction of *APOE Epsilon* genotype and body weight. We also investigated the plasma LC *n-3* PUFA status and plasma inflammatory marker concentrations response to LC *n-3* PUFA supplementation. The FINGEN study was a double-blind placebo controlled cross-over trial, which is considered the gold standard to investigate the effect of nutritional compounds on health outcomes in humans. There is no potential bias from not-blinded volunteers and/or researchers or the placebo-effect. Moreover, in cross-over trials subjects receive a sequence of different treatments in a random order and subjects serve as their own controls. This increased our power to detect a true effect with a similar number of volunteers, as the response in nutritional studies is often considerable different from individual to individual (inter-individual variation).

A limitation of this study is that we assessed circulating fatty acid status and inflammatory markers as biomarkers of whole body fatty acid and inflammatory status, which is routinely conducted. The plasma PL fraction is the only valid marker of short term availability for EPA and one of the valid markers for DHA [Schuchardt and Hahn, 2013]. Thus, we used the correct plasma lipid fraction to study circulating EPA and DHA availability. Although there is generally a good correlation between circulating levels and whole-body status, circulating levels may not fully reflect tissue levels in all instances [Minihane et al., under review]. A strength of our study is that we used a range of soluble markers of inflammation, including acute phase proteins, cytokines and adhesion molecules, which are associated with inflammation in metabolic diseases [Calder et al., 2013]. This provided a more complete measure of inflammation than a single biomarker. There is a considerable between-

individual variation and a number of modifiers influence the concentrations of the soluble inflammatory markers, including age, gender, adiposity, genetics, diet, smoking, physical (in)activity, gut microbiota and use of certain medication [Calder et al., 2013]. In our study, we corrected for many of these modifiers including age, gender, adiposity (BMI) and smoking, which makes our analysis investigating the effect of -219G/T and *APOE Epsilon* genotype on plasma inflammatory markers concentrations more robust. It is uncertain what contribution is made by various organs and tissues to the circulating inflammatory profile. Hence the circulating profile, in addition to being non-tissue specific, may over- or under-represent the inflammatory state of individual organs. This is therefore recognised as a limitation of this study.

An exclusion criteria for the FINGEN was a habitual oily fish intake of > 1 portion per week as determined by the FFQ. Our analysis showed that the average intake was 0.9 and 1.1 portions per week in men and women. Therefore either volunteers were not completely honest at screening or when completing the FFQ, where they likely exaggerated their fish intake given the average fish intake in the UK is 0.33 portions per week. The latter can also explain the high upper level of oily fish intake in the highest quintile of habitual oily fish intake, which was 7.7 portions per week. The high habitual oily fish intake at baseline might have influenced the outcomes on various levels. First habitual oily fish intake could have influenced EPA and DHA status in the plasma PC fraction, as plasma phospholipids and red blood cell EPA and DHA concentrations reflect respectively short-term and long-term dietary intake [Risé et al., 2007]. The plasma phospholipid EPA concentrations increase in a linear dose-dependent manner in response to supplementation reaching a new steady state within one month of supplementation [Arterburn et al., 2006]. The plasma phospholipid DHA concentrations increase in a dose-dependent saturable manner in response to supplementation, with DHA concentration approaching saturation at dose of approximately 2 mg/d. The kinetics of red blood cell EPA and DHA concentrations follow a similar pattern to plasma phospholipids, but reach a steady state after 4 to 6 months of supplementation [Arterburn et al., 2006]. Thus, individuals with a high habitual oily fish intake would have higher plasma phosphatidylcholine and red blood cell EPA and DHA concentrations. Besides, fish oil supplementation in combination with a high oily fish intake could have resulted in saturation of plasma phospholipid DHA concentrations. Several *in vitro* studies suggested DHA has more potent anti-inflammatory effects than EPA in macrophages [Mullen et al., 2010, Oliver et al., 2012, Weldon et al., 2007]. Therefore saturation of plasma phospholipid DHA concentrations may explain the limited changes in plasma inflammatory marker concentrations from baseline.

The proportion of smokers was not similar for the -219G/T genotypes. Smoking induces oxidative stress and inflammation [van der Vaart et al., 2004] and this might have possibly confounded our results.

We have used the ratio of plasma oxidized LDL-C to plasma apolipoprotein B (apoB) concentrations as a biomarker of oxidative status to investigate if the decreased LC *n-3* PUFA concentrations in obese subjects could be attributed to an increased non-enzymatic oxidation of these fatty acids. However, this measure gives us no information about whether there is an effect of BMI on the sequestering of these fatty acid into mitochondrial and/or peroxisomal (enzymatic) fatty acid oxidation.

In conclusion, we demonstrated that the -219G/T * *APOE Epsilon* haplotype is a determinant of plasma apoE concentrations and select plasma inflammatory marker concentrations, without affecting plasma LC *n-3* PUFA concentrations in the PC lipid fraction or plasma inflammatory marker response to LC *n-3* PUFA supplementation. In line with previous findings, our data support an interaction between SNPs in the *APOE* promoter region and *APOE Epsilon* genotype to determine plasma apoE concentrations and potentially disease risk markers.

Chapter 3. *In vivo* studies – diet-induced obesity and metabolic function: The interactive impact of *APOE Epsilon* genotype and fish oil on diet-induced obesity and whole-body glucose handling.

Studies in human APOE3 and APOE4 targeted replacement mice

Introduction

Adipose tissue and metabolic disease

Obesity is associated with metabolic complications, such as metabolic syndrome (MetS) and insulin resistance (IR), which in turn increase the risk of developing Type 2 Diabetes (T2DM) and cardiovascular disease (CVD) [Maury and Brichard, 2010, Van Gaal et al., 2006]. Although the link between obesity and metabolic abnormalities has been well established, approximately one-third of obese people, termed metabolically healthy obese (MHO), have a normal metabolic profile characterised by a low visceral fat (VAT) content, high high-density lipoprotein-cholesterol (HDL-C) levels, low triglyceride (TG) levels and high insulin sensitivity, despite their high fat mass [Wildman et al., 2008, Karelis et al., 2004]. In addition, adipose tissue (AT) inflammation, in which macrophages play a pivotal role, is an important determinant of the metabolic consequences of obesity. For example, Barbarroja *et al.* showed that MHO insulin sensitive individuals (mean BMI 56 ± 1 (SD) kg/m^2) lacked the inflammatory response in VAT that characterised the metabolically unhealthy obese (MUO) insulin resistant individuals [Barbarroja et al., 2010]. Finally, the expansion capacity of the AT has been postulated as the underlying mechanism connecting obesity to IR, which is referred to as the adipose tissue expandability hypothesis [Virtue and Vidal-Puig, 2010]. According to this hypothesis, each individual has a defined maximum AT expansion capacity which is determined by genetic and environmental factors, and as an individual gains weight it eventually reaches its maximum AT expansion capacity. After this point, the AT cannot store TG anymore and excess lipids start to accumulate in other tissues such as liver, muscle and pancreas. This ectopic lipid deposition causes toxic effects in these tissues leading to IR, inflammation and cardiovascular complications. Thus, an impaired AT expansion capacity, excess VAT and macrophage-mediated inflammation in VAT are important determinants of the metabolic consequences of obesity [Virtue and Vidal-Puig, 2010, Karelis et al., 2004, Barbarroja et al., 2010].

Apolipoprotein E and adipose tissue metabolism

There is accumulating evidence indicating that apolipoprotein E (apoE) plays a role in AT development, the development of obesity and its related metabolic complications [Kypreos et al., 2009, Slim & Minihane, 2014]. Zechner *et al*. were the first to show that apoE expression increases linearly with time upon differentiation and correlated with lipid content and free intracellular cholesterol content in 3T3-L1 adipocytes [Zechner et al., 1991]. In line with this, Huang *et al*. showed that endogenous apoE expression is required for lipid accumulation and apoE deficiency decreased the expression of genes involved in adipocyte differentiation in primary adipocytes [Huang et al., 2006]. In addition, freshly isolated adipocytes from apoE$^{-/-}$ mice exhibit an impaired fatty acid uptake, decreased TG synthesis and increased TG lipolysis and apoE$^{-/-}$ mice have an impaired plasma TG clearance, less body fat and smaller adipocytes, yet an improved glucose tolerance compared to wild type mice [Huang et al., 2006, Hofmann et al., 2008]. These studies clearly indicate a role for apoE in AT development, including adipocyte differentiation and adipocyte lipid metabolism.

Secondly, the very low-density lipoprotein (VLDL)-derived TG-rich lipoproteins, which contain apoE, play an important role in AT development by being the main source of fatty acids for AT in both the fasting and fed state [Voshol et al., 2009]. Although VLDL plays an important role in AT development, it could also be argued that AT development plays a crucial role in VLDL metabolism and the clearance of TG-rich VLDL particles from the blood stream. Lastly, the apoE-recognising receptors low-density lipoprotein receptor (LDLR), VLDL receptor (VLDLR) and LDL-related protein 1 (LRP1) have been implicated in AT development [Karagiannides et al., 2008, Goudriaan et al., 2001, Hofmann et al., 2007]. These observations suggest a potential link between apoE, lipoprotein metabolism and the development of obesity, which was first demonstrated by Chiba *et al.* who showed that the VLDL-induced adipogenesis was apoE-dependent [Chiba et al., 2003]. In more detail, apoE-deficient VLDL failed to induce differentiation of apoE$^{-/-}$ bone marrow stromal cells into adipocytes, which could be restored by incubation of apoE-deficient VLDL with apoE. In addition, apoE-deficient *ob*/*ob* mice were protected from high fat diet (HFD)-induced obesity despite their higher plasma VLDL levels [Chiba et al., 2003]. These findings clearly indicate an interactive role of apoE and lipoprotein metabolism in AT development and diet-induced obesity (DIO).

Finally, limited studies investigating the association between *APOE Epsilon* genotype and adiposity in humans have provided accumulating evidence supporting a link between *APOE Epsilon* genotype, adiposity and the metabolic consequences of obesity [Sima et al., 2007, Kolovou et al., 2009, Elosua et al., 2003]. To start, various studies reported a higher prevalence of the *APOE4* allele in obese people, although there is some inconsistency [Sima et al., 2007, Kolovou et al., 2009, Elosua et al., 2003, Volcik et al., 2006, Oh and Barrett-Connor, 2001]. In addition, few studies reported an interaction between *APOE Epsilon* genotype and BMI to determine metabolic and/or inflammatory parameters [Kofler et al., 2012, Marques-Vidal et al., 2003, Pouliot et al., 1990]. For example, Marques-Vidal *et al*.

showed that *APOE Epsilon* genotype interacted with BMI to determine plasma total cholesterol, LDL-cholesterol (LDL-C), total insulin and bioactive insulin in 266 healthy men without hypolipidemic or antidiabetic drug treatment, with *APOE4* carriers having higher levels compared to other *APOE Epsilon* genotypes and the difference enlarging with increasing BMI [Marques-Vidal et al., 2003]. These findings suggest that *APOE Epsilon* genotype may influence adiposity and *APOE Epsilon* genotype may interact with adiposity (BMI) to determine the metabolic consequences of increased adiposity.

APOE Epsilon genotype and metabolic function in obesity

The capacity of AT to buffer excess nutrients and macrophage-mediated AT inflammation are important determinants for maintaining adipocyte function and a metabolic healthy profile in obesity [Virtue and Vidal-Puig, 2010, Barbarroja et al., 2010, Osborn and Olefsky, 2012]. Recent studies in human *APOE3* and *APOE4* targeted replacement (TR) mice have shown that the *APOE4* allele is associated with an impaired AT expandability and a greater predisposition to the metabolic complications of obesity [Arbones-Mainar et al., 2008 and 2010]. In more detail, *APOE4* TR mice had an impaired expandability of the epididymal VAT depot and a greater epididymal adipocyte size, an impaired insulin sensitivity, and an impaired *ex vivo* adipocyte differentiation and lipid storage capacity. *APOE4* TR mice also showed a greater predisposition to develop glucose intolerance upon HFD feeding compared to *APOE3* TR mice. Besides, the *APOE4* genotype has been associated with pro-inflammatory state [Jofre-Monseny et al., 2008A], with AT inflammation being an important determinant of AT dysfunction [Osborn and Olefsky, 2012]. In conclusion, these findings indicate that *APOE Epsilon* genotype impacts on AT function and the metabolic complications of obesity.

Long chain *n-3* PUFA and adipose tissue function

Long chain (LC) *n-3* polyunstaurated fatty acids (PUFA) exert various beneficial effects on AT function that include reducing AT expansion, AT inflammation and improving fatty acid oxidation (Table 3.1) [Flachs et al., 2009]. The ability of LC *n-3* PUFA to reduce the HFD-induced increase in AT mass is dependent on the genetic background of mice [Puglisi 2011]. However, the beneficial effects on systemic IR, dyslipidemia, hepatic steatosis, AT inflammation and IR are independent of changes in AT mass [Puglisi et al., 2011]. The effects of LC *n-3* PUFA in humans are less convincing with a modest beneficial or neutral effect on body weight and body composition in combination with caloric restriction or exercise (Table 1.5, Chapter 1) [Bender et al., 2014, Lorente-Cebrián et al., 2013] and no effect on glycemic control and insulin sensitivity (Table 1.7, Chapter 1) [Akinkuolie et al., 2011, Jafar et al., 2013]. Besides, LC *n-3* PUFA have anti-inflammatory effects and reduced AT inflammation in various strains of mice [Todoric et al., 2006, Saraswathi et al., 2007, Muurling et al., 2003, Oh et al., 2010]. Therefore, LC *n-3* PUFA may provide a potential therapeutic molecule to reverse the unfavourable adipocyte phenotype and hypothesized AT inflammation associated with the *APOE4* allele.

Table 3.1. The effects of long chain *n-3* PUFA on adipose tissue function [Flachs et al., 2009, Puglisi et al., 2011, Moreno-Aliaga et al., 2010].

Adipocyte function	Effect
Adipogenesis	Inhibit adipocyte proliferation and differentiation Induce apoptosis post-confluent pre-adipocytes Reduce adipocyte hypertrophy and hyperplasia
Lipid metabolism	Increase mitochondrial biosynthesis and oxidative capacity Increase β-oxidation Improve triglyceride storage and reduce lipolysis
Adipokine secretion	Increase adiponectin expression
Inflammation and oxidative stress	Decrease inflammatory cytokine production Decrease oxidative stress

Research gaps

Our understanding of how genetic determinants interact with nutrition and other environmental factors to cause metabolic disease is still poor, and the identification of protective and susceptibility genes is crucial to help us, among others, explain why not all people with obesity become insulin resistant [Osborn and Olefsky, 2012]. The AT expandability hypothesis proposes that an inadequate AT expansion capacity, rather than obesity itself, is the key feature linking a positive energy balance to development of T2DM [Virtue and Vidal-Puig, 2010]. In addition, macrophage-mediated AT inflammation causing AT dysfunction is recognised as the underlying pathology causing systemic IR and systemic inflammation in obesity [Osborn and Olefsky, 2012]. Although the AT expansion capacity and AT inflammation can be linked, more knowledge is needed about the factors that determine both AT expansion capacity and macrophage-mediated AT inflammation.

Previous research from our group and others have demonstrated that the *APOE4* allele is associated with an impaired AT expandability and greater predisposition to the metabolic complications of obesity *in vivo* [Arbones-Mainar et al., 2008 and 2010] and a pro-inflammatory macrophage phenotype *in vitro* [Jofre-Monseny et al., 2007A]. LC *n-3* PUFA reduce DIO, improve AT function, decrease AT inflammation and improve insulin sensitivity [Flachs et al., 2009, Puglisi et al., 2011, Lottenberg et al., 2012], providing a potential strategy to reverse the adipocyte dysfunction and metabolic complications associated with the *APOE4* allele. However, the interactive impact of *APOE Epsilon* genotype and fish oil fatty acids on DIO and metabolic function, and the ability of fish oil to reverse the AT dysfunction associated with the *APOE4* allele has never been investigated.

Research question

The aim of this study was to investigate the independent and interactive impact of *APOE Epsilon* genotype and fish oil on the development of obesity and whole-body glucose tolerance in human *APOE3* and *APOE4* TR mice.

Hypotheses

Previous research has demonstrated that the *APOE4* allele is associated with an impaired AT expandability and greater predisposition to the metabolic complications of obesity (i.e. IR and ectopic lipid deposition in the liver). In addition, the *APOE4* allele has been associated with pro-inflammatory state and AT inflammation is an important determinant of AT dysfunction. AT dysfunction is in turn thought to be the driving force behind the systemic IR and low-grade inflammation in obesity. Therefore, we hypothesise that *APOE4* TR mice will gain less weight yet have a greater AT dysfunction and poorer whole-body glucose tolerance upon HFD feeding compared to *APOE3* TR mice. LC *n-3* PUFA eisopentaenoic acid (EPA) and docosahexaenoic (DHA) have beneficial effects on adipocyte function besides their anti-inflammatory effects. Therefore, we hypothesise that dietary fish oil will reduce the AT inflammation and resolve the adipocyte dysfunction associated with the *APOE4* allele in mice upon HFD feeding, thereby improving whole-body glucose tolerance.

Materials & Methods

A mouse model humanised for the human *APOE3* and *APOE4* gene was used to investigate the independent and interactive impact of *APOE Epsilon* genotype and fish oil on the development of obesity and its metabolic complications. Studies in human *APOE3* and *APOE4* TR mice allowed us, to use an experimental design tailored specifically to answer our research question; control for many confounding intrinsic and environmental factors difficult to control for in human studies, such as genetic background and diet; and collect AT biopsies and perform a thorough investigation into the role of apoE in AT development. The specific outcome measures of interest concerning the development of obesity were body weight gain, food intake and food efficiency. Metabolic function was investigated by performing detailed analysis of plasma biochemistry; estimating whole-body IR using the homeostatic model assessment-insulin resistance (HOMA-IR) index; and performing an intra-peritoneal glucose tolerance test (IPGTT) to measure whole-body glucose tolerance. The expression levels of key genes involved in AT development and insulin sensitivity were analysed to obtain further insight into potential underlying mechanisms.

Animals & treatment

This animal experiment was conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under license from the Home Office. All efforts were made to reduce the number of animals and severity of the experiment by following the 3R's. A power calculation was employed to minimise the number of animals without losing statistical power, with 10 mice per genotype * diet group enabling us to detect a standardised mean difference (SMD) of 1.45 with power of 90 % at significance level of $\alpha = 0.05$. The statistical power was calculated using the SMD for a two sample comparison of means with null hypothesis $\mu1 = \mu2$ (Figure 3.1), as no data was available from previous studies in human *APOE3* and *APOE4* TR mice regarding our main outcome of interest, i.e. AT macrophage infiltration and phenotype. Our experimental methods, in particular procedures on living animals, were designed and performed to minimise pain, suffering and distress - that may be suffered by the mice - without losing out on the quality of the data, for example by using appropriate anaesthetics.

Figure 3.1. Statistical power and sample size calculation.

The formula for calculation of the statistical power and sample size

$$SMD = (\mu_1 - \mu_2) / \delta = \sqrt{((2 * (Z_{\alpha/2} + Z_{\beta})^2) / n)}$$

$$Z_{\alpha/2} = Z_{0.025} = 1.960;\ Z_{\beta} = Z_{0.10} = 1.282$$

The standardised mean difference (SMD) for a two sample comparison of means (μ) with a given standard deviation ($\delta = \delta_1 = \delta_2$) and balanced design ($n = n_1 = n_2$) can be calculated by dividing the mean difference ($\mu_1 - \mu_2$) by standard deviation of the means (δ), or by taking the square root of the square of the sum of the Z-value corresponding to the 2-sided significance level $\alpha = 0.05$ ($Z_{\alpha/2}$) and Z-value for a given power of 90%, $\beta = 1 - 0.90 = 0.10$ (Z_{β}) multiplied by two and divided by sample size (n).

Mice

Twenty 5 – 6 week old male human *APOE3* (HomozygousB6.129P2-*Apoe*$^{tm2(APOE*3)Mae}$ N8, Model #1548-M) and twenty 6 – 8 week old male human *APOE4* (HomozygousB6.129P2-*Apoe*$^{tm2(APOE*4)Mae}$ N8, Model #1549-M) TR mice were purchased from Taconic (Germantown, NY, USA). The *APOE3* and *APOE4* TR mice are homozygous for the human *APOE3* or *APOE4* gene by targeted replacement of the endogenous mouse *Apoe* gene. The expression of the human apoE3 or apoE4 isoform is under the control of the murine *Apoe* regulatory sequence and thus apoE3 and apoE4 are produced at physiological levels [Sullivan et al., 1997]. The human *APOE3* TR mice show a similar plasma lipid profile to wild type mice on a standard low fat diet despite their 2-fold lower VLDL remnant clearance rate, but are more susceptible to HFD-induced hypercholesterolemia and atherosclerosis compared to wild type mice [Sullivan et al., 1997]. Compared to the human *APOE3* TR mice, human *APOE4* TR mice in turn have a 2-fold lower VLDL remnant clearance rate but similar VLDL secretion rate, and a greater susceptibility to HFD-induced atherosclerosis [Knouff et al., 1999]. Male mice were used to reduce potential variation arising from the female oestrous cycle.

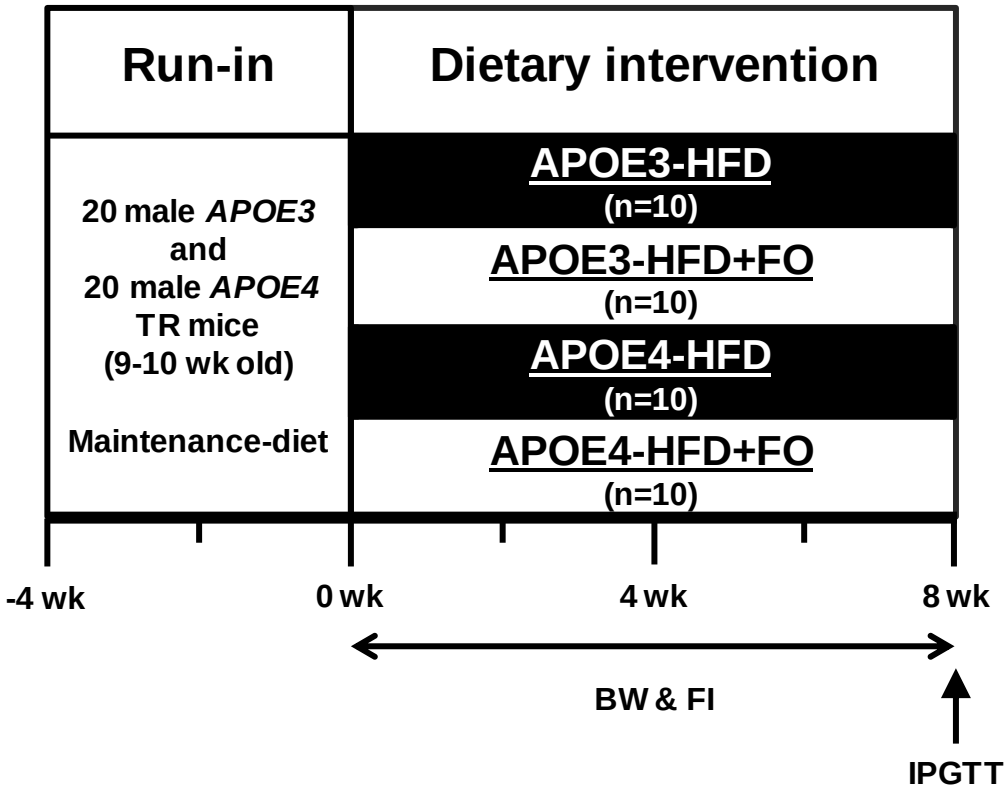


Figure 3.2. Time line of studies in human *APOE3* and *APOE4* targeted replacement (TR) mice. Forty male human *APOE3* and *APOE4* TR mice were purchased and allowed to acclimatise for 4 weeks prior to dietary intervention (Run-in period). At age of 13 – 14 week, *APOE3* and *APOE4* mice were randomly allocated to a high-fat diet (HFD; 45 kcal% from fat) without or with long chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (30 g EPA+DHA / kg of diet with a ratio of 2:3; HFD+FO) for 8 weeks (n = 10 mice per *APOE Epsilon* genotype * diet group). Food and water was provided *ad libitum.* Body weight (BW) and food intake (FI) were monitored every second day. At 8 weeks, whole-body glucose tolerance was assessed using an intra-peritoneal glucose tolerance test (IPGTT). APOE, apolipoprotein E.

Mice were allowed to acclimatise for 4 weeks before the commencement of the dietary intervention (Figure 3.2). During the run-in period mice were fed a palletised maintenance diet (Rat and Mouse No.3 Breeding Expanded diet (RM3); SDS Diets, Essex, UK). The mice were housed in groups of five per cage with two cages per *APOE Epsilon* genotype * diet group, allowed free access to food and water unless stated otherwise, and maintained under a 12 h light – 12 h dark cycle (7 am – 7 pm; lights on 7:00) in a temperature-controlled environment (21 °C, humidity 55 %) in a pathogen-free facility at the University of East Anglia. Mice were identified by ear tagging with miniature lightweight ear tags (Kent Scientific Corporation, Torrington, USA).

Dietary intervention

At the age of 13 – 14 weeks, the *APOE3* and *APOE4* TR mice were randomly allocated to a palletised high-fat diet (45 % calories (kcal%) from fat, 0.02 g / 100 g cholesterol; D07011903, Research Diets, New Brunswick, NJ, USA) without (HFD) or with long chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (30 g EPA+DHA / kg of diet; D12111601, Research Diets) (HFD+FO) for 8 weeks (n = 10 mice per *APOE Epsilon* genotype * diet group). Older mice (13 – 14 weeks) were used rather than younger mice (6 – 8 weeks), because obesity is a stronger trigger of metabolic complications at an older age. The diet compositions are given in Table 3.2. Briefly, D07011903 (HFD) was modified from standard D12451 (Research Diets) to contain no sucrose and D12111601 (HFD+FO) was modified from D07011903 to contain 12 g EPA and 18 g DHA per kg of diet in a ratio of 2:3, by replacing fat from lard with a blend of Menhaden fish oil (In-house Research diets) and DHASCO oil (a generous gift from DSM Nutritional Products, Colombia, USA). The HFD+FO diet would provide an obese mouse (40 g) with a food intake of 5 g/d daily with 60 mg EPA + 90 mg DHA, which corresponds to a dose of 3750 mg EPA+DHA / kg of body weight / d.

The composition of the HFD was designed to specifically induce obesity ('fattening') without triggering diabetes or IR. In particular, sucrose levels of the diets were kept low, because they are not only known to induce IR in mice, but also counteract the anti-inflammatory effect of fish oil in AT [Ma et al., 2011]. The standard D12451 containing 45 kcal% from fat has been shown to induce DIO in wild type C57BL/6 mice [Duffaut et al., 2009] and HFD feeding for 8 to 10 weeks was sufficient to induce considerable macrophage influx into AT (average AT macrophage turnover is 8 weeks) in mice [Lumeng et al., 2007A]. Furthermore, replacement of dietary fat with 27 % Menhaden fish oil (wt/wt; Menhaden oil: 16 % EPA and 9 % DHA) resulting in a daily dosage of 3750 mg EPA + DHA / kg of body weight per mouse has been shown to reduce AT inflammation - an important determinant of metabolic complications in obesity [Osborn and Olefsky, 2012] - in wild type C57BL/6 mice on HFD (60 kcal% from fat) [Oh et al., 2010]. The interactive impact of *APOE Epsilon* genotype and fish oil on AT inflammation is discussed in Chapter 4.

Table 3.2. Dietary components and fatty acid composition of the experimental diets.

	High-fat control diet (HFD)		High-fat fish oil diet (HFD+FO)	
Macronutrients	g / 100 g	kcal%	g / 100 g	kcal%
Protein	24	20	24	20
Carbohydrate	41	35	41	35
Fat	24	45	24	45
Total		100		100
kcal/g	4.73		4.73	
Ingredient	g/kg		g/kg	
Casein	200		200	
L-cysteine	3		3	
Corn starch	246		246	
Maltodextrin	100		100	
Sucrose	0		0	
Cellulose	50		50	
Soybean oil	25		25	
Lard	178		85	
Menhaden oil	0		72	
DHASCO oil	0		20	
Mineral mix	10		10	
Dicalcium phosphate	13		13	
Calcium Carbonate	6		6	
Potassium Citrate	17		17	
Vitamin mix	10		10	
Choline bitartrate	2		2	
LC *n-3* PUFA				
EPA (g / kg of diet)	0		18	
DHA (g / kg of diet)	0		12	
EPA:DHA ratio	0		0.67	
Fatty acid composition				
% of total fatty acids				
14:0	1.1		5.0*	
16:0	21.0		19.2*	
16:1 n-7	1.5		5.9*	
17:0	0.4		1.1*	
18:0	11.4		8.2*	
18:1 n-9	34.7		23.7*	
18:2 n-6	14.2		16.7*	
18:3 n-6	ND		0.1	
18:3 n-3	1.6		2.0*	
18:4 n-3	ND		0.9*	
20:0	0.2		1.0	
20:1	0.6		0.6	
20:2	0.7		0.4*	
20:4 n-6	0.2		0.6*	
20:5 n-3 (EPA)	ND		4.0*	
22:5 n-3	ND		0.1*	
22:6 n-3 (DHA)	ND		6.0*	
24:1	ND		0.8*	

*Fatty acid content (% of total fatty acids) of HFD+FO is different from HFD, Student's *t*-test $p < 0.05$ ($n = 3$). 14:0, myristic acid; 16:0, palmitic acid; 16:1 n-7, palmitoleic acid; 17:0, margaric acid; 18:0, stearic acid; 18:1 n-9, oleic acid; 18:2 n-6, linoleic acid; 18:3 n-6, gamma-linolenic acid; 18:3 n-3, alpha-linolenic acid; 18:4 n-3, stearidonic acid; 20:0, icosanoioc acid; 20:1, gondoic acid; 20:4 n-6, arachidonic acid; 20:5 n-3, eicosapentaenoic acid (EPA); 22:5 n-3, docosapentaenoic acid; 22:6 n-3, docosahexaenoic acid (DHA); 24:1, nervonic acid; HFD, high-fat diet; HFD+FO, high fat diet containing 30 g EPA+DHA per kg of diet; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acids; ND, not detected.

Diet composition

The HFD contained 45 kcal% from fat which resembles the current average UK diet that contains approximately 35 kcal% from fat [Great Britain NDNS Report, 2011]. The major source of fat was lard which is rich in saturated fat (SFA, 44 %) and the SFA:MUFA:PUFA ratio (40:40:10 g / 100 g food) [McCance & Widdowson's The Composition of Foods Integrated Dataset, 2002] corresponds to the average dietary SFA:MUFA:PUFA ratio of the UK population (30:30:15 g/d), i.e. 40 % SFA [Great Britain NDNS Report, 2011]. The EPA:DHA ratio of the HFD+FO diet resembled the EPA:DHA ratio of oily fish, which is the major dietary source of EPA and DHA, and thereby corresponds to the EPA:DHA ratio of the average UK diet [Welch et al., 2010]. The EPA and DHA content of the HFD+FO resulted in an animal dosage of 3750 mg EPA+DHA / kg of body weigt / day which corresponds – based on allometric scaling and body surface (BSA)-based calculation – to a human equivalent dose of 36 g EPA+DHA / d in a normal weight adult (70 kg) (Figure 3.3) [U.S. Department of Health and Human Services, 2005]. This is a supra-physiological dose corresponding to 12 portions of oily fish per day and 12-fold the physiological daily intake achievable with fish oil supplements (3 g EPA+DHA / d). Also, it is beyond physiological tolerable levels previously approached in human intervention trials (≤ 5.6 – 8.4 g EPA+DHA / d) (for review see Calder *et al*. [Calder et al., 2006 and 2011A]). However, this is the first study investigating the impact of fish oil on AT inflammation according to *APOE Epsilon* genotype and a wide array of previous mouse studies investigating the impact of LC *n-3* PUFA on a range of health outcomes and specifically on AT inflammation in a DIO model have used comparable animal dosages in the range of 1763 mg / kg of body weight to 3750 mg / kg of body weight [Oh et al., 2010, Todoric et al., 2006, Saraswathi et al., 2007]. Thus, to prevent null findings resulting from too low dosages, a higher dosage was chosen based on previous studies reporting beneficial effects of fish oil on AT inflammation.

Figure 3.3. Conversion of the Animal Dose (AD) to the Human Equivalent Dose (HED) [U.S. Department of Health and Human Services, 2005].

The formula for conversion of the Animal Dose (AD) to the Human Equivalent Dose (HED), as proposed by the FDA

$$HED\ (mg / kg) = AD\ (mg/kg) * ([W_{animal}\ (kg) / W_{human}\ (kg)]^{0.33})$$

The AD is translated to the HED using a conversion factor. This conversion factor is calculated by raising the division product of the species body weights (W) to the power of 0.33. The 0.33 value is result of 1 – b value, where b is the allometric exponent of 0.67 that scales the bodyweight well with the body surface area [U.S. Department of Health and Human Services, 2005].

The fatty acid composition of the experimental diets is presented in Table 3.2. Diets contained sufficient levels of tert-Butylhydroquinone (tBHQ) and vitamin E to protect the fatty acids from oxidation. The feed pellets were colour marked, packaged per 1 kg, and stored at -80 °C in the dark. Every week a new package was used and stored at 4 °C in the dark.

Feed and water were provided *ad libitum*, unless stated otherwise, and fresh food was provided every 2nd / 3rd day. The fatty acid composition of the experimental diets did not significantly change over three days at room temperature (Appendix 3-1). Body weight and food intake were monitored every 2nd / 3rd day. The dietary intervention started and finished two weeks earlier for the *APOE4* TR mice compared to the *APOE3* TR mice, because the *APOE4* mice were two weeks older upon arrival.

Sample and tissue collection

Following eight weeks of dietary intervention, an IPGTT was performed over two subsequent mornings, testing in a random order ten mice (two cages housing each five mice) per day. Forty-eight hours after the IPGTT, mice were randomly (per cage of five mice) sacrificed by exsanguination during three subsequent mornings. Blood was collected by cardiac puncture under isoflurane anaesthesia in Lithium-Heparin coated microtubes (Sarstedt, Leicester, UK), coagulated for 30 minutes at room temperature, centrifuged for 10 min at 2,000 *g* at 20 °C, after which plasma was collected, frozen on dry-ice and stored at -80 °C. Mice were perfused with 30 – 40 ml ice-cold PBS containing heparin (10 units/ml; Sigma-Aldrich, Dorset, UK) before removing the other tissues and organs. One epididymal AT was collected in DMEM (4500 mg/L glucose; Sigma-Aldrich, Dorset, UK) and placed in an incubator (37 °C, 5% CO_2 - 95% O_2) for isolation of the stromal vascular fraction (see Chapter 4). The second epididymal AT was snap frozen on dry-ice, and stored at -80 °C for RNA isolation and analysis of the fatty acid composition (see Chapter 4). The epididymal gonadal fat depot was chosen for analysis, because it is the largest and anatomically easiest to collect VAT depot, although VAT comprises also of mesenteric and retroperitoneal AT. Throughout this chapter, epididymal AT will be referred to as VAT.

Whole body insulin-resistance

The whole-body IR was estimated using the HOMA-IR index, using the fasting plasma glucose and insulin concentrations and formula “glucose (mmol/L) x fasting insulin (mU/L) / 22.5” [Matthews et al., 1985].

Intra-peritoneal glucose tolerance test

Prior to testing, mice were fasted 10 – 16 hr overnight that is suitable for studies focussing on glucose utilisation [Ayala et al., 2010]. An IPGTT was performed with intra-peritoneal (IP) injection of a fixed glucose load based on a glucose dose of 2 g/kg of body weight [Ayala et al., 2010] and the average body weight of 40 g that provided 8 mg *D*-glucose in saline solution. A fixed glucose load was preferred above the more commonly used glucose dose based on the individual weight of the mice to prevent that the glucose load would be biased by the increased fat mass in obese mice as lean mass is the principal site for glucose disposal [Ayala et al., 2010]. Animals were denied food during the course of the test. Water was provided *ad libitum*. A drop of blood was collected using tail snip blood sampling, which is considered an appropriate method for blood sampling during GTT [Ayala et al., 2010], for

determination of glucose using the AlphaTRAK 2 glucometer calibrated for mice (Abbott Laboratories Ltd., Maidenhead, UK) at time points 0, 10, 20, 30, 60 and 120 min. Whole-blood glucose levels obtained with the AlphaTRAK 2 glucometer correlate relatively well with plasma glucose levels determined with enzymatic assays that are considered the medium of choice for glucose assessment [Ayala et al., 2010]. An additional 30 µl blood sample was collected at time points 0, 15 and 120 min into EDTA-coated microvette tubes (Sarstedt), centrifuged for 10 min at 2,000 *g* at 20 °C to collect plasma, which was stored at -80 °C for determination of insulin levels.

Biochemical analysis

Plasma total cholesterol (TC), HDL-C, TG and glucose concentrations were measured using commercial IL Test™ assays (Instrumentation Laboratory UK Ltd., Warrington, UK). Plasma non-esterified fatty acids (NEFA) levels were measured using the commercial colorimetric assay (Randox Laboratories Ltd., Crumlin, UK). Liver function was investigated by analysing the plasma concentrations of enzymes commonly included in a liver function test which give an indication of liver inflammation and damage [British Liver Trust, 2007], namely alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) using commercial IL Test™ assays (Instrumentation Laboratory UK Ltd.). The plasma levels of ALT and AST, two aminotransferases enzymes present in hepatocytes which leak into the bloodstream when hepatocytes are damaged, can be increased up to 20 – 50 times in liver inflammation (hepatitis). ALT is more specific to the liver, whereas increased AST levels can also be an indication of muscle damage. ALP is an enzyme found in liver bile duct and increased plasma levels can indicate obstructive or cholestatic liver disease. All analyses were performed according to manufacturers' instructions using the ILab-650 analyser (Instrumentation Laboratory UK Ltd.). The ILab-650 analyser was calibrated against specific calibrators with a Coefficient of Variation (CV) < 5 %. Quality control samples (QCs) were included to validate each assay. Plasma non-HDL-C levels, consisting of LDL-C plus VLDL-C, were calculated by subtracting HDL-C from TC. The plasma LDL-C levels cannot be estimated from TC, HDL-C and TG using the Friedewald formula [Friedewald et al., 1972], because the use of this formula is not valid in mice [Emeson et al., 1995].

Plasma samples (5 µl) were analysed in duplicate for insulin using a commercial ELISA kit (Millipore Ltd., Livingston, UK) and BMG Omega plate reader (BMG Labtech, Aylesbury, UK) according to manufacturers' instructions (intra and inter assay CV%; 11.3 and 21.0 %).

Plasma interleukin-10 (IL-10) was analysed in singlet (5-fold dilution) using commercial Mouse IL-10 ELISA Ready-Set-Go (2nd Generation) kit (eBiosciences, Hatfield, UK) and BMG Omega plate reader (BMG Labtech) according to manufacturers' instructions. Thirty-eight out of the 40 samples had values below the sensitivity of the assay (32 pg/ml), indicating that the 5-fold dilution factor was too high. There was not enough sample to rerun the analysis for IL-10. Therefore, the data of 38 samples was extrapolated using the 2nd polynomial regression fit standard curve obtained from the serial dilution of the top standard

(4000 pg/ml) on each plate. Future experiments are recommended to use a 2-fold dilution factor for the samples to fall within the detection range (32 – 4000 pg/ml) of the assay. Four samples (one from each *APOE Epsilon* genotype*diet group) were analysed in duplicate to determine the within assay CV of 28.4 %.

Plasma adiponectin and leptin levels were analysed in singlet (2000-fold and no dilution factor) using commercial ProcartaPlex™ Simplex Immunoassays (eBiosciences) according to manufacturers' instructions using Luminex 200 System (Luminex, Austin, United States). Six samples were analysed in duplicate to determine the within assay CV of < 10 %. The STartStation 3.0 software (Applied Cytometry, Dinnington, UK) was used to analyse the data.

RNA isolation

Total RNA was isolated from epididymal AT using Ambion® TRIzol reagents (Life Technologies, Paisley, UK) followed by Chloroform extraction according to the manufacturers' instructions. Briefly, 150 – 400 mg of tissue was homogenised in 1 ml TRIzol with one pre-cooled stainless steel bead in a pre-cooled tube for 5 min at 50 Hz using the TissueLyser LT (Qiagen, Manchester, UK). After centrifugation at 12,000 *g* for 10 min at 4 °C, the fat layer was removed and the supernatant was collected, mixed with 0.2 ml Phenol-Chloroform-Isoamyl alcohol 25:24:1 (Life Technologies) and incubated for 2 – 3 min at room temperature. The RNA-containing aqueous phase was collected after centrifugation at 12,000 *g* for 15 min at 4 °C. Total RNA was precipitated with 0.5 ml 100 % isopropanol (Fisher Scientific, Loughborough, UK), washed with 1 ml 75 % ethanol, and resuspended in nuclease free water (Promega UK Ltd, Southampton, UK) and incubated at 60 °C for 10 min. RNA was cleaned-up using an overnight ethanol precipitation with 0.1 volumes 3 M sodium acetate (pH 5.2) (Fisher Scientific) and 2.2 volumes 100 % ethanol (Sigma-Aldrich) at -20 °C, centrifugation at 12,000 *g* for 10 min at 4 °C, 70 % ethanol wash and centrifugation for 5 min, and air-dried before resuspension in RNase-free water. The RNA concentration and purity were measured using the NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA USA).

mRNA expression analysis by real-time quantitative PCR

Real-time quantitative PCR (RT-QPCR) was performed on individual samples (n = 10 per group). One µg of total RNA was treated with DNase I (Thermo Scientific Fermantas) and used for cDNA synthesis using Invitrogen™ Oligo (dT) primers and SuperScript® II Reverse Transcriptase (Life Technologies) according to manufacturers' instructions. RT-QPCR reactions were performed with Precission qPCR Mastermix (PrimerDesign Ltd., Southampton, UK) using SYBR green detection technology and the Applied Biosystems 7500 Real-Time PCR system (Life Technologies). Each reaction (20 µl) contained 10 µl Precission qPCR Mastermix, 1 µl forward primer (6 µM), 1 µl reverse primer (6 µM), 3 µl RNase-free water and 5 µl cDNA (10-fold dilution). The following cycles were performed: 1x

10 min 95 °C, 40 amplification cycles (40x 15 sec 95 °C, 1 min 60 °C), followed by a continuous melting curve (15 sec 95 °C, 1 min 60 °C, 30 sec +Δ 1 % up to 95 °C, 15 sec 60 °C). A negative control without cDNA template (NTC) and a negative control without reverse transcriptase (-RT) were taken along with every assay. Data was normalized against the reference genes *actin, beta* (*Actb*), *ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit* (*Atp5b*) and *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) which were chosen for their most stable gene expression levels between mice (Appendix 3-2). The reference gene expression stability and the optimal reference genes were established by screening 3 – 4 samples per group for the expression levels of *Actb*, *Gapdh*, *Rpl13a*, *18S*, *Eif4a2*, and *Atp5B* using the 6-gene geNorm SYBR green detection kit (PrimerDesign Ltd.) with the above described reaction conditions. The primers for *apolipoprotein E* (*Apoe*) were designed using primer-BLAST (www.blast.ncbi.nlm.nih.gov/Blast). Primers for *solute carrier family 2 (facilitated glucose transporter) member 4* (*Slc2a4*, *Glut4*) were designed by PrimerDesign (PrimerDesign Ltd.). Primer sequences are presented in Table 3.3. A standard curve was generated using serial dilutions of a pooled sample (pooled cDNA from all experimental samples) to determine reactions' amplification efficiency and sensitivity. Samples with a non-exponential amplification, indicating poor reaction efficiency, were omitted from analysis. Samples with cycle threshold (C_T) values below the sensitivity limit of the assay but 5 or more C_T's greater than the NTC for that assay (indication of target-specific amplification), were assigned the C_T value corresponding to the detection limit of that assay, in order to minimise the bias resulting from the exclusion of these samples on the end results (Appendix 3-2, Table A3-2.1). The mRNA expression levels were calculated using the ΔΔCT method and gene-specific amplification efficiencies [Hellemans et al., 2007] with the qbase+ software (Biogazelle, Zwijnaarde, Belgium). All mRNA expression levels are presented as relative quantity scaled to the average across all samples per target gene and normalised to the geometric mean of the reference genes *Actb*, *Atp5b* and *Gapdh*.

Table 3.3. Primers used for mRNA expression analysis using RT-QPCR.

Gene	Primer Sequence	Product size
Apoe	F: 5'- TGCGTTGCTGGTCACATTCC -3' R: 5'- CAGGTAATCCCAAAAGCGACC -3'	146
Glut4 (Slc2a4)	F: 5'- CCAGTATGTTGCGGATGCTAT -3' R: 5'- TTTTAGGAAGGTGAAGATGAAGAAG -3'	88

Apoe, apolipoprotein E; Glut4, solute carrier family 2 (facilitated glucose transporter), member 4 (Slc2a4).

Statistical analysis

The independent and interactive impact of *APOE Epsilon* genotype and diet on body weight and food intake was determined using repeated-measures Analysis of Variance (ANOVA)

with body weight or food intake at time point 0 – 8 weeks as dependent variables, time (Week (9 levels)) as a within-subject variable, genotype (G) and diet (D) (main and interaction effect) as independent between-subject variables, and was considered statistically significant when $p < 0.05$. The independent and interactive impact of *APOE Epsilon* genotype and diet on other outcome measures was analysed using 2 x 2 factorial univariate ANOVA with G, D and G*D as independent variables and considered statistically significant when $p < 0.05$. In the presence of a significant *APOE Epsilon* genotype * diet (G*D) interaction effect, post-hoc analysis was performed using the two-tailed Student's *t*-test with Bonferroni correction to analyse differences between the *APOE Epsilon* genotype * diet groups. Differences were considered significant at $p < 0.008$ (= 0.05 / 6). Statistical analysis of the mRNA expression levels in AT was done using log transformed values. Data with a non-normal distribution and/or unequal variances was analysed using a non-parametric Kruskal-Wallis test and was considered statistically significant when $p < 0.05$. Post-hoc Mann-Whitney U test with Bonferroni correction was employed to test for differences between the *APOE Epsilon* genotype * diet groups and was considered significant at $p < 0.008$ (= 0.05 / 6). Various outcome measures had not-normally distributed data and/or unequal variances which was not improved and often worsened by log (^{10}log or ^{e}log), square root ($\sqrt{x}$) or inverse ($1/x$) transformation, and as a consequence for many outcome measures non-parametric testing had to be employed. In the results section, the statistical test employed will be clearly indicated in the tables and figures. All statistical analysis was performed using SPSS 18.0.

Results

Development of diet-induced obesity

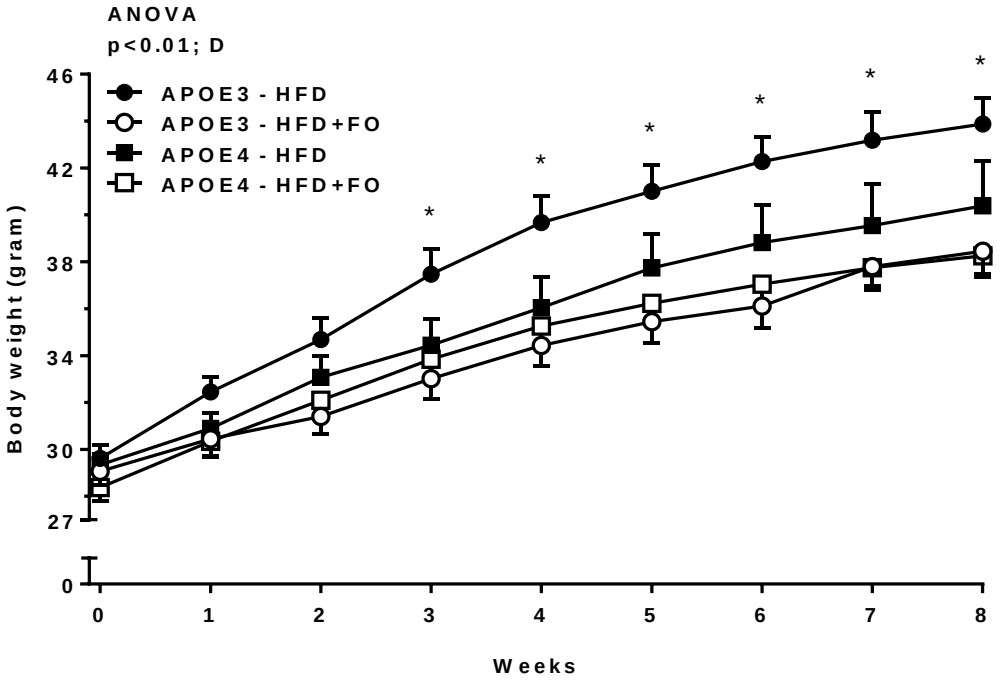


Figure 3.4. The effect of *APOE Epsilon* genotype and diet on the development of diet-induced obesity in human *APOE3* and *APOE4* targeted replacement mice fed a high-fat diet (HFD) or HFD containing 30 g EPA+DHA / kg of diet (HFD+FO). Data is presented as mean ± SEM, n = 10 mice per group. Statistical analysis was done by ANCOVA to test for genotype (G), diet (D) and G*D interaction effect; between-animal analysis revealed a significant effect of diet ($F_{1,36}$ = 7.805, p<0.01) on body weight, with no effect of *APOE Epsilon* genotype ($F_{1,36}$ = 1.781, p = 0.19) or *APOE Epsilon* genotype * diet interaction ($F_{1,36}$ = 2.819, p = 0.10). Group comparisons at each time point were done by Student's *t*-test with Bonferroni correction; *APOE3-HFD is different from APOE3-HFD+FO and APOE4-HFD+FO, p < 0.05. APOE, apolipoprotein E; HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA / kg of diet.

At the start of the dietary intervention, the mice (age 13 – 14 weeks) had a similar body weight of 29.0 gram (range 25.0 – 32.1 g). Between-animal analysis revealed there was a significant effect of diet ($F_{1,36}$ = 7.805, p < 0.01), but not of *APOE Epsilon* genotype ($F_{1,36}$ = 1.781, p = 0.19), on bodyweight at the end of the dietary intervention (week 8), which did not interact with genotype ($F_{1,36}$ = 2.819, p = 0.10). Group comparison revealed that from 3 weeks onwards, *APOE3* mice on HFD had a significantly higher body weight than *APOE3* and *APOE4* mice on HFD+FO (36.6 ± 0.5 (SEM) vs. average of 32.8 ± 0.4 g, p<0.05; Figure 3.4). This difference in body weight persisted over the course of the dietary intervention, with *APOE3* mice on HFD reaching a final body weight of 43.9 ± 1.1 g compared to *APOE4* mice on HFD+FO (the leanest group) reaching only 38.3 ± 0.8 g. There was an overall trend for a difference in final body weight between *APOE3* and *APOE4* mice fed the HFD (43.9 ± 1.1 vs. 40.4 ± 1.9 g, p = 0.36) though this did not reach significance. In summary, HFD feeding resulted in the development of DIO that was prevented by dietary fish oil in *APOE3* mice. Moreover, there was a strong trend for diet to interact with *APOE Epsilon* genotype to determine the development of DIO, with *APOE4* mice being more resistant to DIO.

The accumulation of especially VAT is associated with an increased risk for metabolic complications and disease in obesity. In line with the findings on body weight, there was an effect of diet on VAT weight ($F_{1,35}$ = 410.5, p < 0.05), but surprisingly in the opposite direction (Figure 3.5A). Mice on HFD+FO had a consistent higher VAT weight compared to the control HFD. In addition, there was an effect of *APOE Epsilon* genotype ($F_{1,35}$ = 1517, p < 0.05) with *APOE3* mice having a consistently higher VAT weight compared to *APOE4* mice. However, the effect of diet and *APOE Epsilon* genotype on VAT weight disappeared after correcting for body weight (diet $F_{1,35}$ = 21.709, p = 0.14 and *APOE Epsilon* genotype $F_{1,35}$ = 7.457, p = 0.22), which provides a more robust estimate of adiposity (Figure 3.5B). In summary, the *APOE3* genotype and dietary fish oil were associated with a greater VAT weight.

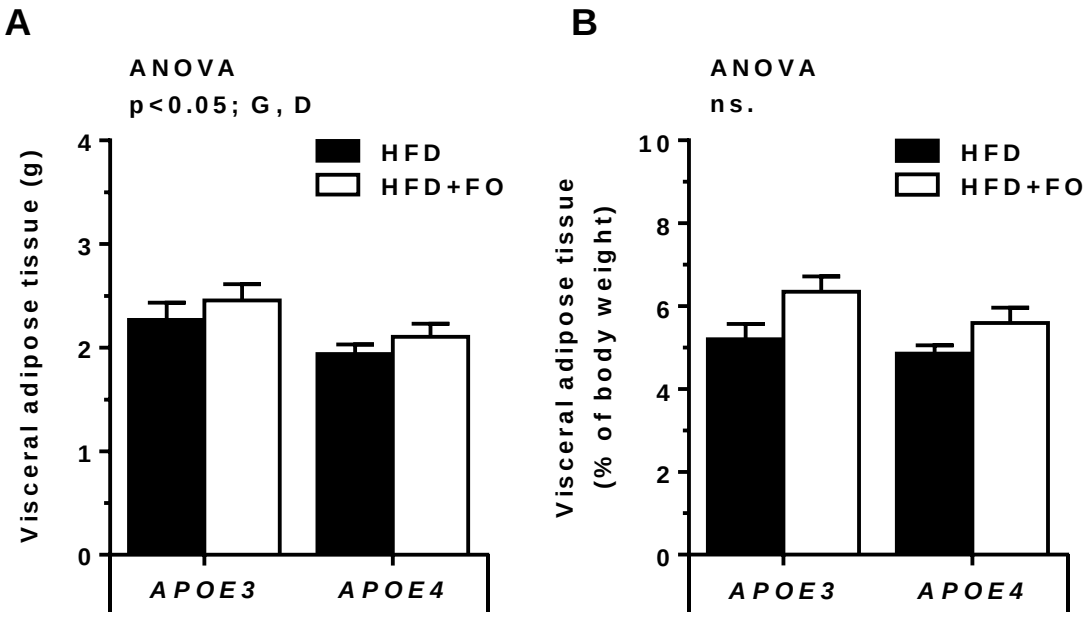


Figure 3.5. The effect of *APOE Epsilon* genotype and diet on epididymal visceral adipose tissue (VAT) in human *APOE3* and *APOE4* targeted replacement mice fed a high-fat diet (HFD) or HFD containing 30 g EPA+DHA / kg of diet (HFD+FO). Epididymal VAT weight in g [A] and % of body weight [B]. Data is presented as mean ± SEM, n = 10 mice per group. Statistical analysis was done by two-way ANOVA to test for genotype (G), diet (D) and G*D interaction effect. Group comparisons by Student's *t*-test with Bonferroni correction revealed no difference between groups. APOE, apolipoprotein E; HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA / kg of diet.

Given the important role apoE plays in AT development, including adipocyte differentiation and adipocyte lipid accumulation, the *Apoe* mRNA expression levels in VAT were investigated. The *Apoe* mRNA expression levels were significantly lower in VAT of *APOE3* mice on HFD compared to all other three groups (p < 0.001) (Figure 3.6).

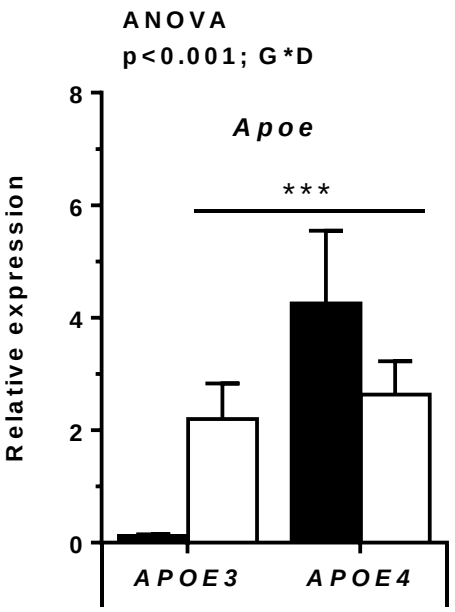


Figure 3.6. The relative mRNA expression of *apolipoprotein E* (*Apoe*) in the epididymal visceral adipose tissue (VAT) of human *APOE3* and *APOE4* targeted replacement mice fed a high-fat diet (HFD) (black bar) or a HFD containing 30 g EPA+DHA / kg of diet (HFD+FO) (white bars). *APOE Epsilon* genotype interacted with diet to determine *Apoe* expression levels ($p < 0.001$), with *APOE3* mice on HFD having significantly lower expression levels than the three other groups ($p < 0.001$). Data is presented as relative quantity normalised to the reference genes *Actb*, *Atp5B* and *Gapdh*, mean ± SEM, n = 9 – 10 mice per group. Statistical differences were tested using ANOVA for the effects of *APOE Epsilon* genotype (G), diet (D) and G*D interaction and considered statistically significant when $p < 0.05$. Only significant effects are displayed on top of the figure. Differences in the mRNA expression between the groups were tested using Student's *t*-test with Bonferonni correction, ***$p < 0.001$.

Food intake and food efficiency

To investigate if the difference in body weight could be related to a differential food intake, we calculated the average food intake and food efficiency (Figure 3.7). *APOE Epsilon* genotype interacted with diet to determine food intake ($p < 0.001$). In more detail, *APOE3* mice on HFD had a consistently higher average food intake compared to the other three groups (APOE3-HFD 3.2 ± 0.1 g / mouse / day vs. APOE3-HFD+FO 2.8 ± 0.0, APOE4-HFD 2.6 ± 0.1, APOE4-HFD+FO, 2.6 ± 0.1 g / mouse / day, $p < 0.05$) (Figure 3.7A), which was most pronounced in the first three weeks of HFD feeding (Figure A3-3.1, Appendix 3-3). In addition, the average food intake of *APOE3* mice on HFD+FO was higher compared to *APOE4* mice on a similar diet (2.8 ± 0.0 vs. 2.6 ± 0.1 g / mouse / day, $p < 0.01$). The overall food efficiency, calculated by dividing the body weight gain (g) by food intake (g), of the mice did not significantly differ between the groups, and on average mice gained 0.7 g of body weight for each gram of food consumed (Figure 3.7B), with no difference in the weekly food efficiencies either (Figure A3-3.2, Appendix 3-3). The higher average food intake, yet similar food efficiencies, of *APOE3* mice on HFD compared to *APOE3* mice on HFD+FO is in line with the greater body weight gain observed in these mice. Noteworthy, we observed anecdotally over the course of the dietary intervention that *APOE3* mice were generally more active than *APOE4* mice and hypothesize that perhaps *APOE3* mice had a higher daily energy expenditure compared to *APOE4* mice. This could explain the observed similar food efficiency and similar body weight of *APOE3* and *APOE4* mice on HFD+FO, despite the higher food intake of *APOE3* mice.

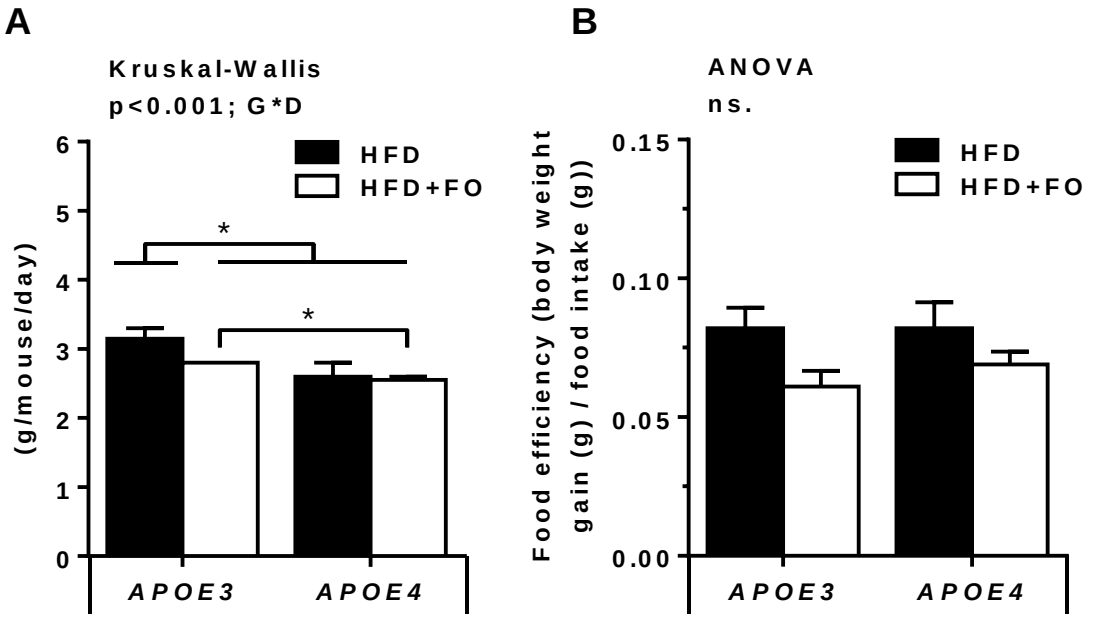


Figure 3.7. The average food intake [A] and food efficiency [B] of human *APOE3* and *APOE4* targeted replacement mice fed a high-fat diet (HFD) without or with 30 g EPA+DHA / kg of diet (HFD+FO). Data is presented as mean ± SEM, n = 10 mice per group. The average food intake (g/day) per mouse was calculated by dividing the joint food intake (g) of 5 mice in a single cage by 5 (accounting for the 5 mice) and then by 56 days. The average food efficiency was calculated by dividing the total body weight gain (g) by the total food intake (g). Statistical analysis was done by ANOVA for *APOE Epsilon* genotype (G), diet (D) and G*D interaction effect or by Kruskal-Wallis test for G*D effect and considered statistically significant when p < 0.05. Significant effects are displayed on top of the figure. Differences between the groups were tested using Mann-Whitney U test with Bonferroni correction, *p < 0.008 (= 0.05 / 6). APOE, apolipoprotein E; HFD, high-fat diet; HFD+FO, HFD containing 30g EPA+DHA/kg of diet; ns, not significant.

Plasma lipid profile

APOE Epsilon genotype interacted with diet to determine plasma TG ($F_{1,35}$ = 14.115, p < 0.01), total cholesterol (TC) ($F_{1,35}$ = 10.566, p < 0.01), non-HDL-C ($F_{1,34}$ = 17.422, p < 0.001), and non-esterified fatty acids (NEFA) ($F_{1,31}$ = 8.284, p < 0.01), but not plasma HDL-C (Table 3.4). Furthermore, there was a significant effect of diet on plasma glucose levels ($F_{1,35}$ = 260.041, p < 0.05), with a borderline non-significant effect of *APOE Epsilon* genotype ($F_{1,35}$ = 146.277, p = 0.05). In line with the differences in body weight, plasma TG levels were significantly higher in *APOE3* mice on HFD compared to the other three groups (p < 0.05). The plasma TG levels (71 – 128 mg/dL) were in general higher than previous reported values in *APOE3* and *APOE4* TR mice on a HFD (48 ± 4 (SD) and 66 ± 31 mg/dL respectively) [Knouff et al., 1999]. Plasma TC levels were higher in mice on HFD compared to those on HFD+FO (119 to 137 mg/dL vs. 91 to 100 mg/dL, p < 0.05), with *APOE3* mice on HFD having the highest levels also compared to *APOE4* mice on HFD (137 ± 3 vs. 119 ± 5 mg/dL, p < 0.05). The plasma TC levels in the mice on HFD (119 to 137 mg/dL) were in line with previous reports in *APOE3* and *APOE4* mice on a HFD (122 ± 43 (SD) and 130 ± 27 mg/dL respectively), whereas the plasma TC levels in the mice on HFD+FO (91 – 100

mg/dL) were more in the direction of plasma levels of *APOE3* and *APOE4* mice on a chow diet (75 ± 26 (SD) and 72 ± 27 mg/dL) [Knouff et al., 1999].

Table 3.4. The effect of *APOE Epsilon* genotype and diet on the plasma biochemistry in human *APOE3* and *APOE4* TR mice fed a high-fat diet (HFD) or HFD containing 30 g EPA+DHA / kg of diet (HFD+FO).

Plasma lipids	**APOE3-HFD**	**APOE3-HFD+FO**	**APOE4-HFD**	**APOE4-HFD+FO**	
TG (mg/dL)	128 ± 10^{a}	71 ± 6^{b}	81 ± 9^{b}	84 ± 5^{b}	ANOVA p<0.01; G*D
TC (mg/dL)	137 ± 3^{a}	91 ± 4^{bd}	119 ± 5^{c}	100 ± 5^{bd}	ANOVA p<0.01; G*D
HDL-C (mg/dL)	116 ± 3	99 ± 7	107 ± 6	82 ± 4	ANOVA ns.
non-HDL-C (mg/dL)	20 ± 2^{a}	10 ± 1^{b}	12 ± 1^{ab}	17 ± 2^{ab}	ANOVA p<0.001; G*D
NEFA (mmol/L)	1.7 ± 0.1^{ac}	0.7 ± 0.1^{b}	1.3 ± 0.2^{ac}	0.9 ± 0.1^{bc}	ANOVA p<0.01; G*D
Glucose (mg/dL)	327±16^{a}	276 ± 6^{ab}	290 ± 13^{a}	241 ± 9^{b}	ANOVA p<0.05; D
Liver enzymes					
ALT (IU/L)	49 ± 8^{a}	21 ± 1^{b}	31 ± 8^{ab}	17 ± 1^{b}	Kruskal-Wallis p<0.001; G*D
AST (IU/L)	106 ± 12	123 ± 21	105 ± 14	79 ± 7	ANOVA ns.
ALP (IU/L)	63 ± 4	49 ± 3	62 ± 5	62 ± 5	ANOVA ns.
Adipokines/ cytokines					
IL-10 (pg/ml)	301 ± 55	378 ± 43	278 ± 35	536 ± 138	ANOVA ns.
Adiponectin (µg/ml)	194 ± 22	183 ± 29	207 ± 22	214 ± 18	ANOVA ns.
Leptin (ng/ml)	6.7 ± 0.5	5.1 ± 0.5	5.5 ± 0.8	4.4 ± 0.4	ANOVA ns.

Values are mean ± SEM in non-fasted plasma samples of 7 – 10 animals per group. Statistical analysis was done by ANOVA for *APOE Epsilon* genotype (G), diet (D) and G*D interaction effect using un- or ^{10}log-transformed data, or by Kruskal-Wallis test for G*D effect and considered statistically significant when $p < 0.05$. Significant effects are indicated in the last column of the table. Differences between the groups were tested using Student's *t*-test or Mann-Whitney U test with Bonferroni correction; different letters indicate differences between the *APOE Epsilon* genotype*diet groups, $p < 0.008$ (= 0.05 / 6). ALP, alkaline phosphatase; ALT, alanine aminotransferase; APOE, apolipoprotein E; ASP, aspartate aminotransferase; HDL-C, high-density lipoprotein cholesterol; HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA / kg of diet; IL-10, interleukin 10; IU, international units; NEFA, non-esterified fatty acids; non-HDL-C, non-HDL-C consisting of low-density lipoprotein and very low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

Plasma HDL-C levels showed a similar trend as plasma TC levels, with higher circulating levels in *APOE3* and *APOE4* mice on HFD (116 ± 3 and 107 ± 6 mg/dL) and lower levels in *APOE3* on HFD+FO (99 ± 7 mg/dL) and lowest levels in *APOE4* mice on HFD+FO (82 ± 4 mg/dL). Plasma HDL-C levels were similar to previous reports of 74 to 107 mg/dL in *APOE3* and *APOE4* mice on a HFD [Knouff et al., 1999]. *APOE Epsilon* genotype interacted with diet to determine plasma non-HDL-C levels, which is the sum of LDL-C and VLDL-C. Briefly, HFD+FO feeding tended to decrease plasma non-HDL-C levels in *APOE3* mice but

increased plasma non-HDL-C levels in *APOE4* mice (Table 3.4). The plasma non-HDL-C levels were increased in *APOE3* mice on HFD compared to *APOE3* mice on HFD+FO (20 ± 2 vs. 10 ± 1 mg/dL, $p < 0.05$). The plasma NEFA levels tended to be lower in mice on HFD+FO compared to those on HFD, with *APOE3* mice on HFD having significantly higher NEFA levels than *APOE3* and *APOE4* mice on HFD+FO (1.7 ± 0.1 mmol/L vs. 0.7 ± 0.1 and 0.9 ± 0.1 mmol/L, $p < 0.05$) (Table 3.4). The non-fasted plasma glucose levels were 241 – 327 mg/dL and much higher than previous reported non-fasted glucose levels (160 – 175 mg/dL) in *APOE3* and *APOE4* TR mice fed a HFD [Arbones-Mainar et al., 2008]. *APOE3* and *APOE4* mice on HFD+FO had significantly lower plasma glucose levels compared to their counterparts on HFD (327 ± 16 vs. 276 ± 6 mg/dL and 290 ± 13 vs. 241 ± 9 mg/dL, $p < 0.05$). In addition, there was a non-significant trend for lower non-fasted glucose levels in *APOE4* mice compared to *APOE3* mice ($p = 0.05$).

Plasma liver enzymes

APOE Epsilon genotype interacted with diet to determine plasma ALT levels (Kruskal-Wallis $p < 0.001$), but not plasma AST and ALP (Table 3.5). Briefly, *APOE3* mice on HFD had increased plasma ALT levels compared to *APOE3* and *APOE4* mice on HFD+FO (49 ± 8 IU/L vs. 21 ± 1 and 17 ± 1 IU/L, $p < 0.05$). The plasma ALT levels in *APOE3* mice on HFD (49 IU/L) were modestly lower than reference values for C57BL/6 mice (50 – 89 IU/L), whereas the other groups had plasma levels (≤ 31 IU/L) far below this reference range. These results suggest that *APOE3* mice on HFD had a slightly higher degree of liver inflammation compared to the mice on HFD+FO, although values were still within the physiological range. Furthermore, the findings were not confirmed by plasma AST and ALP levels, which revealed no significant differences between the *APOE Epsilon* genotype * diet groups. Plasma AST levels were within normal range for C57BL/6 mice (46 – 392 IU/L) and plasma ALP levels were below reference values for C57BL/6 mice (111 – 275 IU/L).

Plasma adipokines and cytokines

Plasma adiponectin levels were on average 200 µg/ml and there was no significant effect of *APOE Epsilon* genotype ($F_{1,31} = 5.737$, $p = 0.25$) or diet ($F_{1,31} = 0.034$, $p = 0.88$) (Table 3.5). Plasma leptin levels ranged from 4.4 to 6.7 ng/ml. Although there was no significant main effect of *APOE Epsilon* genotype ($F_{1,35} = 0.176$, $p = 0.18$) or diet ($F_{1,35} = 0.125$, $p = 0.13$), *APOE4* mice on HFD+FO tended to have lower levels compared to *APOE3* mice on HFD (4.4 ± 0.4 ng/ml vs. 6.7 ± 0.5 ng/ml). These differences in plasma leptin levels corresponded to the difference in body weight (Table 3.4), with lower leptin levels in mice with a lower body weight. The plasma IL-10 levels were approximately 300 – 500 pg/ml. Overall there were no differences in plasma IL-10 levels between *APOE Epsilon* genotypes ($F_{1,36} = 12.295$, $p = 0.18$), although there was a trend for higher plasma IL-10 levels in *APOE3* and *APOE4* mice on HFD+FO diet ($F_{1,36} = 127.461$, $p = 0.06$).

Insulin resistance

To investigate the impact of *APOE Epsilon* genotype and diet on insulin sensitivity whole-body IR was estimated at the end of the 8 week dietary intervention using the HOMA-IR index. This revealed that *APOE3* mice on HFD developed IR compared to the *APOE3* and *APOE4* mice on HFD+FO (HOMA-IR 23.3 ± 4.5 vs. 4.6 ± 0.7 and 5.1 ± 0.8, $p < 0.001$) (Figure 3.8C) associated with higher fasting plasma insulin levels (1.17 ± 0.20 ng/ml vs. 0.30 ± 0.05 and 0.33 ± 0.04 ng/ml, $p < 0.01$) (Figure 3.8B). Fasting plasma glucose levels tended to be higher in *APOE3* mice on HFD, but this did not reach statistical significance (Figure 3.8A).

Given the important role insulin-regulated glucose transporter Glut4 plays in insulin-stimulated glucose uptake, the *Glut4* mRNA expression levels in epididymal VAT were investigated. *APOE Epsilon* genotype interacted with diet to determine *Glut4* expression levels ($F_{1,35} = 7.033$, $p < 0.05$) (Figure 3.8D). *APOE3* mice on HFD had the lowest *Glut4* expression levels in VAT, but HFD+FO feeding resulted in a 5-fold increase in *Glut4* expression in *APOE3* mice. The beneficial effect of fish oil on *Glut4* expression in VAT was however completely absent in *APOE4* mice.

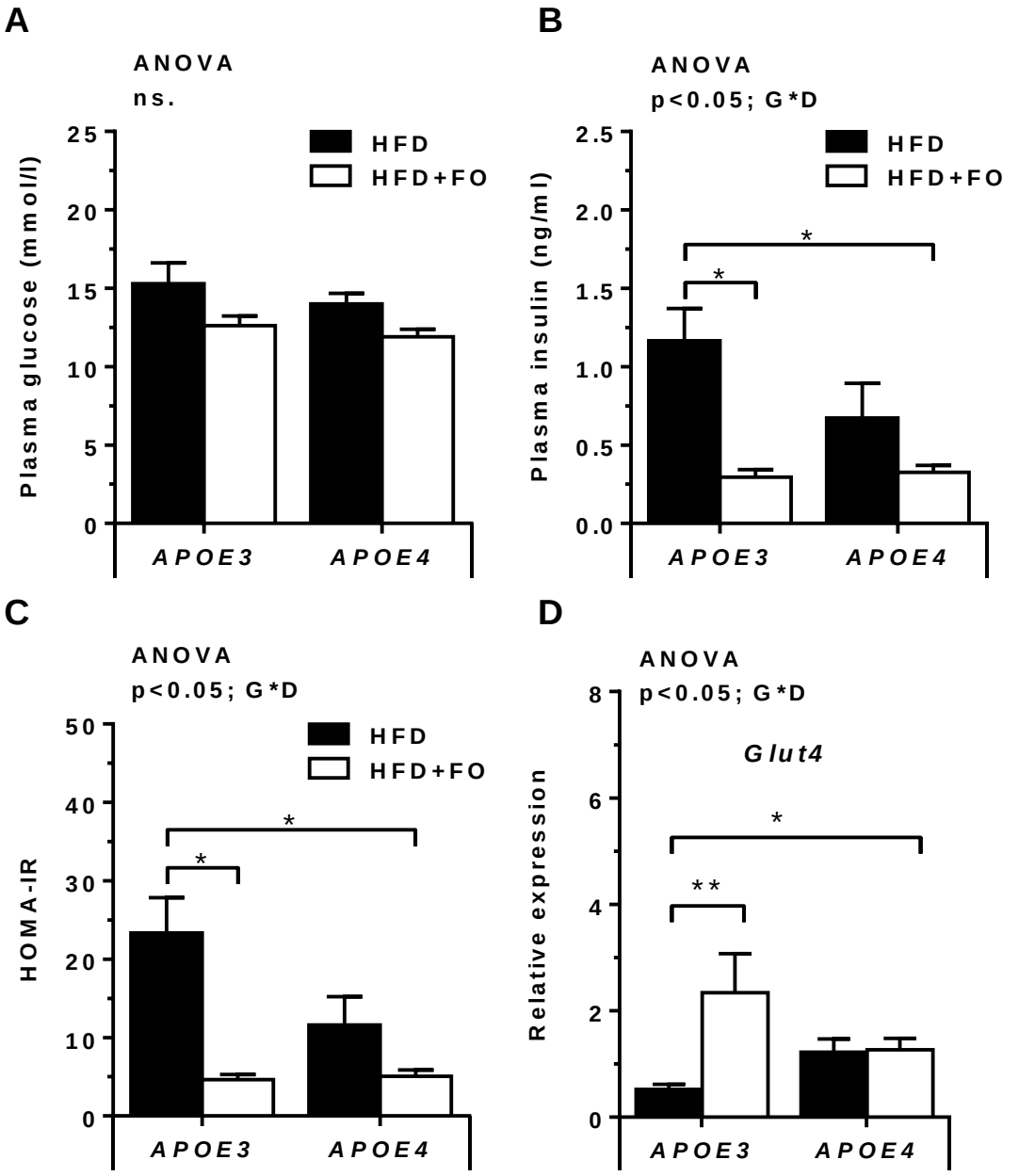
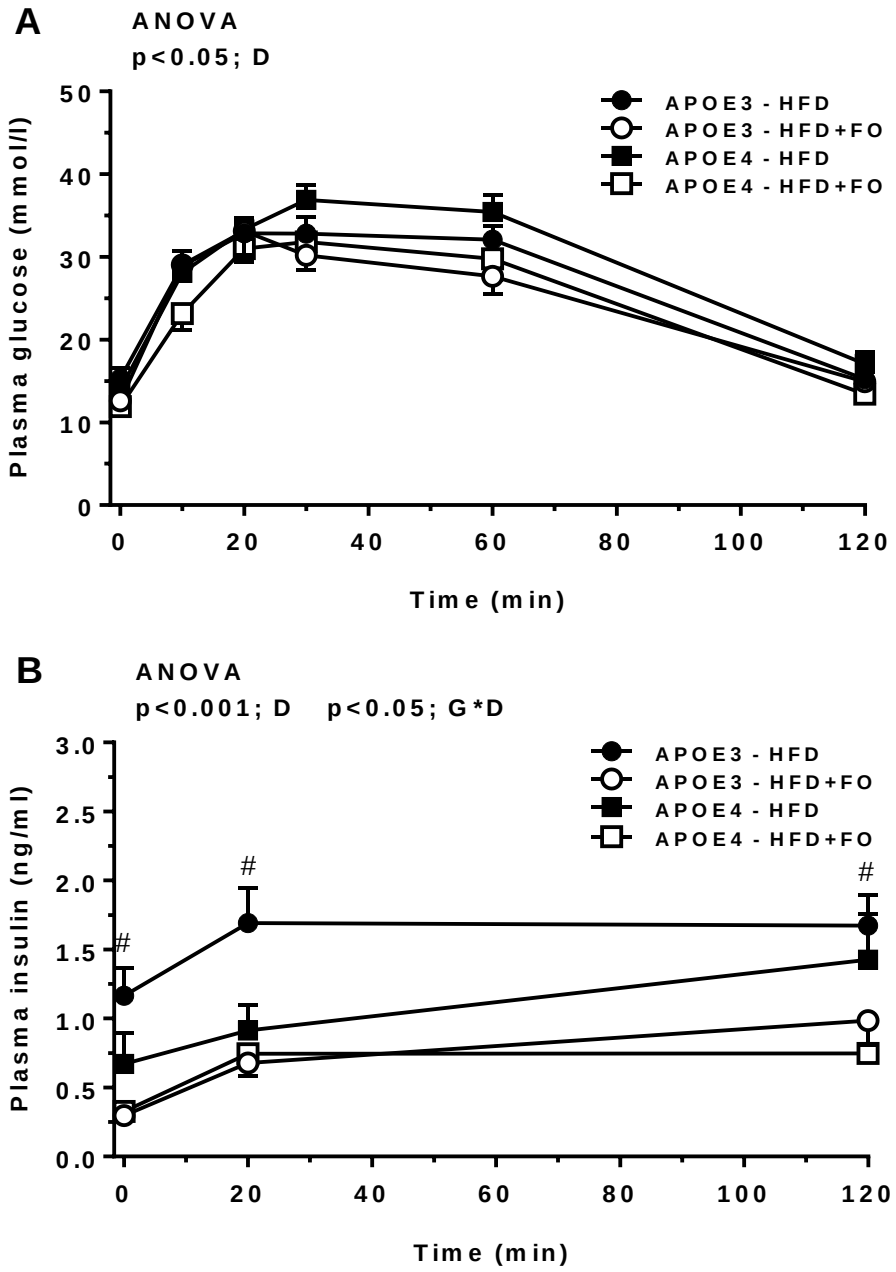


Figure 3.8. Fasting plasma glucose [A] and insulin [B], the homeostatic model assessment – insulin resistance (HOMA-IR) index [C] and mRNA expression of insulin-regulated glucose transporter *Glut4* in epididymal adipose tissue [D] in human *APOE3* and *APOE4* targeted replacement mice after 8 weeks of high-fat diet (HFD) without or with 30 g EPA+DHA / kg of diet (HFD+FO) feeding. Data is presented as mean ± SEM, n = 9 – 10 mice per group. The HOMA-IR was calculated using formula 'glucose (mmol/L) x fasting insulin (mU/L) / 22.5' from Matthews *et al*. Diabetologia (1985). mRNA expression levels are relative quantities normalised to the reference genes *Actb*, *Atp5B* and *Gapdh*. Statistical analysis was done by ANOVA for *APOE Epsilon* genotype (G), diet (D) and G*D interaction effect using un- or ^{10}log-transformed data and considered statistically significant when p < 0.05. Significant effects are displayed on top of the figure. Differences between the groups were tested using Student's *t*-test with Bonferroni correction, *p < 0.05, **p < 0.01. APOE, apolipoprotein E; Glut4, solute carrier family 2 (facilitated glucose transporter) member 4 (Slc2a4); HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA / kg of diet.

Glucose tolerance test

The glucose tolerance after 8 weeks of HFD feeding was investigated using an intra-peritoneal glucose tolerance test (IPGTT). After a 10 – 16 hour fast, mice received a fixed glucose load of 8 mg, corresponding to an average dosage of 2 g glucose / kg of body weight, and plasma glucose and insulin levels were monitored for 120 minutes (Figure 3.9). There was a significant effect of diet on plasma glucose during the IPGTT ($F_{1,25} = 6.488$, $p < 0.05$), with a trend for lower plasma glucose levels in mice on HFD+FO (Figure 3.9A) although plasma glucose area under the curve (AUC) values were similar (Figure 3.9C). There were large differences in the plasma insulin levels during the IPGTT with a significant effect of diet ($F_{1,35} = 23.184$, $p < 0.001$) that interacted with *APOE Epsilon* genotype ($F_{1,35} = 4.398$, $p < 0.05$). Overall, *APOE3* mice on HFD had significantly higher plasma insulin levels and AUC compared to *APOE3* and *APOE4* mice on HFD+FO ($p < 0.05$) (Figure 3.9B and 3.9D). This dramatically increased insulin production to clear plasma glucose in the *APOE3* mice on HFD indicate an impaired insulin sensitivity in these mice, which is in line with the estimated HOMA-IR index. Furthermore, these results reveal that supplementation with dietary fish oil on a HFD background significantly improved glucose clearance and decreased insulin production during a glucose challenge (IPGTT) indicating improved insulin sensitivity, which could be mediated through increased mRNA expression of the insulin-regulated glucose transporter Glut4 (Figure 3.8D).



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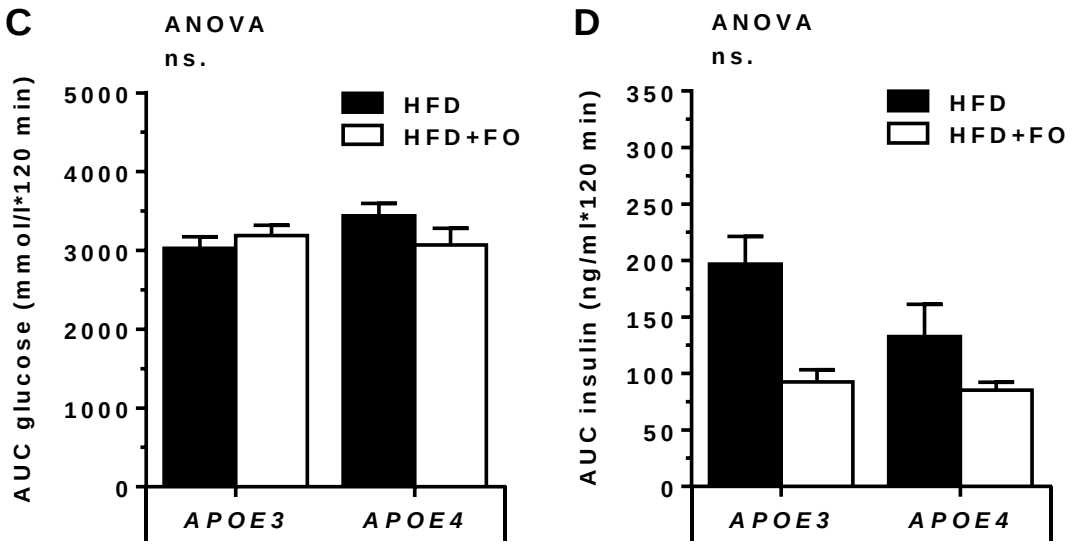

Figure 3.9. Glucose tolerance in human *APOE3* and *APOE4* targeted replacement mice after 8 weeks of high-fat diet (HFD) feeding without or with 30 g EPA+DHA / kg of diet (HFD+FO). After a 10 – 16 hour fast, animals received intra-peritoneal injection providing 8 mg glucose (based on average dose of 2 g / kg of body weight). Plasma glucose [A] and plasma insulin [B] were recorded at the indicated time points and the area under curve (AUC) was calculated [C,D]. Values are mean ± SEM, n = 9 – 10 mice per group. Statistical analysis of plasma glucose and insulin levels during glucose tolerance test [A,B] was done by repeated measures ANOVA with Greenhouse-Geisser correction to test for genotype (G), diet (D) and G*D interaction effect using un- or ^{10}log-transformed data; within-subjects analysis revealed a significant effect of time on plasma glucose levels ($F_{5,125}$ = 145.498, p < 0.001) that did not interact with genotype ($F_{5,125}$ = 1.735, p = 0.16), diet ($F_{5,125}$ = 2.203, p = 0.08) or genotype*diet ($F_{5,125}$ = 0.585, p = 0.66) and a significant effect of time on plasma insulin levels ($F_{2,70}$ = 57.365, p < 0.001) that interacted with diet ($F_{2,70}$ = 5.075, p < 0.01) and genotype*diet interaction ($F_{2,70}$ = 3.657, p < 0.05), but not with genotype ($F_{2,70}$ = 0.145, p = 0.86). Between-subject analysis revealed a significant effect of diet on plasma glucose and insulin levels ($F_{1,25}$ = 6.488, p < 0.05 and $F_{1,35}$ = 23.184 , p < 0.001) that interacted with genotype for plasma insulin ($F_{1,35}$ = 4.398, p < 0.05) but not for plasma glucose ($F_{1,25}$ = 0.242, p = 0.63). There was no effect of genotype on plasma glucose and insulin ($F_{1,25}$ = 0.015, p = 0.90 and $F_{1,35}$ = 3.283, p = 0.08). Differences between the groups were tested using Student's *t*-test with Bonferroni correction; #APOE3-HFD is different from APOE3-HFD+FO and APOE4-HFD+FO at t = 0 min (p < 0.01), APOE3-HFD is different from APOE4-HFD, APOE3-HFD+FO and APOE4-HFD+FO at t = 20 min (p < 0.05) and APOE3-HFD is different from APOE4-HFD+FO at t = 120 min (p < 0.01). Statistical analysis of plasma glucose and insulin area under the curve (AUC) [C,D] was done by ANOVA for *APOE Epsilon* genotype (G), diet (D) and G*D interaction effect using un- or ^{10}log-transformed data and considered statistically significant when p < 0.05. APOE, apolipoprotein E; HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA / kg of diet.

Discussion

In summary, we showed that *APOE3* mice developed DIO and IR upon HFD feeding associated with an increased food intake and body weight gain, an increased HOMA-IR index and increased plasma insulin levels during the glucose tolerance test, and this was prevented by dietary fish oil. However, *APOE4* mice were resistant to the development of DIO and IR upon HFD feeding. Plasma lipid and adipokine levels reflected the differential body weight, the plasma levels of TG, TC, non-HDL-C, HDL-C and NEFA and leptin tended to increase with increasing body weight with no change in plasma adiponectin levels. The highest circulating levels were in general observed in the obese *APOE3* mice on HFD that also showed a trend towards liver inflammation indicated by increased plasma ALT levels.

APOE Epsilon genotype and fish oil determine the development of obesity

In line with a limited number of previous studies in human *APOE3* and *APOE4* TR mice we demonstrated that *APOE3* mice develop DIO and IR upon HFD feeding, with *APOE4* mice being resistant to both DIO and IR [Arbones-Mainar et al., 2008 and 2010]. However, we are the first to show that dietary fish oil can prevent the development of obesity and IR in *APOE3* mice on a HFD background. Our findings from the IPGTT contradict previous reports on glucose tolerance and IR in *APOE3* and *APOE4* mice [Arbones-Mainar et al., 2008 and 2010]. Briefly, body weight of the *APOE3* and *APOE4* mice upon HFD feeding in our study were roughly similar to those reported by Arbones-Mainar *et al*., after taking into account the approximate 7 wk age difference of the animals in our study compared to their study (age at start intervention 13 – 14 wks vs. 7 wks) [Arbones-Mainar et al., 2008 and 2010]. On the contrary, our findings from the IPGTT indicating *APOE3*, but not *APOE4*, mice develop IR after 8 weeks of HFD feeding, were the opposite of Arbones-Mainar *et al*. their findings from an oral GTT (OGTT) showing *APOE4*, but not *APOE3*, mice develop IR upon HFD feeding [Arbones-Mainar et al., 2008]. However, their reported effects of PPAR activation through rosiglitazone treatment on glucose handling during OGTT in *APOE3* and *APOE4* mice were similar to the beneficial effects of dietary fish oil on glucose handling during IPGTT in *APOE3*, but not in *APOE4*, mice in our study [Arbones-Mainar et al., 2010]. These important differences most likely relate to difference in the composition of the diets; our diet contained 24 % fat, 0.02 % cholesterol but no sucrose, whereas their HFD contained 21.1 % fat, 0.2 % cholesterol and 34.1 % sucrose [Arbones-Mainar et al., 2008 and 2010], with sucrose known to promote the development of IR in mice [Neuhofer et al., 2013]. Thus, human *APOE3* TR mice develop DIO along with IR, whereas human *APOE4* TR mice are resistant to DIO and concurrent IR on a high-fat non-diabetogenic diet. Finally, dietary fish oil prevented DIO and IR in *APOE3* TR mice on a high-fat non-diabetogenic background.

To investigate if the differential body weight gain was the result of a differential food intake and food efficiency, we monitored food intake every second day and estimated the average food efficiency over the course of the dietary intervention. Briefly, *APOE3* mice on HFD had a consistent higher average food intake, yet a similar overall food efficiency, compared to

the other three groups, which was in line with their greater body weight gain. However, *APOE3* mice on HFD+FO had a higher food intake, yet similar food efficiency and body weight, compared to *APOE4* mice. This indicates that *APOE3* mice have a higher energy expenditure compared to *APOE4* mice on HFD+FO, which we speculated to be explained by a greater activity resulting in a higher energy expenditure in these mice. However, mice were housed below their thermoneutrality of 30 °C at 21 °C and studies have indicated that at temperature below thermoneutrality (20 °C and 24 °C) energy expenditure from activity, although still contributing to daily energy expenditure, has no effect on total daily energy expenditure in mice [Virtue et al., 2012]. Thus, the observed differences in activity between the *APOE Epsilon* strains are unlikely to lead to differences in total daily energy expenditure or explain our results, although future studies using comprehensive laboratory animal monitoring systems need to confirm this. A potential difference in the metabolic response to fish oil between the *APOE3* and *APOE4* genotype cannot be excluded, as we did not investigate metabolic function using respiratory chambers and fish oil increase β-oxidation [Flachs et al., 2005]. Although a differential responsiveness to fish oil remains to be investigated, the limited studies have found supporting evidence for an increased β-oxidation in *APOE4* compared to *APOE3* mice on either a low or high fat diet [Conway et al., 2014, Huebbe et al., 2014]. Future studies using metabolic chamber systems should elucidate potential differences between the *APOE3* and *APOE4* mice in basal metabolic rate and total daily energy expenditure and their responsiveness to dietary fish oil. Huebbe *et al*. recently investigated energy metabolism, food conversion and fat deposition in human *APOE* TR mice and showed that *APOE4* mice tended to have a higher basal energy expenditure than *APOE3* mice that was associated with increased uncoupling protein (Ucp) and fatty acid binding protein 4 (Fabp4) protein expression in skeletal muscle suggesting an increased uncoupling of mitochondrial respiration through Ucp and increased fatty acid oxidation [Huebbe et al., 2014]. However, the impact of dietary fish oil on energy expenditure was not investigated in this study and thus remains a potential interaction between *APOE Epsilon* genotype and dietary fish oil to determine energy expenditure in human *APOE* TR mice to be elucidated. In addition, they showed that *APOE3* mice had an increased food intake associated with lower plasma leptin levels and a more efficient food conversion and fat storage associated with increased expression of genes involved in TG synthesis in AT (fatty acid syntase (Fasn), diacylglycerol acyltransferase 1 (Dgat1)) compared to *APOE4* mice. Furthermore, they confirmed a greater physical activity in *APOE3* mice compared to *APOE4* mice indicated by a longer running time and longer running distance in voluntary running wheel experiments. Altogether, these findings indicate that *APOE4* mice are less efficient in conversion of food into body fat, they exhibit a lower physical activity level, yet a similar energy expenditure, which cannot be fully accounted for by their lower food intake suggesting a dissipation of energy in *APOE4* mice compared to *APOE3* mice that would explain their differential predisposition to develop obesity. Future studies should investigate the dissipation of energy in *APOE4* mice by looking into mitochondrial density and Ucp

expression in liver, brown AT and epididiymal AT that contains more mitochondria than other white AT [Bjørndal et al., 2011].

Besides, we showed that dietary fish oil prevented DIO and IR in *APOE3* mice on a high-fat non-diabetogenic background, which is in line with various animal studies reporting anti-obesogenic effects of fish oil in mice, though it is becoming clear that this is dependent on the genetic background [Puglisi et al., 2011]. The anti-obesity effects of EPA and DHA are in part attributed to the induction of a metabolic switch resulting in increased β-oxidation and reduced lipogenesis in the metabolically active organs, such as AT, liver and muscle [Kalupahana et al., 2011]. For example, replacement of 15 % of the constitute lipids in a HFD with EPA and DHA reduced the development of obesity and was associated with an increased expression of genes regulating mitochondrial biosynthesis (*PPARg1a* and *Nrf1*), an upregulation of genes involved in the oxidative phosphorylation system and an increase in mitochondrial protein levels accounting for the increased β-oxidation and decreased lipogenesis in white AT of mice [Flachs et al., 2005]. Thus, an increased β-oxidation in AT, liver and muscle likely contributes to the observed anti-obesogenic effects of EPA and DHA in *APOE3* mice. Though dietary fish oil could also have improved satiety in *APOE3* mice resulting in a lower food intake contributing to the prevention of DIO [Buckley and Howe, 2010]. In summary, findings from our study and others suggest that *APOE3* is an 'energy-thrifty' allele and *APOE4* is an 'energy-dissipative' allele [Huebbe et al., 2014]. Future studies using metabolic chamber systems and whole-body imaging techniques should shed light on potential differences in body composition, basal metabolic rate (BMR) and total daily energy expenditure between the *APOE3* and *APOE4* mice and between the HFD and HFD+FO group.

APOE Epsilon genotype and adipose tissue development

There is accumulating evidence that apoE plays a role in AT development and the development of pathologies associated with obesity, such as IR [Kypreos et al., 2009, Slim and Minihane, 2014], paving the way for *APOE Epsilon* genotype to have potential impact on AT development. The development of AT, or adipogenesis, occurs in circumstances of a positive energy balance and involves both an increase in adipocyte number (hyperplasia) and an increase in adipocyte size due to increased fat storage (hypertrophy) [Fruebeck, 2008]. In order for AT to expand and adipocytes to increase in size and number, extracellular matrix remodelling also has to occur [Virtue and Vidal-Puig, 2010, Divoux and Clement, 2011]. An adequate formation of new adipocytes is required for AT expansion, with a limited pre-adipocyte formation from stem cells expecting to contribute to a limited AT expansion capacity [Virtue and Vidal-Puig, 2010]. Arbones-Mainar *et al*. demonstrated that *APOE4* mice have fewer but larger adipocytes after 8 weeks of HFD feeding compared to *APOE3* mice [Arbones-Mainar et al., 2008] suggesting that formation of new adipocytes rather than adipocyte expansion is contributing to the reduced AT expansion in *APOE4* mice. Hence, it would have been very interesting to have measured adipocyte number and

size in this study. Future studies are recommended to investigate adipocyte number and morphology using immunohistochemistry. The formation of new adipocytes or adipogenesis is a complex process involving many transcription factors, cofactors and signalling intermediates from various pathways (Figure 3.10) [Rosen and MacDougald, 2006]. It is split into two phases; 'determination' involving commitment of pluripotent stem cells to the adipocyte lineage (i.e. pre-adipocyte), and 'terminal differentiation' involving differentiation of the pre-adipocyte into mature adipocyte [Rosen and MacDougald, 2006]. PPAR-γ is both sufficient and essential for adipogenesis, and is required for both the initiation and maintenance of the differentiated state in adipocytes [Rosen and MacDougald, 2006]. Besides, several members of the CCAAT-enhancer-binding protein (C/EBP) family are involved in adipogenesis, with C/EBPα being an important inducer of many adipocyte genes and crucial for the attainment of insulin sensitivity [Rosen and MacDougald, 2006].

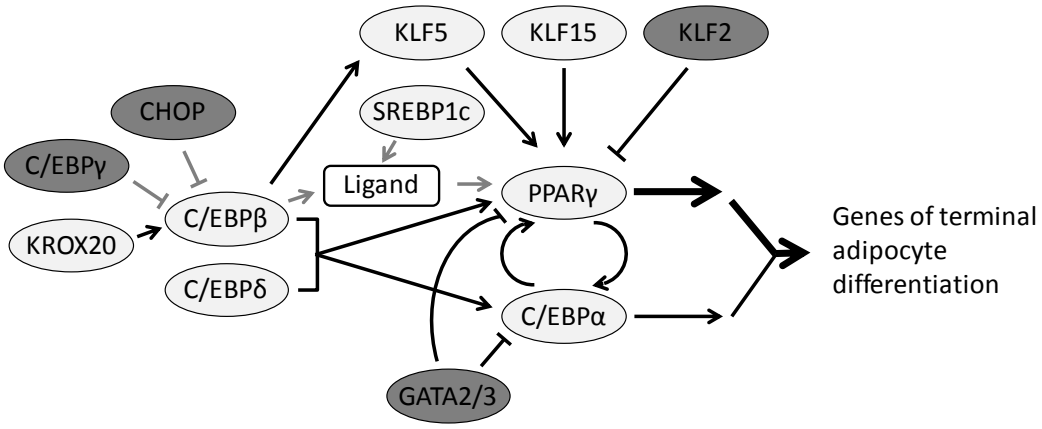

Figure 3.10. A simplified overview of the complex transcriptional cascade regulating adipogenesis. Peroxisome proliferator-activated receptor γ (PPARγ) is the core transcription factor regulating adipogenesis, with its expression being regulated by variety of pro-adipogenic (white) and anti-adipogenic (dark grey) factors. In addition, a yet-unidentified ligand is able to activate PPARγ, with the expression of this ligand proposed to to be induced by the transcription factor sterol response element-binding protein-1c (SREBP1c). A second important transcription factor regulating adipogenesis is CCAAT-enhancer binding protein α (C/EBPα), which is regulated through various inhibitory protein-interactions. Other members of the C/EBP family also either stimulate (C/EBPβ, C/EBPδ) or suppress (C/EBPγ, CHOP) adipogenesis. Black lines indicate effects on gene expression and grey lines indicate effects on protein activity. Figure has been modified from Rosen and MacDougald (2002). C/EBP, CCAAT-enhancer binding protein; CHOP, transcription factor homologous to CCAAT-enhancer binding protein; GATA2/3, GATA transcription factors 2 and 3; KLF, Krüppel-like factor; PPARγ, peroxisome proliferator-activated receptor γ; SREBP1c, sterol response element-binding protein-1c.

A potential mechanism linking *APOE Epsilon* genotype to adipogenesis signalling pathways is endoplasmic reticulum (ER)-stress that is increasingly recognised as one of the underlying causes of metabolic dysfunction in obesity [Hotamisligil, 2010]. Nutritional excess and an accumulation of unfolded and misfolded proteins within the ER are two known causes of ER-stress triggering the unfolded protein response (UPR) which activates metabolic and inflammatory signalling pathways [Hotamisligil, 2010]. The apoE4 protein is known to have a greater tendency to form a molten globule state (a destabilization of the protein

conformation), show a lower protein stability and is increasingly subjected to intracellular degradation compared to the apoE3 protein [Hatters et al., 2006, Heeren et al., 2004, Rellin et al., 2008]. These protein characteristics could predispose the apoE4 protein to be increasingly recognised as misfolded protein leading to the UPR response and ER-stress. Indeed, Zhong *et al*. showed that murine astrocytes expressing murine apoE with similar biophysical properties as human apoE4 (Arg-61 apoE) showed an increased expression of UPR markers (including CHOP) indicative of increased ER-stress that was associated with astrocyte dysfunction [Zhong et al., 2009]. Moreover, they concluded that the Arg-61 apoE, having similar characteristics to apoE4 isoform, is recognized as an abnormally folded protein in the ER, eliciting an ER-stress response [Zhong et al., 2009]. In line with this, Cash *et al*. showed an increased ER-stress response in *APOE4* compared to *APOE3* macrophages with increased expression of CHOP (a marker of UPR response) after incubation with oxidized-LDL and concluded that apoE4 provides a second hit to exacerbate oxidized-LDL-induced ER stress in macrophages [Cash et al., 2012]. Although never investigated, this makes it tempting to speculate that also in adipocytes the apoE4 protein is recognised as an abnormally folded protein triggering UPR response and increasing ER stress and CHOP expression that in turn would negatively impact on adipogenesis. As shown in Figure 3.10, CHOP is a negative regulator of adipogenesis by inhibiting C/EBPβ, an important pro-adipogenic transcription factor that induces PPARγ and C/EBPα. Moreover, C/EBPβ and sterol response element-binding protein-1c (SREBP-1c) increase the production of a to-be-indentified ligand of PPARγ, which has been shown to be essential for initiation of adipogenesis, but not for maintaining PPARγ-dependent gene expression in mature adipocytes [Rosen and MacDougald, 2006]. Thus, increased ER-stress resulting in an increased CHOP expression inhibits the PPARγ ligand production through C/EBPβ, thereby inhibiting the initiation of adipogenesis. Although obesity is known to be associated with increased ER-stress in liver and AT, the mechanism as to how ER-stress and related signalling pathways impact on the regulation of adipogenesis and adipocyte function *in vivo* remains an important unanswered question [Hotamisligil, 2010]. Future studies should investigate the potential impact of the *APOE4* genotype on the determination and terminal differentiation phases of adipogenesis process and the potential involvement of ER-stress.

Adipose tissue *Apoe* mRNA expression is decreased in obese *APOE3* mice

As mentioned earlier, the expression of apoE increases upon differentiation and correlates with lipid content in adipocytes [Zechner et al., 1991]. Thus, *Apoe* mRNA expression levels in AT could provide a hint towards the level of adipocyte differentiation. In addition, *Apoe* expression in AT also appear to be modulated by the state of feeding in mice. In more detail, *Apoe* expression in AT of mice was reduced in DIO and hyperphagia, but was increased by fasting [Huang et al., 2007]. These findings were interpreted by Kypreos *et al*. as an intrinsic defence mechanism existing in AT which limits adipogenesis by reducing the expression of apoE in the fed state [Kypreos et al., 2009]. In fact, we showed that the expression of *Apoe*

in AT was significantly lower in *APOE3* mice on HFD compared to *APOE3* mice on HFD+FO, as well as to the *APOE4* mice on either of the two diets. Thus, this proposed mechanism could explain the lower *Apoe* expression in the AT of *APOE3* mice on a HFD, as these mice gained more weight compared to the other mice and developed DIO. Our findings are similar to those reported by Arbones-Mainar *et al*., who showed that HFD feeding decreased the *Apoe* expression in the epididymal AT by almost 40 % in *APOE3*, but not in *APOE4*, mice compared to 8 weeks chow feeding and this was associated with a 30 % greater weight gain in *APOE3* mice upon HFD feeding compared to *APOE4* mice [Arbones-Mainar et al., 2008]. Furthermore, another study by Arbones-Mainar *et al*. showed that treatment with ROSI, a PPAR-γ agonist, in the last 4 weeks of a 16-week HFD feeding intervention increased the *Apoe* expression in the epididymal AT of *APOE3* mice, but this was completely blunted in *APOE4* mice. Similarly *APOE3* mice showed an 80 % greater weight gain upon HFD feeding compared to *APOE4* mice, with no effect of ROSI treatment on body weight in both *APOE3* and *APOE4* mice [Arbones-Mainar et al., 2010]. However, only the fold difference in mRNA expression levels were given making it difficult to make a direct comparison to relative expression levels reported in our study. Nevertheless, these results indicate a role for apoE in the process of adipogenesis and suggest that obesity is a negative regulator of AT *Apoe* expression.

Fish oil prevents the development of insulin resistance in *APOE3* mice

In this study, we measured fasting glucose and insulin levels and performed an IPGTT in *APOE3* and *APOE4* mice after 8 weeks of dietary intervention with HFD or HFD+FO as initial screening test of glucose metabolism. Briefly, there was no significant effect of diet or genotype on glucose levels both in the fasting state and during the IPGTT. However, *APOE3* mice on HFD had in both the fasting state and during the IPGTT significantly higher insulin levels compared to all other three groups, indicating an impaired insulin action and reduced insulin sensitivity in these mice. The reduced insulin sensitivity was accompanied by an increase in plasma free fatty acid (NEFA) and TG levels. These results are in line with previous findings showing that diet-induced obesity leads to the development of IR in human *APOE3* targeted replacement mice [Kleemann et al., 2010, Arbones-Mainar et al., 2010].

The LC *n-3* PUFA EPA and DHA have been shown to exert various beneficial effects on obesity and its complications. They have anti-obesogenic effects in both humans [Bender et al., 2014, Mori et al., 1999] and rodents [Puglisi et al., 2011], lower plasma TG levels [Harris, 1997, Minihane et al., 2000], have anti-inflammatory effects [Itariu et al., 2012] and potentially improve insulin sensitivity [Akinkuolie et al., 2011, Kalupahana et al., 2011]. However, many of these effects, including the anti-obesogenic, anti-inflammatory and insulin sensitising effects, have not been investigated according to *APOE Epsilon* genotype. As discussed above, we are the first to report anti-obesogenic effects of fish oil in *APOE3* mice, but not *APOE4* mice. In addition, this is the first study investigating the insulin sensitising effects of fish oil in human *APOE* TR mice. We observed a strong trend that dietary fish oil

improves insulin sensitivity, indicated by the lower plasma insulin levels, in *APOE3* and *APOE4* TR mice, despite causing a slight increase in VAT mass. These insulin sensitising effects are in line with a recent report that the effects of dietary fish oil on metabolic parameters, including systemic IR, are independent of changes in AT mass in other strains of mice [Puglisi et al., 2011]. However, LC *n-3* PUFA failed to consistently improve insulin sensitivity in humans (standard mean difference (SMD) = 0.08, 95% CI -0.11 – 0.28) although improvements are seen in HOMA-IR (SMD = 0.30, 95% CI 0.03 – 0.58) [Akinkuolie et al., 2011] possibly due to a modest decrease in fasting insulin levels [Hartweg et al., 2008]. Fish oil fatty acids EPA and DHA exert various beneficial effects in liver, muscle and AT that together account for their insulin sensitising effects in mice [Kalupahana et al., 2011]. Briefly, EPA and DHA promote hepatic fatty acid oxidation (β-oxidation) and suppress lipogenesis through inhibition of the enzymes involved in TG synthesis, thereby reducing hepatic TG accumulation [Kalupahana et al., 2011]. Besides, EPA and DHA increase β-oxidation in AT and muscle preventing fatty acid intermediate accumulation in these tissues [Kalupahana et al., 2011]. Finally, EPA and DHA alleviate AT inflammation through the production of special proresolving mediators (e.g. resolvins and protectins) and activation of G-coupled receptors, whilst increasing the secretion of adiponectin, leptin and visfatin, which all contribute to a better AT function improving systemic insulin sensitivity [Kalupahana et al., 2011].

Given the important role insulin-regulated glucose transporter Glut4 plays in insulin-stimulated glucose uptake, the *Glut4* mRNA expression levels in the epididymal VAT were investigated. We demonstrated that EPA and DHA increased the *Glut4* mRNA expression levels in VAT of *APOE3* mice that could account for the observed beneficial effects on insulin sensitivity and glucose tolerance. However surprisingly, the beneficial effects of EPA and DHA on *Glut4* expression in VAT were not observed in *APOE4* mice. Glut4 is an insulin-regulated glucose transporter mainly expressed in muscle and AT that is responsible for insulin-stimulated glucose uptake into these tissues [Bell et al., 1990]. The binding of insulin to its receptor leads to phosphorylation of the insulin receptor substrate (IRS) proteins, subsequent activation of the type I phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB), also known as Akt, which results in Glut4 translocation from intracellular vesicles to the cell membrane increasing glucose uptake [Elmendorf, 2004].

EPA and DHA exert their beneficial effect through various signalling pathways. For instance, in the liver the effects on oxidative processes are mediated through activation of PPAR-α and 5' AMP-activated protein kinase (AMPK) and inhibition of the carbohydrate-responsive element-binding protein (ChREBP) and sterol regulatory element-binding proteins (SREBP), such as SREBP-1a and SREBP-1c, resulting in the suppression of lipogenesis [Lottenberg et al., 2012, Kalupahana et al., 2011]. In AT, activation of PPAR-γ and AMPK are responsible for the increased β-oxidation, with PPAR-γ also being responsible for mediating the beneficial effects on adipocyte function and insulin sensitivity [Lottenberg et al., 2012, Kalupahana et al., 2011]. Finally, EPA and DHA exert their anti-inflammatory and insulin

sensitising effects through G-coupled receptors, such as GPR40 and GPR120, which are receptors that can be activated by long chain fatty acids [Oh et al., 2010]. For example, GPR120 is highly expressed in mature adipocytes and has been shown to be essential in mediating the anti-inflammatory and insulin sensitising effects of EPA and DHA [Oh et al., 2010]. In adipocytes, EPA and DHA have been shown to improve glucose uptake via activation of $G_{q/11}$-coupled protein receptors (e.g. GPR120) and PI3K, which inhibit down-stream signalling to IKKβ / NF-κB (inhibitor of nuclear factor kappa-B kinase / nuclear factor kappa-B) and JNK / AP1 (c-Jun N-terminal kinase / activator protein-1) pathways (that otherwise impair insulin receptor signalling by disrupting serine phosphorylation of IRS-1) and increase Glut4 translocation to the cell surface resulting in increased glucose uptake [Oh et al., 2010, Kalupahana et al., 2012]. This poses a mechanism via which EPA and DHA can improve insulin signalling in adipocytes, although EPA and DHA can also influence the plasma membrane Glut4 concentration by improving membrane fluidity [Lottenberg et al., 2012].

A strength of this study is the use of a mouse model humanised for the human *APOE3* and *APOE4* gene variant. Human *APOE* TR mice express human *APOE* under control of the murine regulatory sequence and produce apoE at physiological levels. This gives us a complete *in vivo* model for direct comparison of *APOE Epsilon* isoform-specific effects. In addition, this mouse model allowed us to use an experimental design tailored specifically to answer our research question while controlling for many confounding intrinsic and environmental factors difficult to control for in human studies, such as genetic background and diet. This provided us significant power to evaluate the effects of *APOE Epsilon* genotype and fish oil on the development of obesity and its metabolic complications. Furthermore, in human studies it is difficult to study internal organs, such as the liver and visceral AT, whereas a mouse model provides free access to our tissue of interest (i.e. AT). This enabled us to perform a thorough investigation into the role of apoE in AT development, which is a strength of this study.
Achieving obesity in mice is not easy as they are resistant to obesity on a low fat diet, and require a HFD containing usually at least 40 – 45 kcal% from fat. Diets with 60 kcal% from fat have also been used to induce DIO in mice, but we think this is rather unrepresentative of the human diet that on average contains 35 kcal% from fat. The EPA to DHA ratio of the HFD+FO diet was 2:3 and similar to that of oily fish - the main dietary source of EPA and DHA - and corresponded to the ratio observed for the average UK diet.

Our study was designed to compare the *APOE3* with the *APOE4* genotype and the HFD+FO with HFD, i.e. compare the effect of *APOE Epsilon* genotype and fish oil on a HFD background. We did not have the financial resources to include a low-fat diet group for both *APOE3* and *APOE4* TR mice, which would have been another appropriate control. Similarly the inclusion of wild type C57BL/6 mice carrying murine *apoe* would have provided a control

condition allowing to discriminate between the effect of human *APOE* and murine *apoe*. However, the latter would be less relevant than the low-fat diet control.

The dose of EPA and DHA used in this study was supra-physiological, but typical of a dose used in comparable published studies. The HFD+FO diet provided each mouse with a food intake of 3 g/d daily 36 mg EPA + 54 mg DHA, which corresponds to a dose of 2250 mg EPA+DHA per kg of body weight per day in an obese mouse (40 g). This corresponds to human equivalent dose of 21.6 g EPA+DHA per day in a normal weight adult (70 kg) [U.S. Department of Health and Human Services, 2005], which corresponds to 7.2 portions of oily fish per day and is beyond physiological tolerable levels previously approached in human intervention trials (≤ 5.6 – 8.4 g EPA+DHA / d) (for review see Calder *et al*. [Calder et al., 2006 and 2011A]). The energy density of the diets was 3.75 kcal per gram and mice ate 3 grams per day, hence their energy intake was 14.2 kcal. The HFD+FO provided the mice with 90 mg EPA+DHA per day and thus the dose of EPA and DHA per unit of energy was 6.3 mg/kcal. The daily energy intake in the UK is approximately 2,300 kcal [HSCIC, 2012] which would adequate to 14.5 grams of EPA+DHA per day. Thus the dose of EPA and DHA, either expressed per unit of body weight or per unit of energy intake, in this study was beyond physiological tolerable levels approached in humans. The dose-response curve of EPA and DHA may not been linear. It could be saturable or U-shaped with adverse effects at either ends of the spectrum. Hence, it would have been helpful to include a group with a more physiological dose in this study to aid the dose-response comparisons.

An oral or IPGTT are widely used in the published literature to assess glucose tolerance in mice and are recognised as a robust method to evaluate glucose handling, however the gold standard to assess IR in mice is the euglycemic clamp method [Ayala et al., 2010]. We showed that *APOE3* mice on HFD had impaired insulin action and reduced insulin sensitivity using IPGTT, these findings should be corroborated with an insulin tolerance test (ITT) or hyperinsulinemic-euglycemic clamp [Ayala et al., 2010]. A strength of this study is the use of a fixed glucose load in the IPGTT. This reduced the chance of misdiagnosing obese mice as being glucose intolerant due to giving them more glucose for the same amount of lean mass, which would have occured when the glucose load was based on body weight [Ayala et al., 2010]. However, using a glucose load based on lean body mass is most correct when body composition data is available [Ayala et al., 2010]. An oral GTT is a more physiological test than an IPGTT, but the first is also influenced by other factors, such as gastric emptying and incretin secretion, which are bypassed by the IPGTT [Ayala et al., 2010].

A limitation of this study was that we did not measure adipocyte number and size, which would have provided important information on adipocyte morphology and important clues towards the potential impact of *APOE4* genotype on formation of new adipocytes. Future studies in human *APOE3* and *APOE4* TR mice are recommended to investigate adipocyte number and morphology using immunohistochemistry.

Future studies should also investigate liver TG levels in human *APOE3* and *APOE4* TR mice, which would provide a measure of fatty liver. This would give us a greater insight into any potential disturbance in metabolism in the liver of *APOE3* mice on a HFD that could explain the severe IR observed in these mice. Furthermore, LC *n-3* PUFA promote fatty acid oxidation and suppress lipogenesis in liver thereby reducing hepatic TG accumulation [Kalupahana et al., 2011], which could contribute to their insulin sensitising effects in *APOE3* and *APOE4* mice indicated by the improved HOMA-IR and improved glucose tolerance during the IPGTT.

In conclusion, we showed that the *APOE3*, but not the *APOE4* genotype, is associated with the development of DIO, dylipidemia and IR in human *APOE* TR mice upon HFD feeding and this can be prevented by dietary fish oil. The potential involvement of AT inflammation in the development of IR in *APOE3* and *APOE4* mice will be explored in the next chapter.

Chapter 4. *In vivo* studies – inflammation: The interactive impact of *APOE Epsilon* genotype and fish oil on adipose tissue inflammation.

Studies in human APOE3 and APOE4 targeted replacement mice

Introduction

Adipose tissue inflammation

In obesity, the expansion of adipose tissue (AT) is associated with structural and functional changes together with changes in cellular composition and cell phenotype [Dalmas et al., 2011, Ouchi et al., 2011]. The adipocytes increasingly take up triglycerides becoming hypertrophic and start to secrete mediators that attract macrophages, which increasingly infiltrate the AT. These recruited AT macrophages (ATM) will predominantly acquire a pro-inflammatory (M1) phenotype and start to outnumber the resident ATM that have an anti-inflammatory (M2) phenotype [Weisberg et al., 2003, Wentworth et al., 2010]. The infiltration of ATM adopting a M1 phenotype is thought to be the fundamental step in AT inflammation, which results in AT dysfunction and subsequent systemic insulin resistance (IR) [Osborn and Olefsky, 2012]. In addition to the phenotypic heterogeneity of ATM in both lean and obese states, ATM can also 'switch' their phenotypes [Morris et al., 2011]. Although ATM can adopt a range of phenotypes between M1 and M2, the simplified M1/M2 model of macrophage polarisation (Table 4.1) is most frequently used [Morris et al., 2011]. Thus, obesity is not only characterised by quantitative increase in ATM, but also with qualitative changes ('phenotype switch') in ATM from the anti-inflammatory M2 phenotype to the pro-inflammatory M1 phenotype. These cellular alterations in AT are associated with an increased production of pro-inflammatory mediators and reduced expression of anti-inflammatory mediators by ATM and hypertrophic adipocytes, resulting in a state of systemic chronic low-grade inflammation [Fain et al., 2004, Maury and Brichard, 2010]. This is in turn contributes to systemic IR and dyslipidemia [Lumeng et al., 2007A and 2007B] that are part of the metabolic syndrome (MetS), and contribute to an increased risk of cardiovascular disease (CVD) [Barbarroja et al., 2010, Hotamisligil, 2006, Van Gaal et al., 2006].

Table 4.1. Phenotypic and functional characteristics of adipose tissue macrophages (ATM) according to the M1/M2 model of macrophage polarisation [Morris et al., 2011].

	M1 (pro-inflammatory)	M2 (anti-inflammatory)
Activators	LPS, IFN-γ	IL-4, IL-13
Surface markers (human)	$CD11c^+$ $CD206^-$ $CD40^+$ $CD86^+$	$CD11c^-$ $CD206^+$ $CD150^+$
Surface markers (mice)	$CD11c^+$ $CD206^-$ $MGL1^-$	$CD11c^-$ $CD206^+$ $MGL1^+$
Change with obesity	increased	decreased
Gene expression	*Tnfα* *Il1β* *Il6* *Nos2*	*Il10* *Mgl1/2* *Arginase 1* *Mrc2* *Ym1/Chi3l3*
Production	TNF-α, IL-6, IL-12, ROS	IL-10, IL-1RA, arginase

M1, classically activated pro-inflammatory macrophages; M2, alternatively activated anti-inflammatory macrophages; CD11c, integrin alpha X chain; CD206, mannose receptor; CD40, co-stimulatory protein CD40; CD86, co-stimulatory protein Cluster of Differentiation 86; IFN, inteferron; iNOS, inducible nitric oxide synthase IL, interleukin; IL-1RA, Interleukin-1 receptor antagonist; LPS, lipopolysaccharide; Mgl1/2, macrophage galactose N-acetyl-galactosamine-specific lectins 1 and 2; MGL, macrophage galactose type C-type Lectin; Mrc2, mannose receptor C type 2; ROS, reactive oxygen species; TNFα / TNF-α, tumor necrosis factor alpha; Ym1/Chi3l3, Ym1/chitinase 3-like 3.

APOE Epsilon genotype and adipose tissue inflammation and function

In summary, the quantitative and qualitative properties of ATM determine AT inflammation, which together with the capacity of AT to 'buffer' excess nutrients, are important determinants for maintaining adipocyte function and a metabolic healthy profile in obesity [Blüher, 2010, Lumeng et al., 2007A and 2007B, Ouchi et al., 2011]. The *apolipoprotein Epsilon 4* (*APOE4*) allele is associated with a pro-inflammatory status in macrophages and human *APOE* TR mice [Jofre-Monseny et al., 2007A, Lynch et al., 2003, Maezawa et al., 2006, Ophir et al., 2005, Tsoi et al., 2007]. In addition, our group has demonstrated that the *APOE Epsilon* genotype interacts with adiposity to determine plasma inflammatory marker concentrations [Kofler et al., 2012] suggesting that *APOE Epsilon* genotype may exert it effects on inflammation through AT. This is supported by two recent studies in human *APOE Epsilon* targeted replacement (TR) mice showing that the *APOE4* allele is associated with an impaired expandability of the visceral (epididymal) fat depot and greater epididymal adipocyte size, an impaired insulin sensitivity, and an impaired *ex vivo* adipocyte differentiation and lipid-storage capacity [Arbones-Mainar et al., 2008 and 2010]. Furthermore, *APOE4* TR mice were more susceptible to the metabolic complications of obesity, where *APOE4* TR mice developed impaired glucose tolerance earlier (4 wks vs. 12 wks) and had greater predisposition to developing a fatty liver than *APOE3* TR mice upon high fat diet (HFD) feeding with rosiglitazone, a peroxisome proliferator-activated receptor(PPAR)-γ ligand. However, we found that *APOE4* TR mice were protected from HFD-induced obesity and impaired glucose tolerance (Chapter 3). Also, we found no indication for an impaired liver function after 8 weeks of HFD feeding with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two long chain (LC) *n-3* polyunsaturated fatty acids (PUFA) that are ligands for PPAR-γ (Chapter 3). In conclusion, these findings show that the *APOE4* allele is associated with a reduced AT expansion. The effects of the *APOE4*

allele on predisposition to the metabolic complications of obesity, that is IR and ectopic lipid deposition in the liver, are less clear. Moreover, the impact of *APOE Epsilon* genotype on AT inflammation and ATM phenotype is unknown, although a similar ATM infiltration in *APOE3* and *APOE4* TR mice was reported in a single previous study [Arbones-Mainar et al., 2008].

Long chain *n-3* PUFA and adipose tissue function

LC *n-3* PUFA exert various beneficial effects on AT function including increased mitochondrial biosynthesis and β-oxidation [Flachs et al., 2009]. In addition, LC *n-3* PUFA have been shown to reduce AT mass upon HFD feeding, which is dependent on the genetic background in mice [Puglisi et al., 2011]. However, the beneficial effects of LC *n-3* PUFA on systemic IR, dyslipidemia, hepatic steatosis, AT inflammation and AT IR are independent of the change in AT mass [Puglisi et al., 2011]. Finally, LC *n-3* PUFA have been shown to reduce ATM infiltration and/or markers of AT inflammation in *db/db* mice [Todoric et al., 2006], *LDLR*$^{-/-}$ mice [Saraswathi et al., 2007], *APOE*3-Leiden* mice [Muurling et al., 2003] and wild type C57BL/6 mice [Oh et al., 2010]. LC *n-3* PUFA exert anti-inflammatory effects in macrophages, they lower the pro-inflammatory cytokine and eicosanoids production and promote a phenotype switch towards the anti-inflammatory M2 phenotype [Calder, 2012, Oliver et al., 2010, Oliver et al., 2012, Oh et al., 2010, Weldon et al., 2007]. Therefore, LC *n-3* PUFA may provide a potential therapeutic molecule to reverse the unfavourable ATM and adipocyte phenotype.

Research gaps

Our understanding of how genetic determinants interact with the environment and lead to the development of metabolic disease is still poor [Osborn and Olefsky, 2012]. The macrophage-mediated AT inflammation leading to AT dysfunction has been recognised as the underlying pathology causing systemic IR and systemic inflammation in obesity. However, more knowledge is needed about the factors that direct the polarisation state of ATM to either the pro- or anti-inflammatory phenotype [Osborn and Olefsky, 2012].

Previous research from our group and other research groups has demonstrated that the *APOE4* allele is associated with a pro-inflammatory macrophage phenotype *in vitro* [Jofre-Monseny et al., 2008A] and an impaired AT expandability and greater predisposition to the metabolic complications of obesity *in vivo* [Arbones-Mainar et al., 2008 and 2010]. However, the impact of the *APOE4* allele on AT inflammation and ATM phenotype is unknown. LC *n-3* PUFA have been shown to promote an anti-inflammatory M2 phenotype in macrophages, decrease AT inflammation and improve AT function [Oh et al., 2010, Puglisi et al., 2011], providing a potential strategy to reverse the adverse adipocyte and ATM phenotype associated with the *APOE4* allele. However, the interactive impact of *APOE Epsilon* genotype and LC *n-3* PUFA on AT inflammation and ATM phenotype has never been investigated.

Research question

The aim of this study was to investigate the independent and interactive impact of *APOE Epsilon* genotype and fish oil on the ATM infiltration rate and phenotype in human *APOE3* and *APOE4* TR mice using a HFD diet-induced obesity (DIO) model.

Hypotheses

Previous limited research has demonstrated that the *APOE4* allele is associated with an impaired AT expansion and perhaps a differential predisposition to the metabolic complications of obesity (i.e. IR and ectopic lipid deposition in the liver). Though a similar ATM infiltration in *APOE3* and *APOE4* TR mice was reported in a single study, its effect on AT inflammation has never been thoroughly investigated. Therefore, I hypothesise that ATM infiltration in human *APOE3* and *APOE4* TR mice upon HFD feeding will be similar but the ATM phenotype will be different, with a more pro-inflammatory ATM phenotype in *APOE4* TR mice.

The LC *n-3* PUFA EPA and DHA have beneficial effects on adipocyte function in addition to their anti-inflammatory effects in macrophages. Therefore, I hypothesize that EPA and DHA will improve adipocyte function and lower ATM infiltration. In addition, I hypothesize that EPA and DHA exert anti-inflammatory effects in resident ATM stimulating polarisation towards the M2 phenotype. Altogether this will reduce AT inflammation.

Materials & Methods

We used a mouse model humanised for human *APOE3* and *APOE4* gene to investigate the independent and interactive impact of *APOE Epsilon* genotype and fish oil on ATM infiltration rate and phenotype in diet-induced obesity. Studies in human *APOE3* and *APOE4* TR mice allowed us to characterize the macrophages present in AT and investigate AT inflammation using an experimental design tailored specifically to answer our research question controlling for many confounding intrinsic and environmental factors difficult to control for in human studies, such as genetic background and diet. The ATM phenotype was investigated using flow cytometry, which quantifies the expression of one or more cell surface markers simultaneously in individual cells. Using cell type-specific markers, a specific cell type (e.g. macrophages) can be identified within a heterogeneous cell population (e.g. all cells from AT). Adding additional markers (e.g. M1 and M2 phenotypic markers) to the panel allows further characterisation of the phenotype of the cell type of interest, i.e macrophages. Although rare cell types can be identified within large heterogeneous cell populations using flow cytometry, it is recommended to have a high concentration of your cell type of interest. AT consist of mainly mature adipocytes and some other cells collectively called stromal-vascular fraction (SVF) that include monocytes and macrophages. Therefore, the SVF was isolated and separated from mature adipocytes using tissue digest procedures. The number of ATM was investigated using gene expression levels of ATM markers in AT. The ATM phenotype data obtained with flow cytometry was corroborated by performing a detailed analysis of gene expression levels of previously used additional M1 and M2 phenotype marker genes. AT fatty acid status was determined by gas-liquid chromatography and established in order to examine the association between tissue fatty acid changes, in particular EPA and DHA enrichment in response to dietary fish oil, and AT inflammation.

Animals & treatment

For details regarding the animals, diets and tissue collection please see Chapter 3 Materials & Methods section (page 95 – 100).

Isolation of stromal-vascular fraction

The isolation and flow cytometry analysis of the SVF of white AT was based on the methodology described by Brake and Smith (2008). One epididymal fat pad was collected in DMEM (with 4500 mg/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate) (Sigma Aldrich, Dorset, UK) and kept at 37 °C, 95 % O_2 – 5 % CO_2 until further analysis within 4 hours. Tissues were rinsed and minced in sterile (0.2 µm) Krebs-Ringer bicarbonate (KRB) buffer (0.77 M NaCl, 0.77 M KCl, 0.77 M KH_2PO_4, 0.77 M $MgSO_4$, 0.77 M $NAHCO_3$, 0.275 M $CaCl_2$, 4 % bovine serum albumin (BSA), 0.3 M D-glucose, gassed with 95 % O_2 – 5 % CO_2) using 3 ml KRB buffer / 1 g tissue. The mature adipocytes and SVF were isolated by digesting the minced fresh tissue with 1 mg/ml Collagenase Type II (from *Clostridium*

histolyticum, Sigma Aldrich) in KRB buffer for 30 minutes at 37 °C in a shaking water bath. The SVF cells were obtained by filtering the digested tissue through a 40 µm cell strainer (Fisher Scientific, Loughborough, UK), washing twice with 2x initial volume KRB buffer, followed by centrifugation (500 x g, 4 °C, 5 min) to separate from floating mature adipocytes. Next, red blood cells (RBC) were removed by incubation with 1 ml RBC lysis buffer (Sigma Aldrich) for 1 min at room temperature. Finally, SVF cells were washed with 15 ml KRB buffer and centrifuged before being resuspended in 200 µl PBS (Oxoid, Fisher Scientific) with 10 mM D-glucose and 1 % BSA (Sigma Aldrich). The cell number was determined using Cellometer Auto T4 Cell Counter (Nexcelom Bioscience, Peqlab Ltd., Sarisbury Green, UK).

Flow cytometry analysis

SVF cells were resuspended in 100 µl at 1 x 10^6 cells/ml in PBS with 10 mM D-glucose and 1 % BSA. Next, the cells were incubated with antibodies at the recommended concentrations; CD11b-PE, 0.2 mg/ml (BD Biosciences, Oxford, UK); CD11c-Alexa700, 0.2 mg/ml (eBiosciences, Hatfield, UK); CD206-FITC, 0.1 mg/ml (AbD Serotec, Kidlington, UK), for 30 min at 4 ºC in the dark. Cells were washed with PBS (10 mM glucose and 1 % BSA) and centrifuged (300 x g, 2 min) twice before being resuspended in 100 µl PBS (10 mM glucose and 1 % BSA). For each antibody used, a control sample was incubated with isotype control Rat igG2b, kappa, isotype-PE (BD Biosciences), Armenian Hamster IgG isotype-Alexa700 (eBiosciences) and Rat igG2a-FITC (AbD Serotec) at similar concentrations to determine non-specific binding. A single stain and fluorophore minus-one (FMO) controls were taken along to set gates and compensation. A representative sample was incubated with 7-AAD (BD Biosciences) for 20 min at 4 ºC, to assess cell viability. Samples were kept on ice and immediately analysed using BD Accuri™ C6 (BD Biosciences). For each sample, data of 30,000 events was collected using a gate excluding debris (Appendix 4-1, Figure A4-1.1) as follows; CD11b-PE, 488 nm laser with 585/40 BP filter; CD11c-Alexa700, 640 nm laser with 675/25 BP filter; CD206-FITC, 488 nm laser with 533/30 BP filter; 7-AAD, 488 nm laser with 670/LP filter. The instrument settings for detecting CD11c-Alexa700 were suboptimal as the emission peak of Alexa700 fell outside of the 675/25 BP filter detection range and only a small proportion of the emission spectra of Alexa700 was detected using the 675/25 BP filter. The data was further analysed as described in the next paragraph.

Flow cytometry data analysis

The SVF of AT contains pre-adipocytes, mesenchymal stem cells, endothelial progenitor cells, dendritic cells, lymphocytes, neutrophils, monocytes, macrophages and mast cells [Ouchi et al., 2011]. Therefore, first a gate (gate E) including ATM but excluding debris, small cells (i.e. red blood cells) and cell clumps was applied to the original data of 30,000 collected events (Figure A4-1.1 and A4-1.2, for more details see Appendix 4-1), which was based on the methodology described by Brake and Smith (2008). This gate E, containing the ATM cell population of interest, yielded on average 32.7 ± 6.0 (SD) % of the total collected

events for the *APOE Epsilon* genotype * diet groups (Appendix 4-1, Table A4-1.1). Next, the threshold to define the positive and negative cell populations (within gate E) for the markers CD11b-PE, CD11c-Alexa700 and CD206-FITC were set using FMO control samples (Appendix 4-1, Figure A4-1.3). A single stained sample was used to confirm that the positive cell population could be distinguished with this threshold. The threshold for the cell viability stain 7-AAD was set using an unstained sample. Next, single stains controls were used to determine spillover of CD206-FITC into 585/40 BP and 670/LP filter; and CD11b-PE into 533/30 BP filter and 670/LP filter, to apply appropriate compensation as presented in the spillover table (Table 4.2). The compensation was setup using our cell population of interest (gate E), which contains besides ATM also autofluorescent eosinophils, neutrophils and granulocytes that posed a challenge to setting compensation (for more details see Appendix 4-1). The results regarding the number of $CD11b^+$ cells was expressed as % of cells in gate E, whereas the number of $CD11b^+CD11c^+$ and $CD11b^+CD206^+$ cells were expressed % of $CD11b^+$ cells (gate A). A graphical representation of the results from a representative sample of the *APOE Epsilon* genotype * diet groups can be found in Appendix 4-2. The non-specific binding was determined using the isotype controls which was for CD11b 17.6 %, for CD11c 5.8 % and for CD206 8.1 % cells in gate E (Appendix 4-1, Figure A4-1.8). The viability of the samples determined using representative samples stained with the cell viability stain 7-AAD was 83 ± 8.3 (SD) % (Appendix 4-1, Figure A4-1.9).

Table 4.2. Spillover table.

Spillover (%)	CD206-FITC 533/30	CD11b-PE 585/40	7-AAD 675/25	CD11c-Alexa700 670/LP
CD206-FITC 533/30		2.70	N/A	1.30
CD11b-PE 585/40	1.90		N/A	0.00
7-AAD 675/25	N/A	N/A		N/A
CD11c-Alexa700 670/LP	0.80	0.00	N/A	

mRNA expression analysis by real-time quantitative PCR

For details of RNA isolation and mRNA expression analysis by real-time quantitative PCR (RT-QPCR) see Chapter 3 Materials & Methods section (page 102 – 103). The primers for *integrin alpha X* (*Itgax*), *interleukin 6* (*Il6*), *C-type lectin domain family 10, member A* (*Clec10a*, also known as *Mgl1*) and *macrophage galactose N-acetyl-galactosamine specific lectin 2* (*Mgl2*), *arginase 1* (*Arg1*) and *interleukin 10* (*Il10*) were designed using primer-BLAST (www.blast.ncbi.nlm.nih.gov/Blast). The primers for *EGF-like module containing mucin-like hormone receptor-like sequence 1* (*Emr1*), *integrin alpha M* (*Itgam*), *mannose receptor, C type 1* (*Mrc1*), *nitric oxide synthase 2, inducible* (*Nos2*), and *tumor necrosis*

factor alpha (*Tnfa*) were designed by PrimerDesign (PrimerDesign Ltd., Southampton, UK). Primer sequences are shown in Table 4.3.

Table 4.3. Primers used for mRNA expression analysis using RT-QPCR.

Gene	Primer Sequence	Product size
Arg1	F: 5'- TGGCTTGCGAGACGTAGAC -3' R: 5'- GCTCAGGTGAATCGGCCTTTT -3'	160
Cd11b (*Itgam*)	F: 5'- ACAGAGCAGGGGTCATTCG -3' R: 5'- ATGGTATTCAGGGCTTCAAAGTT -3'	145
Cd11c (*Itgax*)	F: 5'- ACGTCAGTACAAGGAGATGTTGGA -3' R: 5'- ATCCTATTGCAGAATGCTTCTTTACC -3'	190
Cd206 (*Mrc1*)	F: 5'- TGGCTACTGGAGAACATCATAC -3' R: 5'- AAGGAATCCACGCAGTCTGT -3'	134
F4/80 (Emr1)	F: 5'- GAAGATGAAGATGAATGTGTGACTC -3' R: 5'- GCCCTCCTCCACTAGATTCAA -3'	118
Il6	F: 5'- CTTCCATCCAGTTGCCTTCTTG -3' R: 5'- AATTAAGCCTCCGACTTGTGAAG -3'	142
Il10	F: 5'- CTGGACAACATACTGCTAACCG -3' R: 5'- GGGCATCACTTCTACCAGGTAA -3'	108
Mgl1/2	F: 5'- GCATCCTCTCTTGGACCCAC -3' R: 5'- GGTGCCTAGGTCCCTCCTTA-3'	116
Nos2	F: 5'- CACCTACTTCCTGGACATTACG -3' R: 5'- TACTCTGAGGGCTGACACAA -3'	120
Tnfα	F: 5'- AGCCAGGAGGGAGAACAGA -3' R: 5'- CAGTGAGTGAAAGGGACAGAAC -3'	96

Arg1, arginase 1; *Cd11b*, integrin alpha M (*Itgam*); *Cd11c*, integrin alpha X (*Itgax*); *Cd206*, mannose receptor, C type 1 (*Mrc1*); *F4/80*, EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (*Emr1*); *Glut4*, solute carrier family 2 (facilitated glucose transporter), member 4 (*Slc2a4*); *Il6*, interleukin 6; *Il10*, interleukin 10; *Mgl1/2*, C-type lectin domain family 10, member A (*Clec10a*, also known as *Mgl1*) and macrophage galactose N-acetyl-galactosamine specific lectin 2 (*Mgl2*); *Nos2*, nitric oxide synthase 2, inducible; *Tnfα*, tumor necrosis factor alpha.

Lipid extraction and fatty acid analysis

High-performance liquid chromatography (HPLC) grade solvents for chromatography and solvents for extraction (pro analysis quality) (Fisher Scientific, Loughborough, UK) were used throughout the lipid extraction and fatty acid analysis. Total lipids were extracted from 50 – 200 mg of epididymal AT with chloroform/methanol (2:1 v/v) containing 0.01 % butylated hydroxytoluene (BHT) (Sigma-Aldrich, Dorset, UK) as antioxidant [Folch et al., 1957]. The organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically. Two mg of the total lipid fraction was subjected to acid-catalyzed transmethylation for 16 h at 50 °C, using 1 ml of toluene and 2 ml of 1% sulphuric acid (v/v) in methanol. Prior to transmethylation, nonadecanoic acid (19:0) (Sigma-Aldrich, Dorset, UK) was added as internal standard (5 % of the total lipid analysed) to the lipid extracts. The resultant fatty acid methyl esters (FAME) were separated and purified from TG skeletons (i.e. glycerol), phospholipid skeletons, cholesterol and cholesterol wax on TLC (20

x 20 cm) plates, pre-coated with silica gel (without fluorescent indicator) (Macherey-Nagel, Düren, Germany) by thin layer chromatography (TLC) and visualised under spraying with 1 % iodine in chloroform [Christie, 2003]. After elution, FAME were separated and quantified by gas-liquid chromatography (GC) using a Hewlett Packard 5890 GC and a SGE BPX70 capillary GC column (30 m x 0.22 mm I.D.; SGE UK Ltd) with helium as carrier gas and using on-column injection. The temperature gradient started at 115°C for 3 min, then went to 200°C at 2°C / min, 2 min at 200°C, and then to 240°C at 60°C / min. After 5 min at 240°C, it cooled down to 115°C and equilibrated for 3 min before the next injection. Individual methyl esters were identified by reference to authentic standards (BSA (fatty-acid free), α-linolenic acid, docosahexaenoic acid, eicosapentaenoic acid, tridecanoic acid, nonadecanoic acid, gallic acid, procatechuic acid, and syringic acid; Sigma-Aldrich, Dorset, UK) and to well-characterised fish oil standard (PUFA-3 from menhaden oil, SUPELCO, Supelco Park, Bellefonte, USA). Data were collected and processed using GC Chemstation (version B04-02).

Statistical analysis

The independent and interactive impact of *APOE Epsilon* genotype and diet was analysed using 2 x 2 factorial univariate Analysis of Variance (ANOVA) with *APOE Epsilon* genotype and diet as independent between-subject variables and considered statistically significant when $p < 0.05$. In the presence of a significant *APOE Epsilon* genotype * diet interaction effect, post-hoc analysis was performed using the two-tailed Student's *t*-test with Bonferroni correction to analyse differences between the *APOE Epsilon* genotype * diet groups. Differences were considered significant at $p < 0.008$ (= 0.05 / 6). Statistical analysis of the mRNA expression levels in AT was done using log transformed values. Data with a non-normal distribution and/or unequal variances was analysed using a non-parametric Kruskal-Wallis test and untransformed data, and was considered statistically significant when $p < 0.05$. Post-hoc Mann-Whitney U test with Bonferroni correction was employed to test for differences between the *APOE Epsilon* genotype * diet groups and was considered significant at $p < 0.008$ (= 0.05 / 6). Various outcome measures had not-normally distributed data and/or unequal variances which was not improved and often worsened by log (^{10}log or ^{e}log), square root ($\sqrt{x}$) or inverse ($1/x$) transformation, and as a consequence for several outcome measures non-parametric testing had to be employed. In the results section, the statistical test employed will be clearly indicated in the tables and figures. All statistical analysis was performed using SPSS 18.0.

Results

Adipose tissue inflammation

Flow cytometry analysis

The ATM infiltration and phenotype in epididymal AT of *APOE3* and *APOE4* mice fed a HFD or a HFD containing 30 g EPA+DHA per kg of diet (HFD+FO) was first characterised by analysing the cell surface expression of CD11b (total ATM), CD11c (M1 marker) and CD206 (M2 marker) in the SVF of disaggregated AT using flow cytometry. The flow cytometry data was analysed as depicted in Figure 4.1. Briefly, the cell population of interest (containing ATM) within the SVF was first selected using a specific gate (Figure 4.1A). Next, ATM in the cell population of interest were identified by the cell surface expression of CD11b (Figure 4.1B). The M1 and M2 phenotype of the $CD11b^+$ ATM was then determined by the cell surface expression of CD11c (M1) and CD206 (M2) (Figure 4.1C).

We found that the abundance of ATM in the SVF of disaggregated epididymal AT was determined by *APOE Epsilon* genotype ($F_{1,35}$ = 227.139, $p < 0.05$), but not diet ($F_{1,35}$ = 0.048, p = 0.86), and this effect was independent of diet (genotype * diet $F_{1,35}$ = 0.033, p = 0.86; Table 4.4). *APOE4* mice had significantly higher percentage of $CD11b^+$ ATM (% of total cell events in cell population of interest) in the epididymal SVF compared to *APOE3* mice ($p < 0.05$) (Figure 4.2A). Taking a closer look at the ATM cell population ($CD11b^+$ cells) revealed that we failed to identify any pro-inflammatory M1 ATM (Table 4.4; Figure 4.2B). Diet had a borderline non-significant impact on the percentage of anti-inflammatory M2 ATM ($F_{1,35}$ = 137.617, p = 0.05; Figure 4.2C), with higher percentage of M2 ATM in mice on HFD compared to HFD+FO. There was a trend for lower percentage of M2 ATM in *APOE4* mice compared to *APOE3* mice, but this did not reach significance (p = 0.09). Diet interacted with *APOE Epsilon* genotype to determine the percentage of double positive ATM expressing both M1 marker CD11c and M2 marker CD206 ($F_{1,35}$ = 5.518, $p < 0.05$; Figure 4.2D). The percentage double positive ATM was significantly higher in mice on the HFD+FO compared to HFD ($p < 0.05$). The ATM cell surface expression ratio of CD11c (M1) to CD206 (M2) was not significantly different between the *APOE Epsilon* genotypes ($F_{1,35}$ = 1.144, p = 0.48) or diet groups ($F_{1,35}$ = 66.243, p = 0.08; Figure 4.2E). Though there was a trend for a higher ratio in mice on HFD+FO compared to those on control HFD (p = 0.08), which was attributed to the increase in double positive ATM ($CD11b^+CD11c^+CD206^+$ cells) (Table 4.4; see also Appendix 4-2, Figure A4-2.3 and Table A4-2.1).

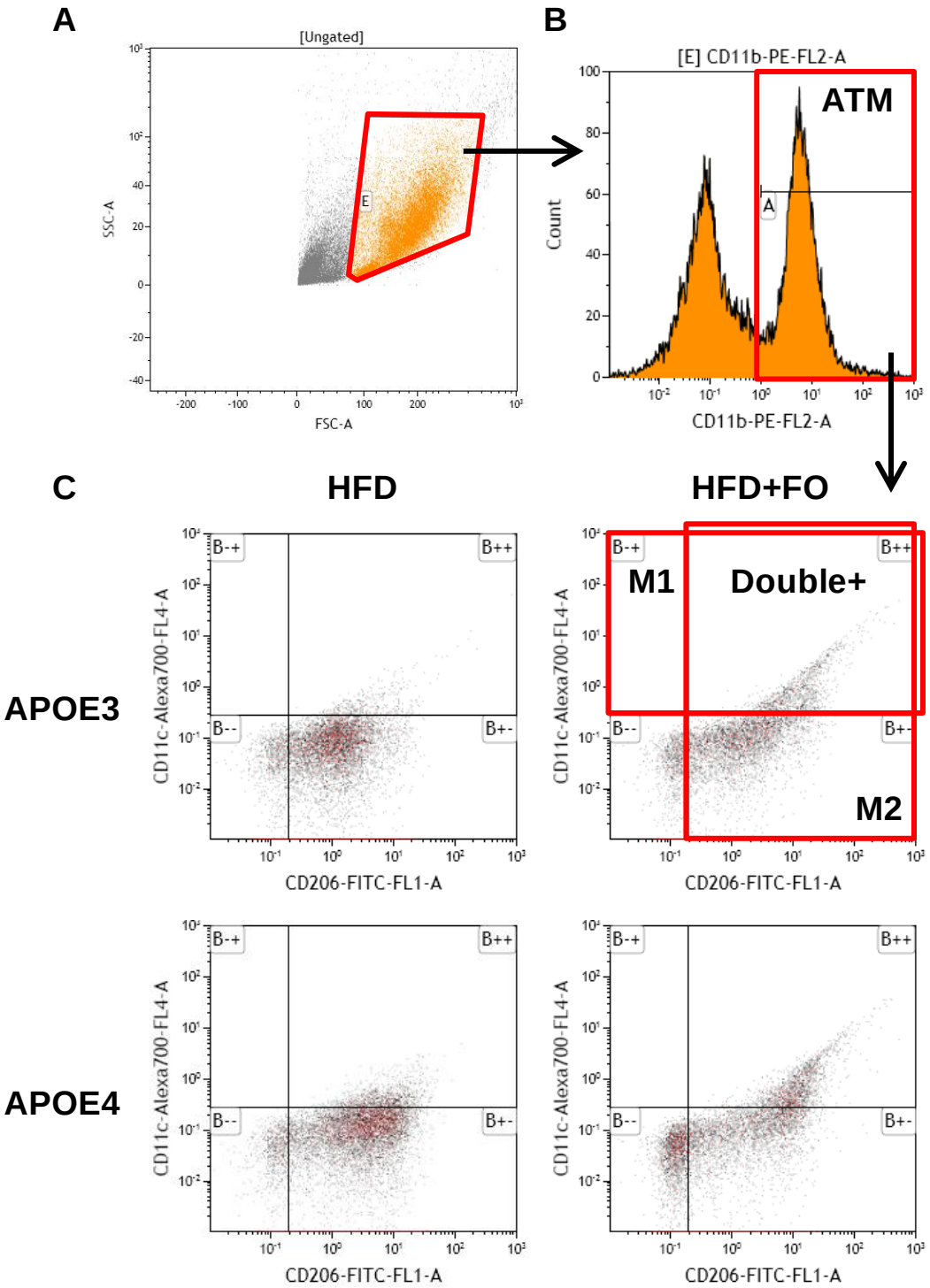

Figure 4.1. Analysis of the adipose tissue macrophage (ATM) content and phenotype using flow cytometry. [A] First, the cell population of interest (containing macrophages) within the stromal vascular fraction was selected (gate E, orange dots). [B] Next, the ATM in the cell population of interest were identified by the cell surface expression of CD11b. [C] Finally, M1 and M2 phenotype of the CD11b$^+$ ATM cell population was determined by the cell surface expression of CD11c (M1) and CD206 (M2), with CD11b+CD11c+CD206– (M1), CD11b+CD11c–CD206+ (M2) cells and CD11b+CD11c+CD206+ (double+) cells. Representative plots are given for *APOE3* and *APOE4* mice fed a high-fat diet (HFD) and HFD containing 30 g EPA+DHA / kg of diet (HFD+FO). APOE, apolipoprotein E; CD11b, integrin alpha M; CD11c, Integrin alpha X; CD206, mannose receptor C type 1.

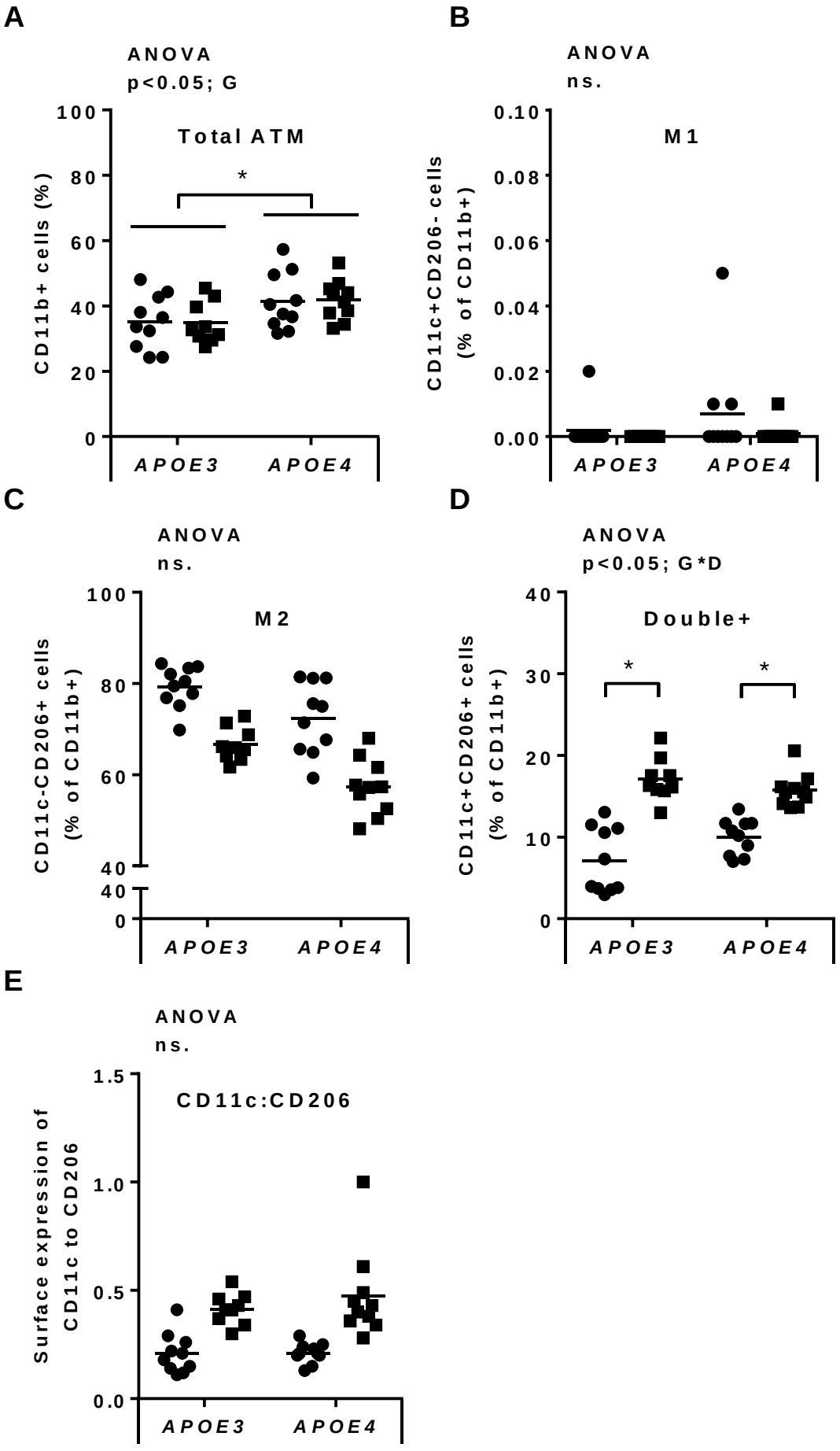
A
ANOVA
p<0.05; G
Total ATM
CD11b+ cells (%)
100
80
60
40
20
0
*
APOE3
APOE4
B
ANOVA
ns.
M1
CD11c+CD206- cells
(% of CD11b+)
0.10
0.08
0.06
0.04
0.02
0.00
APOE3
APOE4
C
ANOVA
ns.
M2
CD11c-CD206+ cells
(% of CD11b+)
100
80
60
40
40
0
APOE3
APOE4
D
ANOVA
p<0.05; G*D
Double+
CD11c+CD206+ cells
(% of CD11b+)
40
30
20
10
0
*
*
APOE3
APOE4
E
ANOVA
ns.
CD11c:CD206
Surface expression of
CD11c to CD206
1.5
1.0
0.5
0.0
APOE3
APOE4

Figure 4.2. Characterisation of the adipose tissue macrophage (ATM) content and phenotype using flow cytometry. The percentage of ATM and their phenotype in the stromal vascular fraction (SVF) of disaggregated epididymal adipose tissue was determined for *APOE3* and *APOE4* mice fed a high-fat diet (HFD) (circles) and HFD with 30 g EPA+DHA / kg of diet (HFD+FO) (squares) by the cell surface expression of CD11b (total ATM), CD11c (M1 marker) and CD206 (M2 marker). [A] The total number of ATM ($CD11b^+$ cells, % of total cell events in gate E confining the cell population of interest) in SVF was higher in *APOE4* mice compared to *APOE3* mice ($p < 0.05$), but similar between the diets. [B] No ATM with a pro-inflammatory M1 phenotype ($CD11b^+CD11c^+CD206^-$ cells) were identified. [C] The percentage of ATM with an anti-inflammatory M2 phenotype ($CD11b^+CD11c^-CD206^+$ cells) was not significantly different between the *APOE Epsilon* genotypes or diets ($p > 0.05$), although there was a trend for a higher percentage M2 ATM in mice on HFD compared to HFD+FO ($p = 0.05$) and *APOE3* mice compared to *APOE4* mice ($p = 0.09$). [D] *APOE Epsilon* genotype interacted with diet to determine the percentage of double positive ATM (Double+; $CD11b^+CD11c^+CD206^+$ cells; $p < 0.05$), with higher percentage of double positive ATM in mice on HFD+FO compared to HFD ($p < 0.05$). [E] The ratio of the CD11c to CD206 ATM cell surface expression was not significantly different between the *APOE Epsilon* genotypes or diets ($p > 0.05$), although there was a trend for a higher ratio in mice on HFD+FO compared to HFD ($p = 0.08$). Data is presented as mean ± SEM for n = 9 – 10 mice per group. Statistical analysis was done by ANOVA for *APOE Epsilon* genotype (G), diet (D) and G*D interaction effect and considered statistically significant when $p < 0.05$. Significant effects are displayed on top of each figure. Differences between the groups were tested using Student's *t*-test with Bonferonni correction, *$p < 0.008$ (= 0.05 / 6). APOE, apolipoprotein E; CD11b, integrin alpha M; CD11c, Integrin alpha X; CD206, mannose receptor C type 1.

Table 4.4. Adipose tissue macrophage (ATM) content and phenotype in the stromal vascular fraction of disaggregated epididymal adipose tissue of human *APOE3* and *APOE4* TR mice fed a high-fat diet (HFD) or HFD containing 30 g EPA+DHA / kg of diet (HFD+FO).

	APOE3-HFD	APOE3-HFD+FO	APOE4-HFD	APOE4-HFD+FO	
$CD11b^+$ cells (ATM) (% of total)	35.2 ± 2.6^a	34.8 ± 2.1^a	41.3 ± 2.8^b	41.8 ± 1.9^b	ANOVA p<0.05; G
$CD11b^+CD11c^+CD206^-$ cells (M1) (% of $CD11b^+$ cells)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ANOVA ns.
$CD11b^+CD11c^-CD206^+$ cells (M2) (% of $CD11b^+$ cells)	79.3 ± 1.4	66.6 ± 1.2	72.3 ± 2.5	57.3 ± 1.9	ANOVA ns.
$CD11b^+CD11c^+CD206^+$ cells (double positive) (% of $CD11b^+$ cells)	7.1 ± 1.3^a	17.1 ± 0.9^b	10.0 ± 0.7^a	15.7 ± 0.6^b	ANOVA p<0.05; G*D
CD11c:CD206 ratio	0.21 ± 0.03	0.42 ± 0.02	0.21 ± 0.01	0.47 ± 0.07	ANOVA ns.

Values are mean ± SEM for 9 – 10 animals per group. The $CD11b^+$ cells (ATM) are presented as percentage of total cell events in gate E that confines the cell population of interest. The $CD11b^+CD11c^+CD206^-$ (M1), $CD11b^+CD11c^-CD206^+$ (M2) and $CD11b^+CD11c^+CD206^+$ (double positive) cells are presented as percentage of $CD11b^+$ cells, i.e. total ATM. The CD11c:CD206 ratio presents the ratio of the cell surface expression of CD11c to CD206. Statistical analysis was done by ANOVA for *APOE Epsilon* genotype (G), diet (D) and G*D interaction effect and considered statistically significant when $p < 0.05$. Significant effects are indicated in the last column of the table. Differences between the groups were tested using Student's *t*-test with Bonferroni correction; different letters indicate differences between the *APOE Epsilon* genotype * diet groups, $p < 0.008$ (= 0.05 / 6). APOE, apolipoprotein E genotype; CD11b, integrin alpha M; CD11c, Integrin alpha X; CD206, mannose receptor C type 1; HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA / kg of diet; n/a, not applicable.

In summary, *APOE4* mice had relatively more ATM in disaggregated epididymal SVF compared to *APOE3* mice, but the ATM phenotype distribution was similar. On the other hand, fish oil determined the ATM phenotype distribution by increasing the number of ATM that coexpress the M1 and M2 phenotypic marker CD11c and CD206, without affecting the relative number of ATM in the disaggregated epididymal SVF in both *APOE3* and *APOE4* mice. The greater abundance of ATM in the epididymal SVF of *APOE4* mice compared to *APOE3* mice could reflect a higher 'absolute' number of ATM in epididymal AT; something which needs to be confirmed using a quantitative technique, such as mRNA expression analysis.

mRNA expression analysis

For a more quantitative analysis of the ATM number and phenotype in the epididymal AT of *APOE3* and *APOE4* mice fed a HFD or a HFD containing 30 g EPA+DHA / kg of diet (HFD+FO), the mRNA expression of *F4/80* (total ATM), *Cd11b* (total ATM), *Cd11c* (M1 marker), *Cd206* (M2 marker) and additional M1 (*Tnfa*, *Nos2*, *Il6*) and M2 (*Mgl1/2*, *Arg1*, *Il10*) markers was investigated.

Statistical analysis revealed a significant effect of diet ($F_{1,35}$ = 358.861, $p < 0.05$), but not *APOE Epsilon* genotype ($F_{1,35}$ = 15.523, $p = 0.16$), on the *F4/80* mRNA expression, with dietary fish oil increasing the expression levels in epididymal AT although group comparison revealed no significant differences (Figure 4.3A). There was no significant effect of *APOE Epsilon* genotype or diet on the mRNA expression levels of the ATM markers analysed in the flow cytometry experiment *Cd11b*, *Cd11c* and *Cd206* ($p > 0.05$) (Figure 4.3A, 4.3B). However, when correcting for body weight there was a borderline significant effect of *APOE Epsilon* genotype ($F_{1,33}$ = 41.824, $p = 0.05$) and a significant effect of diet ($F_{1,33}$ = 15.688, $p < 0.05$) on the expression of *Cd11b*. Subsequent group comparisons revealed that *APOE4* mice on HFD+FO had significantly higher *Cd11b* expression than *APOE3* mice on HFD and HFD+FO ($p < 0.01$). These results indicate there was a trend for a greater number of ATM in AT of *APOE4* mice compared to *APOE3* mice, which was supported by the flow cytometry results showing a greater abundance of ATM in the SVF of *APOE4* mice compared to *APOE3* mice, but not by the *F4/80* mRNA expression levels. Furthermore, fish oil increased the number of ATM based on the *F4/80* and *Cd11b* expression in AT, something that was not reflected in the flow cytometry results of the SVF. There was some inconsistency between the mRNA expression levels of *F4/80* and *Cd11b*, especially for the *APOE4* mice on HFD+FO group (Figure 4.3A). F4/80 is a specific marker for tissue resident macrophages, whereas Cd11b is also expressed by neutrophils, natural killer cells and subsets of T- and B-cells, therefore F4/80 provides a more reliable measure of the resident ATM in AT. Regarding the ATM phenotype, the whole-tissue mRNA expression levels of *Cd11c* and *Cd206* did not entirely reflect the flow cytometry results from the SVF. Briefly, there were no differences in *Cd11c* and *Cd206* mRNA expression levels between the *APOE Epsilon* genotypes and diets (Figure 4.3B), which was in line with the flow cytometry results.

However, the suggestive increase in the CD11c:CD206 ratio by fish oil observed in the flow cytometry experiment was not confirmed in the mRNA expression level (Figure 4.4).

To corroborate the ATM phenotype data, mRNA expression levels of six additional M1 and M2 markers were investigated (Figure 4.5). There was no significant effect of *APOE Epsilon* genotype or diet on the expression of M1 markers *Tnfa*, *Nos2* and *Il6* ($p > 0.05$), although a trend was observed for higher expression levels in mice on HFD+FO (Figure 4.5A). In addition, there was no significant effect of *APOE Epsilon* genotype or diet on the expression of M2 markers *Mgl1/2* and *Arg1* ($p > 0.05$). However, *APOE Epsilon* genotype interacted with diet to determine IL-10 mRNA expression levels ($F_{1,34} = 6.477$, $p < 0.05$) (Figure 4.5B). *APOE4* mice on HFD+FO had significantly higher IL-10 expression than *APOE4* mice on HFD ($p < 0.05$). The mRNA expression results of the three M1 markers (*Tnfa*, *Nos2* and *Il6*) are mostly in line with the gene expression and cell surface expression of Cd11c, namely there is no effect of *APOE Epsilon* genotype or diet. If we can speak of any trend, then fish oil tended to increase, rather than decrease, the number of M1 ATM. The mRNA expression results of the M2 markers (*Mgl1/2, Arg1* and *Il10*) are also fairly consistent with the gene expression and cell surface expression of Cd206, namely there is either no significant effect of *APOE Epsilon* genotype or diet, or *APOE Epsilon* genotype interacts with diet to determine its effect. In more detail, the M2 marker expression was not significantly different between *APOE3* and *APOE4* mice on HFD, although flow cytometry data suggested different. Furthermore, there was no effect of fish oil on the expression of M2 markers in both *APOE3* and *APOE4* mice, with exception of IL-10. For this marker the most strikingly effect was observed, with fish oil increasing the mRNA expression of IL-10 in *APOE4*, but not *APOE3*, mice.

In summary, *APOE3* and *APOE4* mice had a similar number of ATM in epididymal AT and fish oil increased the number of ATM. There was no effect of *APOE Epsilon* genotype or diet on the M1 ATM phenotype expression and if we can speak of any subtle trend, then fish oil tends to increase, rather than decrease, the number of M1 ATM. Likewise, there was no consistent effect of *APOE Epsilon* genotype or diet on the M2 ATM phenotype expression indicating the mice had a similar number of M2 ATM in epididymal AT. However, fish oil increased IL-10 mRNA expression and these effects were confined to *APOE4* mice.

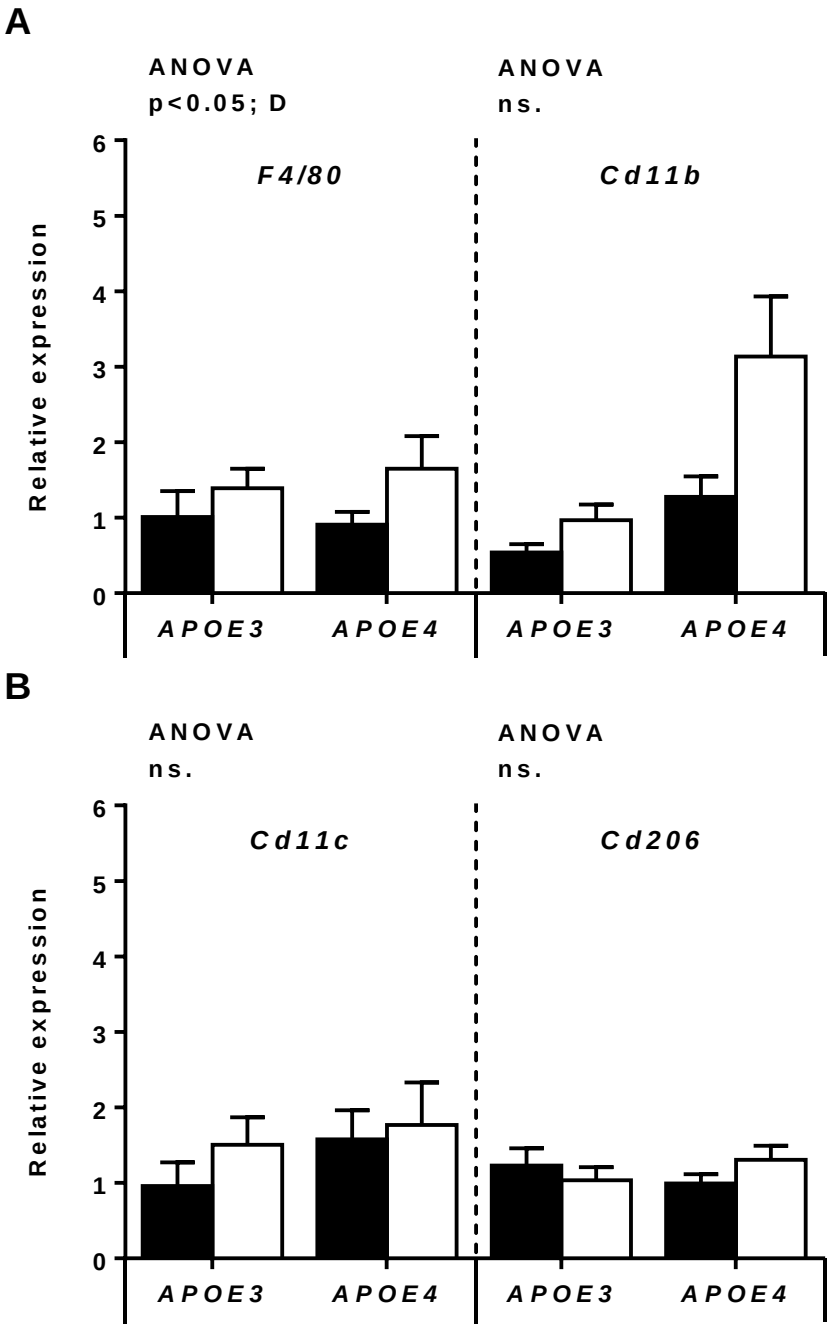

Figure 4.3. The relative mRNA expression of adipose tissue macrophage (ATM) markers *F4/80* (total ATM), *Cd11b* (total ATM), *Cd11c* (M1 marker) and *Cd206* (M2 marker) in epididymal adipose tissue of human *APOE3* and *APOE4* targeted replacement mice fed a high-fat diet (HFD) (black bars) or a HFD containing 30 g EPA+DHA / kg of diet (HFD+FO) (white bars). Statistical analysis revealed a significant effect of diet on *F4/80* expression ($F_{1,35}$ = 358.861, p < 0.05), but no differences between the *APOE Epsilon* genotypes [A]. There was no significant effect of *APOE Epsilon* genotype, diet or *APOE Epsilon* genotype * diet on the expression of *Cd11b*, *Cd11c* or *Cd206* (p > 0.05) [A,B]. Data is presented as relative quantity normalised to the reference genes *Actb*, *Atp5B* and *Gapdh*, mean ± SEM for n = 8 – 10 mice per group. Statistical analysis was done by ANOVA for *APOE Epsilon* genotype (G), diet (D) and G*D interaction effect and considered statistically significant when p < 0.05. Differences between the groups were tested using Student's *t*-test with Bonferroni correction and considered statistically significant when p < 0.008 (= 0.05 / 6). APOE, apolipoprotein E; Cd11b, integrin alpha M (Itgam); Cd11c, Integrin alpha X (Itgax); Cd206, mannose receptor C type 1 (Mrc1); F4/80, EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (Emr1).

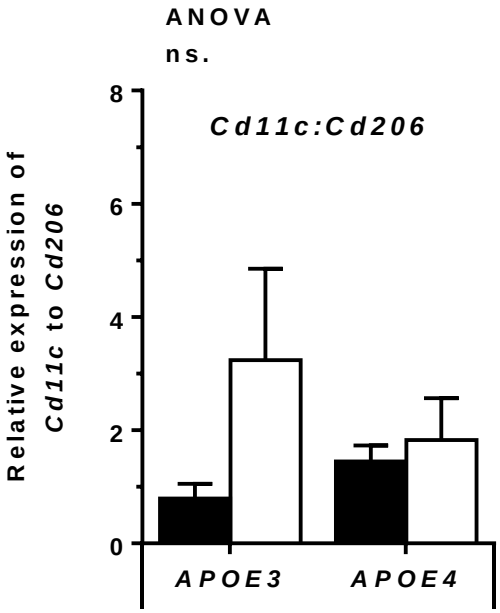

Figure 4.4. The ratio of the relative mRNA expression of the adipose tissue macrophage (ATM) marker *Cd11c* (pro-inflammatory M1 phenotype) to *Cd206* (anti-inflammatory M2 phenotype) in epididymal adipose tissue of human *APOE3* and *APOE4* targeted replacement mice fed a high-fat diet (HFD) (black bars) or a HFD containing 30 g EPA+DHA / kg of diet (HFD+FO) (white bars). Statistical analysis revealed no significant effect of *APOE Epsilon* genotype, diet or *APOE Epsilon* genotype * diet on the ratio of *Cd11c* to *Cd206* expression ($p > 0.05$). Data is presented as ratio of the relative quantity normalised to the reference genes *Actb*, *Atp5B* and *Gapdh* of *Cd11c* to *Cd206*, mean ± SEM for n = 8 – 10 mice per group. Statistical analysis was done by ANOVA for *APOE Epsilon* genotype (G), diet (D) and G*D interaction effect and considered statistically significant when $p < 0.05$. APOE, apolipoprotein E; Cd11c, Integrin alpha X (Itgax); Cd206, mannose receptor C type 1 (Mrc1).

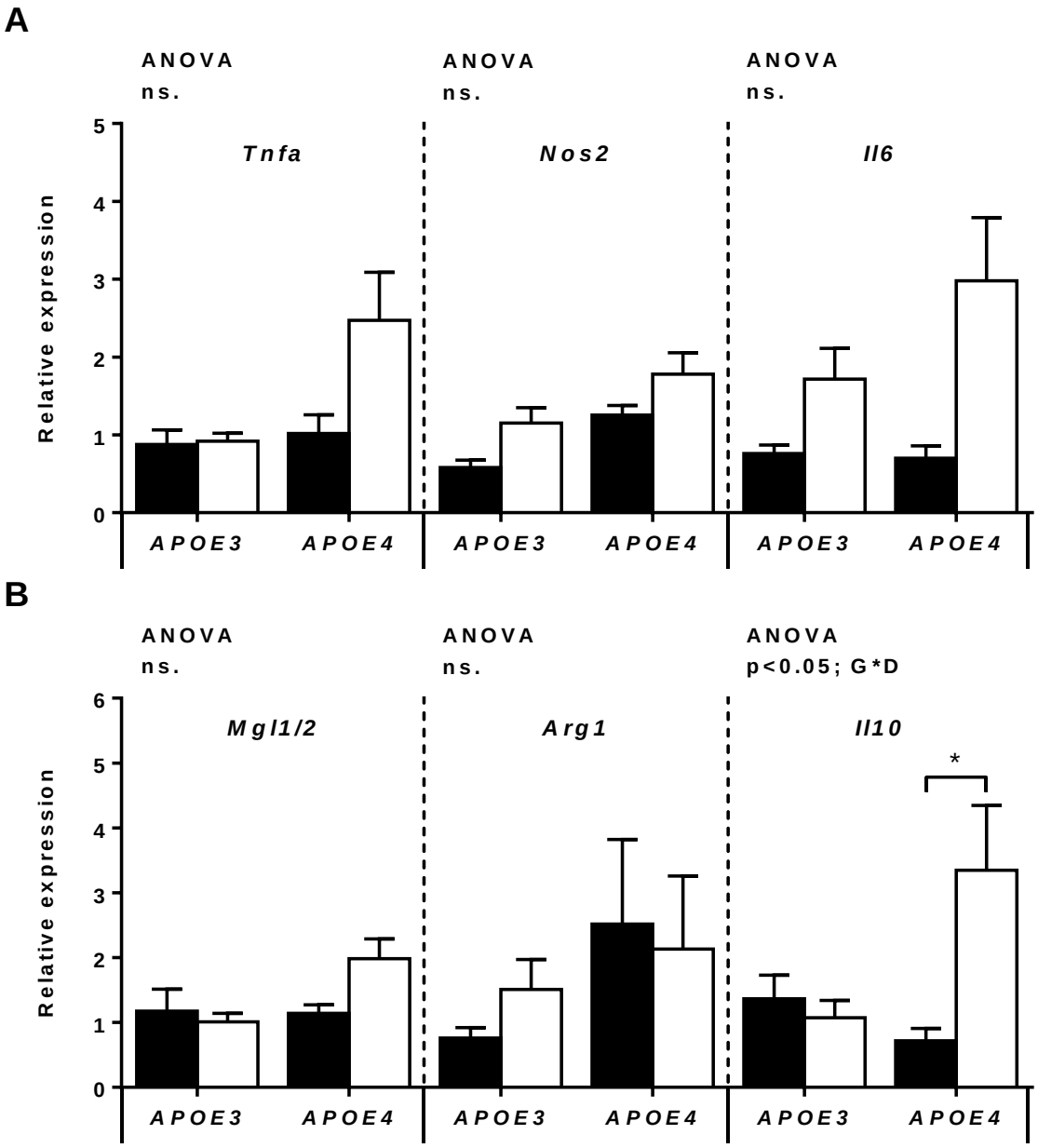

Figure 4.5. The relative mRNA expression of adipose tissue macrophage (ATM) phenotypic markers of the pro-inflammatory M1 phenotype [A] and anti-inflammatory M2 phenotype [B] in epididymal adipose tissue of human *APOE3* and *APOE4* targeted replacement mice fed a high-fat diet (HFD) (black bar) or a HFD containing 30 g EPA+DHA / kg of diet (HFD+FO) (white bars). There was no significant effect of *APOE Epsilon* genotype or diet on the expression of M1 markers *Tnfa*, *Nos2* and *Il6* ($p > 0.05$). Though, a trend for higher expression levels in mice fed HFD+FO compared to control HFD was observed [A]. *APOE Epsilon* genotype interacted with diet to determine mRNA expression levels of M2 marker IL-10 ($F_{1,34} = 6.477$, $p < 0.05$), but there was no significant effect of *APOE Epsilon* genotype or diet on the expression of the other two M2 markers *Mgl1/2* and *Arg1* ($p > 0.05$) [B]. Post-hoc analysis revealed that *APOE4* mice on HFD+FO had significantly higher IL-10 mRNA expression than *APOE4* mice on HFD ($p < 0.05$). Data is presented as relative quantity normalised to the reference genes *Actb*, *Atp5B* and *Gapdh*, mean ± SEM for n = 8 – 10 mice per group. Statistical analysis was done by ANOVA for *APOE Epsilon* genotype (G), diet (D) and G*D interaction effect or by Kruskal-Wallis test for G*D effect and considered statistically significant when $p < 0.05$. Significant effects are displayed on top of each figure. Differences between the groups were tested using Student's *t*-test with Bonferroni correction, *$p < 0.008$ (= 0.05 / 6). APOE, apolipoprotein E; Il6, interleukin 6; Il10, interleukin 10; Mgl1/2, C-type lectin domain family 10, member A (Mgl1) and macrophage galactose N-acetyl-galactosamine specific lectin (Mgl2); Nos2, inducible nitric oxide synthase; Tnfa, Tumor necrosis factor alpha.

Adipose tissue fatty acid composition

To determine if a differential LC *n-3* PUFA enrichment of AT could underlie the differential response to fish oil supplementation in *APOE3* and *APOE4* mice fed a HFD or a HFD containing 30 g EPA+DHA / kg of diet (HFD+FO), the fatty acid composition of the epididymal AT was investigated.

Statistical analysis revealed there were significant differences in the fatty acid composition in epididymal AT of *APOE3* and *APOE4* mice on HFD or HFD+FO (Table 5.5). As expected the largest differences were observed for EPA, DHA and total LC *n-3* PUFA between the HFD and HFD+FO diet in *APOE3* and *APOE4* mice. The levels of EPA (20:5 n-3) in AT were significantly different between the *APOE Epsilon* genotype * diet groups (Kruskal-Wallis, $p < 0.001$). EPA levels were negligible (0.00 and 0.01 %) in mice on HFD, but increased to about 1 % of the total fatty acids in mice on HFD+FO ($p < 0.05$). The levels of DHA in AT were determined by diet ($F_{1,36} = 738.778$, $p < 0.05$), but not *APOE Epsilon* genotype ($F_{1,36} = 0.758$ $p = 0.54$), and the effect of diet was not different according to *APOE Epsilon* genotype ($F_{1,36} = 2.763$ $p = 0.11$). DHA levels were 0.13 and 0.14 % of the total fatty acid of mice on HFD and increased to about 3 % in mice on HFD+FO ($p < 0.05$). The HFD did not contain EPA and DHA, thus the 0.13 and 0.14 % DHA in AT of mice on HFD likely reflect endogenous synthesis of DHA from α-linolenic acid (ALA). The EPA:DHA ratio in AT was 0.33 whereas the EPA:DHA ratio of the diet was 0.67, indicating that DHA was more conserved within AT. The total LC *n-3* PUFA content in AT was determined by diet ($F_{1,36} = 1114.135$, $p < 0.05$), but not *APOE Epsilon* genotype ($F_{1,36} = 3.030$, $p = 0.33$), and the effect of diet was independent of *APOE Epsilon* genotype ($F_{1,36} = 1.590$, $p = 0.22$). Briefly, total LC *n-3* PUFA increased from 0.18 % in mice on HFD to 3.97 % of total fatty acids in mice on HFD+FO ($p < 0.05$). The levels of arachidonic acid (AA, 20:4 n-6), *n-6* PUFA pre-cursor linoleic acid (LA, 18:2 n-6) and *n-3* PUFA pre-cursor ALA (18:3 n-3) were not significantly different between the *APOE Epsilon* genotypes and diets ($p > 0.05$). For ALA (18:3 n-3), the effect of diet ($F_{1,36} = 46.639$, $p = 0.09$) and its interaction with *APOE Epsilon* genotype ($F_{1,36} = 2.783$, $p = 0.10$) were borderline non-significant. There was a trend for lower LA (23 - 24 % vs. 21 %) and higher ALA (1.5 - 1.7 % vs. 1 %) levels in AT of mice on HFD+FO compared to those on HFD. In line with this, total *n-3* PUFA was higher in AT at the cost of total *n-6* PUFA in *APOE3* and *APOE4* mice on HFD+FO compared to those on HFD (total *n-3* PUFA, 6 % vs. 1 %; total *n-6* PUFA, 22 % vs. 24.5 %). Furthermore, the levels of the saturated fatty acids myristic acid (14:0) and palmitic acid (16:0) in AT were higher in *APOE3* and *APOE4* mice on HFD+FO compared to mice on HFD ($p < 0.05$). Whereas, the *APOE3* and *APOE4* mice on HFD had higher levels of the monounsaturated fatty acids vaccenic and oleic acid (18:1 *n-7* and C18:1 *n-9*) compared to mice on HFD+FO ($p < 0.05$).

Besides, the fatty acid composition in epididymal AT was not significantly different between the *APOE Epsilon* genotypes in mice fed a similar diet, either HFD or HFD+FO. Briefly, there were no significant differences in levels of EPA (20:5 n-3), DHA (22:6 n-3), ALA (18:3 n-3), LA (18:2 n-6) or AA (20:4 n-6) in AT between *APOE3* and *APOE4* mice on HFD+FO diet (p

> 0.05). The composition of the saturated fatty acids in AT showed minor differences between the *APOE Epsilon* genotypes. On the HFD+FO diet, *APOE3* mice had slightly higher levels of lauric acid (12:0) and myristic acid (14:0) compared to *APOE4* mice (0.21 vs. 0.16 % and 3.23 vs. 2.82 %, $p < 0.05$).

To explore if the LC *n-3* PUFA, EPA and DHA enrichment of epididymal AT determined the response to fish oil supplementation in *APOE3* and *APOE4* mice, we investigated the correlation between LC *n-3* PUFA, EPA and DHA levels and several outcome measures related to AT inflammation and IR using the Pearson's correlation coefficient (Appendix 4-3).
Regarding the measures of AT inflammation, we found that the level of LC *n-3* PUFA, EPA and DHA in epididymal AT was negatively associated with the expression of M2 ATM ($CD11b^+CD11c^-CD206c^+$ cells) ($r = -0.661$, $r = -0.644$ and $r = -0.668$, $p < 0.001$) and positively associated with the percentage of double positive ATM ($CD11b^+CD11c^+CD206c^+$ cells) ($r = 0.809$, $r = 0.805$ and $r = 0.810$, $p < 0.001$) and the ATM surface expression of CD11c to CD206 ($r = 0.680$, $r = 0.685$ and $r = 0.677$, $p < 0.001$) in SVF of disaggregated epididymal AT. There was no correlation between LC *n-3* PUFA, EPA and DHA levels and the percentage of ATM ($CD11b^+$ cells) and M1 ATM ($CD11b^+$ $CD11c^+CD206^-$ cells) in SVF of disaggregated epididymal AT. The *F4/80* mRNA expression levels correlated with EPA levels ($r = 0.339$, $p < 0.05$) and *Cd11b* mRNA expression levels correlated with LC *n-3* PUFA, EPA and DHA levels in AT ($r = 0.344$, $r = 0.348$ and $r = 0.337$, $p < 0.05$). There was no relationship between *Cd11c*, *Cd206*, *Il10* mRNA expression and LC *n-3* PUFA, EPA and DHA levels in AT. These findings support that there is a positive relation between LC *n-3* PUFA and the number of ATM in epididymal AT of *APOE3* and *APOE4* mice.
Regarding the measures related to IR (see Chapter 3), the level of LC *n-3* PUFA, EPA and DHA levels in AT were negatively associated with the homeostatic model assessment - insulin resistance (HOMA-IR) ($r = -0.540$, $r = -0.534$ and $r = -0.541$, $p < 0.001$), fasting plasma glucose ($r = -0.416$, $r = -0.420$ and $r = -0.410$, $p < 0.01$) and fasting plasma insulin ($r = -0.520$, $r = -0.514$ and $r = -0.522$, $p < 0.01$) and positively associated with *Glut4* mRNA expression in epididymal AT ($r = 0.401$, $r = 0.459$ and $r = 0.376$, $p < 0.05$). These findings indicate there is a negative relationship between LC *n-3* PUFA and measures related to IR in *APOE3* and *APOE4* mice.

Table 4.5. Fatty acid composition (% of total fatty acids) of the epididymal adipose tissue of human *APOE3* and *APOE4* TR mice fed a high-fat diet (HFD) or HFD containing 30 g EPA+DHA / kg of diet (HFD+FO).

Saturated	**APOE3-HFD**	**APOE3-HFD+FO**	**APOE4-HFD**	**APOE4-HFD+FO**	
12:0	0.02 ± 0.01^{a}	0.21 ± 0.01^{b}	0.05 ± 0.03^{a}	0.16 ± 0.01^{c}	Kruskal-Wallis p<0.001; G*D
14:0	0.87 ± 0.03^{a}	3.23 ± 0.04^{b}	0.98 ± 0.04^{a}	2.82 ± 0.06^{c}	ANOVA p<0.001; G*D
16:0	19.36 ± 0.31^{a}	22.64 ± 0.27^{b}	20.07 ± 0.41^{a}	23.40 ± 0.70^{b}	Kruskal-Wallis p<0.001; G*D
18:0	3.90 ± 0.14	4.20 ± 0.14	3.19 ± 0.20	3.70 ± 0.22	ANOVA ns.
20:0	0.00 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.06 ± 0.05	Kruskal-Wallis ns.
Monounsaturated					
14:1	0.01 ± 0.01^{a}	0.07 ± 0.01^{b}	0.00 ± 0.00^{a}	0.10 ± 0.02^{b}	Kruskal-Wallis p<0.001; G*D
16:1 n-7	4.25 ± 0.15	6.52 ± 0.20	5.48 ± 0.29	6.77 ± 0.39	ANOVA ns.
18:1 (n-7 & n-9)	44.66 ± 0.45^{a}	33.18 ± 0.19^{b}	44.33 ± 0.49^{a}	33.86 ± 0.35^{b}	ANOVA p<0.05; D
20:1 n-9	0.60 ± 0.03	0.54 ± 0.01	0.59 ± 0.03	0.51 ± 0.02	ANOVA ns.
22:1 n-9	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.09 ± 0.03	Kruskal-Wallis ns.
24:1 n-9	0.05 ± 0.01^{a}	0.46 ± 0.02^{b}	0.01 ± 0.01^{a}	0.47 ± 0.02^{b}	Kruskal-Wallis p<0.001; G*D
Polyunsaturated					
18:2 n-6 (LA)	24.00 ± 0.21	21.25 ± 0.20	23.13 ± 0.47	21.33 ± 0.42	ANOVA ns.
18:3 n-6 (GLA)	0.07 ± 0.01	0.12 ± 0.02	0.06 ± 0.03	0.10 ± 0.01	Kruskal-Wallis p<0.05; G*D
18:3 n-3 (ALA)	1.03 ± 0.05	1.68 ± 0.03	1.00 ± 0.06	1.47 ± 0.06	ANOVA ns.
18:4 n-3	0.00 ± 0.00^{a}	0.48 ± 0.05^{b}	0.00 ± 0.00^{a}	0.36 ± 0.03^{b}	Kruskal-Wallis p<0.001; G*D
20:2 n-6	0.44 ± 0.01^{a}	0.28 ± 0.01^{b}	0.41 ± 0.01^{a}	0.27 ± 0.01^{b}	Kruskal-Wallis p<0.001; G*D
20:3 n-6	0.16 ± 0.01^{a}	0.14 ± 0.00^{b}	0.19 ± 0.01^{a}	0.14 ± 0.00^{b}	ANOVA ns.
20:3 n-3	0.09 ± 0.04^{a}	0.09 ± 0.01^{ab}	0.01 ± 0.01^{a}	0.07 ± 0.01^{b}	ANOVA ns.
20:4 n-6 (AA)	0.28 ± 0.02	0.35 ± 0.01	0.33 ± 0.01	0.34 ± 0.01	ANOVA ns.
20:4 n-3	0.00 ± 0.00^{a}	0.33 ± 0.01^{b}	0.00 ± 0.00^{a}	0.27 ± 0.01^{c}	Kruskal-Wallis p<0.001; G*D
20:5 n-3 (EPA)	0.01 ± 0.01^{a}	1.19 ± 0.05^{b}	0.00 ± 0.00^{a}	0.97 ± 0.07^{b}	Kruskal-Wallis p<0.001; G*D
22:5 n-3 (DPA)	0.05 ± 0.02	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	Kruskal-Wallis ns.
22:6 n-3 (DHA)	0.13 ± 0.01^{a}	3.02 ± 0.11^{b}	0.14 ± 0.02^{a}	2.71 ± 0.13^{b}	ANOVA p<0.05; D
LC n-3 PUFA	0.20 ± 0.03^{a}	4.22 ± 0.15^{b}	0.16 ± 0.02^{a}	3.71 ± 0.19^{b}	ANOVA p<0.05; D

Values are mean ± SEM for n = 10 animals per group. Statistical analysis was done by ANOVA for *APOE Epsilon* genotype (G), diet (D) and G*D interaction effect using untransformed or $\sqrt{(x+0.001)}$ transformed data or by Kruskal-Wallis test for G*D effect using untransformed data and considered statistically significant when $p < 0.05$. Continues on the next page..

…continued from previous page. The data was normally distributed for all outcome measure but many outcome measure had unequal variances Therefore, Student's *t*-test assuming equal variances or Student's *t*-test not assuming equal variances with Bonferroni correction was used to test for differences between APOE3 HFD and APOE3-HFD+FO, APOE4-HFD and APOE4-HFD+FO, APOE3-HFD and APOE4-HFD, APOE3-HFD+FO and APOE4-HFD+FO group and considered statistically significant when $p < 0.0125$ (= 0.05 / 4), different letters indicate different means. 12:0, lauric acid; 14:0, myristic acid; 14:1, myristoleic acid; 16:0, palmitic acid; 16:1 n-7, palmitoleic acid; 17:0, margaric acid; 18:0, stearic acid; 18:1 (n-7 & n-9), vaccenic acid and oleic acid; 18:2 n-6, linoleic acid (LA); 18:3 n-6, gamma-linolenic acid (GLA); 18:3 n-3, alpha-linolenic acid (ALA); 18:4 n-3, stearidonic acid; 20:0, icosanoioc acid; 20:1 n-9, gondoic acid; 20:2 n-6, eicosadienoic acid; 20:3 n-6, dihomo-gamma-linolenic acid; 20:3 n-3, eicosatrienoic acid; 20:4 n-6, arachidonic acid (AA); 20:5 n-3, eicosapentaenoic acid (EPA); 22:1 n-9, erucic acid; 22:5 n-3, docosapentaenoic acid; 22:6 n-3, docosahexaenoic acid (DHA); 24:1 n-9, nervonic acid; APOE, apolipoprotein E; HFD, high-fat diet; HFD+FO, high fat diet containing 30 g EPA+DHA / kg of diet; LA, linoleic acid; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acids.

Discussion

In summary, *APOE3* and *APOE4* TR mice had a similar number of ATM and a similar ATM phenotype distribution in epididymal AT. Fish oil increased the number of ATM, but their effect on the ATM phenotype was less clear. Flow cytometry data suggested that fish oil increased the number of double positive ATM expressing both M1 and M2 phenotypic marker, which was not confirmed by mRNA expression analysis. In addition, mRNA analysis revealed that fish oil increased IL-10 expression in AT of *APOE4*, but not *APOE3*, mice. The LC *n-3* PUFA enrichment of the epididymal AT was not significantly different between *APOE3* and *APOE4* mice. Mice on HFD without LC *n-3* PUFA had negligible levels and dietary fish oil significantly increased the LC *n-3* PUFA levels in epididymal AT. There was a trend towards lower EPA and DHA levels in *APOE4* mice compared to *APOE3* mice on the HFD+FO.

APOE Epsilon genotype and adipose tissue macrophages

We are the first to thoroughly investigate the ATM number and phenotype in epididymal AT in *APOE3* and *APOE4* mice, and show that they had a similar ATM number and phenotype distribution upon HFD feeding. Surprisingly, *APOE3* mice on HFD showed a greater weight gain but had a similar *F4/80* and *Cd11b* expression level compared to *APOE4* mice on HFD, implying a similar ATM infiltration. These findings are in line with Arbones-Mainar *et al*. (2008), who reported no differences in the mRNA expression levels of the ATM markers *Cd68* and *F4/80* in epididymal AT between *APOE3* and *APOE4* mice fed a Western-diet (21% w/w fat, 0.2% w/w cholesterol, 34% w/w sucrose) for 8 weeks, implying no differential ATM infiltration [Arbones-Mainar et al., 2008]. Similarly, Cash *et al*. observed no difference in the *F4/80* mRNA expression in epididymal AT of *APOE3* and *APOE4* mice fed a similar Western-diet for 4 weeks [Cash et al., 2012]. Moreover, they reported a similar HFD-induced increase in *F4/80* mRNA expression in *APOE3* and *APOE4* mice fed the Western-diet compared to chow [Cash et al., 2012]. These studies together with our findings indicate that on a HFD *APOE3* and *APOE4* mice have a similar ATM infiltration rate.

Second, this is the first study thoroughly investigating the ATM phenotype distribution in epididymal AT of *APOE3* and *APOE4* mice. A study by Baitsch *et al*. has showed that apoE promoted macrophage polarisation toward M2 phenotype *in vitro* and *in vivo*, but the isoform differences remained to be elucidated [Baitsch et al., 2011]. We showed here that there were no differences in the M1:M2 ATM phenotype distribution in epididymal AT between the *APOE3* and *APOE4* mice fed a HFD or HFD containing fish oil. These results were supported by the mRNA expression level of six additional M1 (*Tnfa*, *Nos2*, *Il6*) and M2 (*Mgl1/2*, *Arg1*, *Il10*) genes, for which all but IL-10, were similar between the genotypes and diets. These findings are also in line with Cash *et al*., who investigated the mRNA expression of two M1 ATM markers, *Nos2* and *Tnfa*, in epididymal AT of *APOE3* and *APOE4* mice fed a Western-diet or control chow diet for 4 weeks and reported no differences between the genotypes [Cash et al., 2012]. Moreover, the *APOE3* and *APOE4*

mice showed a similar HFD-induced increase in *Nos2* and *Tnfa* compared to mice on chow diet. These results clearly indicate that *APOE3* and *APOE4* mice have a similar ATM phenotype distribution in epididymal AT upon HFD feeding.

Fish oil increases adipose tissue macrophages

We are the first to investigate the effect of dietary fish oil on ATM number and phenotype in the epididymal AT in *APOE3* and *APOE4* mice and show that fish oil increased the number of ATM, but did not affect the ATM phenotype. There was a significant effect of diet on the *F4/80* expression levels with 40 – 80% higher expression levels in mice on HFD+FO compared HFD, indicating dietary fish oil increases the number of ATM in the epididymal AT on a HFD background. The *Cd11b* expression levels showed a similar picture to the *F4/80* expression levels except for the *APOE4* mice on HFD+FO, who had a high *Cd11b* expression level that was not fully reflected by the *F4/80* expression level. F4/80 is a specific marker for tissue resident macrophages, whereas CD11b is also expressed by neutrophils, natural killer cells and subsets of T- and B-cells that have all been identified within AT, therefore F4/80 provides a more reliable measure of the resident ATM in AT [Lee and Lee, 2014]. The presence of neutrophils, natural killer cells and subsets of T- and B-cells in epididymal AT was not determined in this study, so we can only speculate that the *Cd11b* expression levels also reflects the expression from these cells. Thus, dietary fish oil augmented the ATM infiltration in *APOE3* and *APOE4* mice resulting in a greater ATM accumulation in these mice. These results are surprising in the context of previous studies in mice reporting beneficial effects of fish oil on AT inflammation [Muurling et al., 2003, Neuhofer et al., 2013, Saraswathi et al., 2007], ATM infiltration [Oh et al., 2010, Saraswathi et al., 2007, Todoric et al., 2006] and ATM phenotype [Neuhofer et al., 2013, Oh et al., 2010]. For example, Oh *et al*. showed using flow cytometry and immunohistochemistry a lower ATM content, a decreased number of M1 macrophages and increased number of M2 macrophages in AT of C57BL/6 wild type mice fed a HFD providing 150 mg EPA+DHA per day per mouse, compared to mice fed a control HFD [Oh et al., 2010]. In line with this, a recent randomised placebo controlled fish oil intervention trial reported a similar effect on ATM in humans [Spencer et al., 2013]. In more detail, fish oil supplementation (4 g/day) for 12 weeks decreased the number of ATM in the abdominal AT with the greatest response in subjects with a high baseline ATM count, along with a decrease in plasma and AT monocyte chemoattractant protein (MCP)-1 levels in non-diabetic subjects with MetS and IR. These studies demonstrate that fish oil can have direct beneficial effects on ATM in both mice and humans. However, although using a similar dose to Oh *et al*. we observed a modest increase in ATM number and failed to observe an effect on ATM phenotype in *APOE3* and *APOE4* mice. The modest increase in ATM number is relative to the total cell population within the tissue as mRNA was extracted from all tissue cells, with mature adipocytes being the main cell type within AT. One could speculate that the increase in ATM number upon fish oil supplementation may reflect a relative increase in ATM resulting from a slight decrease in

the number of mature adipocytes with the number of ATM remaining stable. This is a reasonable explanation given that LC *n-3* PUFA inhibit adipocyte proliferation and differentiation and reduce adipocyte hypertrophy and hyperplasia [Flachs et al., 2009]. Future studies using immunohistochemistry techniques allowing visual inspection of the relative abundance of ATM and appearance of mature adipocytes are needed to verify the effects of fish oil on ATM infiltration, their localisation and phenotype in *APOE3* and *APOE4* mice. The importance of this future research is emphasized by a previous report of an increased adipocyte size and reduced number of adipocytes in epididymal AT of *APOE4* mice upon HFD feeding compared to *APOE3* mice, although the effect of dietary fish oil was not investigated [Arbones-Mainar et al., 2008].

The impact of dietary fish oil on the ATM phenotype distribution is less clear. If we can speak of any trend, the flow cytometry data suggests fish oil tended to increase ATM coexpressing M1 and M2 phenotypic marker, which was not entirely supported by the mRNA expression analysis. The different results found for the different approaches, i.e. flow cytometry and mRNA expression analysis, could relate to different methodology. The cell surface expression of ATM markers for individual cells was investigated in the flow cytometry analysis, whereas the gene expression level of ATM markers in whole adipose tissue was investigated in the mRNA expression analysis. The whole tissue ATM marker expression level is the pooled expression of tissue macrophages and non-macrophage cells, and may not fully reflect the individual cells ATM marker expression. Furthermore, mRNA expression levels may not fully reflect protein and cell surface expression levels. Protein levels are the balance between its synthesis and degradation, and in general mRNA expression correlate well with protein levels. However, for some proteins their levels are not determined by its gene expression levels but are controlled at the translation and/or protein degradation level. Therefore, mRNA expression levels always have to be verified with protein expression levels. This could explain the discrepancies between our flow cytometry and mRNA expression results. Of note, the CD11c flow cytometry data was collected using suboptimal instrument settings allowing the detection of only a small proportion of the CD11c-Alexa700 signal. It reduced our power to detect a small difference in the cell surface expression of the M1 phenotypic marker CD11c and explains why we failed to identify any M1 ATM in the epididymal AT using flow cytometry. This is a limitation of the current study and asks for some caution when interpreting these results. Besides, our findings are in sharp contrast with the findings from Oh *et al.*, who used a similar dose of EPA and DHA and reported a reduction in M1 macrophages and an increase in M2 macrophages using flow cytometry and immunohistochemistry, with a concomitant decrease in mRNA expression levels of M1 markers (*Il6*, *Mcp1*, *Il1β*, *Nos2*, *Cd11c*) and increase in M2 markers (*Arg1*, *Il10*, *Mgl1*, *Ym1*, *Mmr*) in AT of C57BL/6 wild type mice upon fish oil supplementation [Oh et al., 2010]. Therefore, more studies in *APOE3* and *APOE4* mice are required to verify the effect of dietary fish oil on the ATM M1:M2 phenotype distribution in epididymal AT.

Fish oil increases IL-10 mRNA expression in *APOE4* mice

Our most striking finding was that the beneficial effect of fish oil on IL-10 mRNA expression in epididymal AT was confined to mice with the *APOE4* genotype. Various *in vitro* studies have demonstrated that DHA specifically, and not EPA, is responsible for these beneficial effects on IL-10 expression levels in macrophages and adipocytes [Bradley et al., 2008, Oliver et al., 2012, Weldon et al., 2007]. However, we did not find any relation between AT LC *n-3* PUFA, EPA or DHA content and IL-10 expression levels (Appendix 3-4). The effect of DHA on IL-10 expression in adipocytes can be either direct [Bradley et al., 2008] or mediated by ATM [Oliver et al., 2012]. The changes in IL-10 production have been associated with concomitant changes in insulin sensitivity in adipocytes [Lumeng et al., 2007B, Oliver et al., 2012]. The important role for IL10 in maintaining insulin sensitivity in adipocytes was demonstrated by Lumeng *et al*., who showed first that the IL-10 receptor (IL10R) was expressed by adipocytes, but not by SVF cells in epididymal AT of mice, and secondly that IL-10 prevented the TNF-α-induced IR by preventing the TNF-α-induced suppression of GLUT4 and insulin receptor protein expression and insulin signalling in 3T3-L1 adipocytes [Lumeng et al., 2007B]. Thus, IL-10 is an important insulin sensitising cytokine in adipocytes. A previous study showed increased adipocyte hypertrophy in epididymal AT of *APOE4* mice fed a Western-diet that was associated with local and systemic IR [Arbones-Mainar et al., 2008]. Therefore, the increased IL-10 mRNA expression in epididymal AT of *APOE4* mice upon fish oil supplementation could be a defensive response to the increased adipocyte stress resulting from adipocyte hypertrophy in these mice, which is aimed at maintaining adipocyte insulin sensitivity in these mice.

APOE Epsilon genotype and long chain *n-3* PUFA status

To determine if a differential DHA enrichment in AT could underlie the differential response to fish oil supplementation in *APOE3* and *APOE4* mice, the fatty acid composition of epididymal AT was investigated. There were no significant differences in the fatty acid composition, including EPA and DHA levels, between the *APOE3* and *APOE4* mice fed a similar diet. The changes in the fatty acid composition between the diet groups reflected in most cases the differential dietary fatty acid intake. Dietary fish oil increased total *n-3* PUFA in epididymal AT from 1% to 6% with a concomitant 2% reduction in total *n-6* PUFA content. EPA and DHA levels were negligible in mice on HFD diet deficient in EPA and DHA, but increased to approximately 1% and 3% upon feeding the HFD+FO containing 30 g EPA+DHA / kg of diet. The EPA and DHA enrichment in AT in our study was similar to previous reported EPA and DHA concentrations of approximately 1% and 3% of total lipids in white AT in C57BL/6 mice fed a HFD containing 30 g EPA+DHA / kg of diet for 9 weeks [Kopecky et al., 2009, Rossmeisl et al., 2012]. DHA was more conserved within AT than EPA, as the EPA:DHA ratio in epididymal AT of mice on HFD+FO was 0.33 compared to dietary EPA:DHA ratio of 0.67. This ratio is in line with previous reports [Kopecky et al., 2009, Rossmeisl et al., 2012]. Besides, we observed a trend for almost 20 % lower EPA and

10 % lower DHA levels in *APOE4* mice compared to *APOE3* mice on HFD+FO. This finding is in line with previous reports of an increased β-oxidation and altered DHA metabolism and suboptimal *n-3* PUFA availability in *APOE4* carriers [Chouinard-Watkins et al., 2013, Conway et al., 2014, Plourde et al., 2009, Vandal et al., 2014]. Briefly, DHA concentrations in plasma triglycerides were increased by only 75 % in *APOE4* carriers after a 6 week dietary supplementation with 3 g EPA+DHA / d compared to a 240 % increase in non-carriers [Plourde et al., 2009]. Moreover, in a kinetics study using isotopically labelled DHA *APOE4* carriers had 31 % lower DHA levels in plasma total lipids and a more rapid β-oxidation of DHA 1 – 28 days post-dose (40 mg [^{13}C]DHA) compared to non-carriers [Chouinard-Watkins et al., 2013]. In line with this, Vandal *et al*. showed that *APOE4* mice had higher plasma DHA levels, lower cortex DHA levels and impaired transport of DHA across the blood-brain barrier (BBB) compared to *APOE2* mice at age of 13 months after 9 months of high *n-6* / low *n-3* PUFA diet feeding (*n-3* PUFA, 0.36 g/kg of diet; no EPA or DHA) [Vandal et al., 2014]. The impaired DHA transport across the BBB in *APOE4* mice manifested itself already at the age of 4 month prior to the high *n-6* / low *n-3* PUFA feeding intervention [Vandal et al., 2014]. Thus, the *APOE4* genotype appears to be associated with an increased β-oxidation of DHA in humans and an impaired DHA transport across the BBB resulting in a suboptimal DHA bioavailability in brain of these mice. The LC *n-3* PUFA availability in liver and AT of *APOE2*, *APOE3* and *APOE4* TR mice was investigated recently by Conway *et al.* (2014) [Conway et al., 2014]. They showed that total *n-3* PUFA concentrations were 46 % lower in AT and 47 % lower in liver in *APOE4* mice compared to *APOE3* mice. In addition, the levels of DHA were 75 % lower in AT and 34 % lower in liver in *APOE4* mice compared to *APOE3* mice. However, plasma levels of total n-3 PUFA and DHA were not different between the *APOE3* and *APOE4* genotype. The lower total *n-3* PUFA and DHA levels in *APOE4* mice were associated with higher fatty acid binding protein (FABP) and carnitine palmitoyltransferase 1 (CPT1) levels in liver and higher fatty acid transport protein (FATP) levels in AT. CPT1 is the rate limiting enzyme in initiation of fatty acid oxidation, whereas FATPs and FABPs regulate transport, uptake and release of fatty acids by cells. We demonstrated that the *n-3* PUFA enrichment of epididymal AT did not significantly differ between *APOE3* and *APOE4* mice, although levels tended to be lower in *APOE4* mice. The lower *n-3* PUFA levels could be the result of the previously described increased β-oxidation in *APOE4* carriers [Chouinard-Watkins et al., 2013] resulting in an increased oxidation of EPA and DHA, although a reduced *n-3* PUFA uptake [Vandal et al., 2014] by AT cannot be ruled out as plasma *n-3* PUFA status was not investigated in this study. Furthermore, the failure to show a significant *APOE Epsilon* genotype effect on the availability of *n-3* PUFA in AT could be caused by the high dose of EPA and DHA added to the experimental diet in this study, with perhaps significant genotype-mediated differences emerging following consumption of a more modest dose of EPA and DHA. We fed mice a HFD without or with 30 g EPA+DHA / kg of diet that also contained approximately 4 g ALA / kg of diet, whereas Vandal *et al*. investigating the bioavailability of DHA in the brain used a

high *n-6* / low *n-3* PUFA diet containing only 0.36 g *n-3* PUFA / kg of diet, of which mainly ALA and no EPA or DHA [Vandal et al., 2014]. Similarly, Conway *et al*. used a low fat (5.0 % w/w) and high *n-6* / low *n-3* PUFA diet containing 0.4 g ALA / kg of diet and no EPA or DHA [Conway et al., 2014]. Thus, by providing a dietary excess of EPA and DHA it is possible that we might have overcome the potential impact of *APOE4* genotype on LC *n-3* PUFA bioavailability in tissues, such as AT, something that remains to be elucidated. Besides, the EPA and DHA levels in epididymal AT were negligible in *APOE3* and *APOE4* mice on the HFD deficient in EPA and DHA and there was no *APOE Epsilon* genotype effect indicating that the bioavailability of EPA and DHA in AT is reflecting dietary fatty acid intake and *do novo* synthesis of EPA and DHA from its precursor ALA is unlikely to play an important role in epididymal AT. The 20 % and 10 % lower EPA and DHA levels in AT of *APOE4* mice compared to *APOE3* mice did not reach statistical significance but might be of clinical relevance. Overweight subjects and metabolically unhealthy obese (MUO), but not metabolically healthy obese (MHO), have lower plasma levels of EPA and DHA compared to lean healthy subjects [Fernandez-Real et al., 2003, Perrault et al., 2014]. This suggests a lower whole-body bioavailability of LC *n-3* PUFA in overweight and MUO individuals that might also persist at tissue levels.

In summary, we showed that epididymal AT fatty acid composition is reflecting dietary intake and *APOE4* mice tend to have a lower bioavailability of EPA and DHA in epididymal AT. Future studies should confirm the impact of *APOE Epsilon* genotype on LC *n-3* PUFA bioavailability in plasma, brain, AT and liver using different dietary LC *n-3* PUFA dosages, e.g. deficient, low and high dose. Besides, studies using isotopically labelled fatty acids should be used to verify the impact of *APOE Epsilon* genotype on the uptake and metabolism of LC *n-3* PUFA within these tissues.

Anti-inflammatory effects of long chain *n-3* PUFA

The anti-inflammatory effects of LC *n-3* PUFA on cell and tissue function were originally suggested to be mediated via four general mechanisms; 1) transcription factor function and cell signalling; 2) cell membrane and lipid raft fatty acid composition influencing membrane fluidity, protein composition and receptor function; 3) cell membrane fatty acid composition influencing production of fatty acid-derived bioactive mediators; 4) oxidative status [Calder, 2012]. Recently, a fifth mechanism has been identified that involves signalling via lipid sensing G-protein coupled receptors (GPR), such as GPR120 and GPR40, that are activated by long chain fatty acids [Oh et al., 2010].

To start, the LC *n-3* PUFA EPA and DHA are known to influence the peroxisome proliferator-activated receptor (PPAR) and nuclear factor kappaB (NF-κB) signalling pathways. Specifically, LC *n-3* PUFA serve as endogenous ligands for PPARα and PPARγ, which are important regulators of fatty acid metabolism, glucose homeostasis, cellular proliferation, differentiation and apoptosis and inflammation [Calder, 2012, Chinetti et al., 2000, Delerive et al., 2001]. PPARα is mainly expressed by hepatocytes and PPARγ is mainly expressed by

adipocytes, whereas macrophages express both [Chinetti et al., 2000, Delerive et al., 2001]. The activation of these PPARs by LC *n-3* PUFA is in part responsible for their anti-inflammatory and metabolic effects in adipocytes and macrophages, such as decreased inflammation and increased β-oxidation in macrophages and improved adipocyte differentiation, lipid storage and insulin sensitivity in adipocytes [Calder, 2012]. In addition, PPAR activation by LC *n-3* PUFA inhibits the NF-κB signalling pathway which plays an important role in inflammation associated metabolic diseases, via direct binding of the PPAR-fatty acid complex to NF-κB thereby preventing the expression of NF-κB target genes [Baker et al., 2011]. Our group has shown that the basal NF-κB transcription factor activity was 80 % higher and the LPS-induced increase in NF-κB activity was 1.5 – 2 fold greater in APOE4 macrophages compared to APOE3 macrophages [Jofre-Monseny et al., 2007A]. Hence, the NF-κB signalling pathway provides a potential link between the *APOE Epsilon* genotype and their associated inflammatory and metabolic phenotype, which potentially can be reversed by LC *n-3* PUFA. Although the interactive impact of *APOE Epsilon* genotype and fish oil on NF-κB signalling pathway remains to be investigated, Oliver *et al*. demonstrated that DHA decreased LPS-induced NF-κB activity in macrophages that in turn resulted in decreased adipocyte inflammation and maintained adipocyte insulin sensitivity [Oliver et al., 2012]. Noteworthy, the reduction in NF-κB activity was accompanied by a greater IL-10 production in both macrophages and adipocytes [Oliver et al., 2012], suggesting that suppression of the NF-κB pathway by LC *n-3* PUFA is one mechanism via which LC *n-3* PUFA exert their anti-inflammatory effects, such as increasing AT IL-10 expression as we observed in the current study.

The second important mechanism through which LC *n-3* PUFA EPA and DHA exert anti-inflammatory effects is through the formation of bioactive mediators, such as eicosanoids, resolvins and protectins, through a series of reactions involving cyclooxygenase (COX) and lipoxygenase (LOX) enzymes [Calder, 2009]. It is increasingly recognised that these mediators, in particular resolvins, play an important role in governing the inflammatory tone in obese AT [Claria et al., 2012, Titos and Clària, 2013]. For example, obese AT showed an increased production of the pro-inflammatory *n-6* AA-derived leukotriene B4 (LTB_4), with LTB_4 triggering NF-kB activation and increasing production of MCP-1, TNF-α and IL-6 in AT explants [Horrillo et al., 2010]. Furthermore, the production of anti-inflammatory *n-3* DHA-derived mediators resolvin D1 (RvD1), resolvin D1 precursor (17-hydroxydocosahexaenoic acid, 17-HDHA) and protectin D1 (PD1) were decreased in obese AT, together with a marked decrease in markers of resolvin biosynthesis (18-HEPE, 17-HDHA) [Claria et al., 2012, Neuhofer et al., 2013]. In other words, there appears to be a disturbed balance in the pro-inflammatory and anti-inflammatory bioactive mediator levels in obese AT. Using an elegant study design, Neuhofer *et al*. demonstrated that dietary EPA and DHA supplementation was able to restore the obesity-associated deficit in 17-HDHA and PD1 levels together with mRNA levels of 12/15-LOX, an essential enzyme for 17-HDHA and PD1 biosynthesis in mice, in epididymal AT [Neuhofer et al., 2013]. Moreover, Claria *et al.*

showed that RvD1 and RvD2 not only decreased inflammatory cytokine production and restored adiponectin levels in AT *ex vivo*, but also decreased the *ex vivo* MCP-1-induced monocyte adhesion to adipocytes and monocyte transmigration [Claria et al., 2012]. In line with this, Hellmann *et al*. showed that RvD1 treatment decreased the number of crown-like structures rich in pro-inflammatory M1 macrophages and the M1:M2 ratio in epididymal AT, and improved insulin sensitivity in *db*/*db* mice [Hellmann et al., 2011]. These findings clearly indicate an important role for eicosanoids and resolvins in AT inflammation in obesity, that provides a pivotal mechanism through which EPA and DHA exert their anti-inflammatory effects in AT.

Finally, EPA and DHA can exert their anti-inflammatory effects through GPR such as GPR40 and GPR120, which can be activated by long chain fatty acids [Hirasawa et al., 2005, Itoh et al., 2003]. GPR120 is the only receptor that is highly expressed in mature adipocytes and $CD11c^+$ pro-inflammatory macrophages and has been shown to be essential in mediating the anti-inflammatory and insulin sensitising effects of EPA and DHA [Oh et al., 2010]. Briefly, Oh *et al*. demonstrated that fish oil supplementation reduced the HFD-induced accumulation of pro-inflammatory M1 ($Cd11b^+Cd11c^+$) ATM and improved insulin sensitivity in wild type C57BL/6 mice, but not in *GPR120*$^{-/-}$ mice [Oh et al., 2010]. Also, fish oil induced the mRNA expression of M2 markers *Arg1*, *Il10*, *Mgl1*, *Ym-1*, *MMR* and decreased expression of M1 markers *Il6*, *Mcp1*, *Il1β*, *Nos2*, *Cd11c* in AT solely in wild type mice. These results illustrate the important role GPR120 plays in mediating the anti-inflammatory effects of fish oil. GPR120 activation has been shown to inhibit IKKβ / NF-κB (inhibitor of nuclear factor kappa-B kinase / nuclear factor kappa-B) and JNK / AP1 (c-Jun N-terminal kinase / activator protein-1) signalling pathways, providing a potential mechanism via which GPR120 activation reduces inflammation [Oh et al., 2010].

A strength of this study is that the human *APOE* TR mice express human *APOE* under control of the murine regulatory sequence and produce apoE at physiological levels. This gives us a complete *in vivo* model for direct comparison of *APOE Epsilon* isoform-specific effects. In addition, this mouse model allowed us to use an experimental design tailored specifically to answer our research question while controlling for many confounding intrinsic and environmental factors difficult to control for in human studies, such as genetic background and diet. This provided us significant power to evaluate the effects of *APOE Epsilon* genotype and fish oil on AT inflammation and LC *n-3* PUFA status. Besides, in human studies it is difficult to study internal organs, such as the liver and visceral AT, because they are difficult if not impossible or unethical to collect. This mouse model provided free access to our tissue of interest (i.e. visceral AT) and enabled us to perform a thorough investigation into the role of apoE in AT biology, which is a strength of this study.

In this study the number and phenotype of ATM were evaluated using flow cytometry and mRNA expression analysis. The first provided a qualitative measure of the cell surface

expression of phenotypic markers for every single cell, whereas the second provided a quantitative measure of the mRNA expression levels of these markers in whole tissue. Therefore, both methods complement each other. Immunohistochemistry is considered the gold standard providing quantitative data on the number, phenotype and localisation of macrophages in AT. This technique also allows the measurement of adipocyte size that provides insight into adipocyte hypertrophy, which is associated with macrophage infiltration. Future studies in human *APOE* TR mice should investigate adipocyte and macrophage phenotype using immunohistochemistry.

As mentioned above, the CD11c flow cytometry data was collected using suboptimal instrument settings allowing the detection of only a small proportion of the CD11c-Alexa700 signal and reduced our power to detect a small difference in the cell surface expression of the M1 phenotypic marker CD11c. It explains why we failed to identify any M1 ATM in the epididymal AT using flow cytometry and is a key limitation of the current study.

Another limitation of this study is that we did not include a low-fat diet control group. The findings of this study suggest LC *n-3* PUFA do not alter AT inflammation status and if we can speak of any trend, then LC *n-3* PUFA selectively increase anti-inflammatory gene expression in AT in human *APOE4* TR mice. Since this study did not include a low-fat diet control group, the HFD-induced inflammatory marker response in AT could not be determined. If the HFD-induced inflammatory response was only small or absent, it may explain the overall neutral effect of LC *n-3* PUFA on AT inflammation in this study.

We investigated the effects of LC *n-3* PUFA on AT inflammation in a humanised mouse model which may not fully represent the response in humans, and is considered a limitation of this study.

This study was most likely underpowered for flow cytometry analysis, mRNA expression analysis and analysis of the fatty acid composition in epididymal AT. Our study with n = 10 mice per *APOE Epsilon* genotype * diet group was designed to detect a standardised mean difference (SMD) of 1.45 with power of 90 % at significance level of $\alpha = 0.05$ (see Chapter 3 Material & Methods). Using the variation data (i.e. standard deviation) obtained from this study for the outcomes ATM infiltration and phenotype and LC *n-3* PUFA status in AT, we retrospectively determined the effect size (absolute mean difference) our study could detect with a power of 90 %. Our study only had the power to detect a 10 % absolute difference in percentage of percentage of M2 ATM in the SVF of the epididymal AT, whereas the difference between the *APOE Epsilon* genotypes was approximately 7 – 9%. Moreover, our study only had a power to detect a difference in *Cd11c* and *Cd206* mRNA expression levels of 0.7 or larger, whereas the approximate difference between *APOE Epsilon* genotypes and diets was 0.5 or smaller. Thus overall our study was underpowered to detect an effect of *APOE Epsilon* genotype on ATM phenotype in flow cytometry and mRNA expression analysis. The absolute difference in EPA and DHA concentrations in the epididymal AT between *APOE3* and *APOE4* mice was approximately 0.2 and 0.3 %, whereas our study

only had the power to detect an absolute difference of 0.9 % and 2.0 % or larger for EPA and DHA concentrations. Hence our study was also underpowered to detect an effect of *APOE Epsilon* genotype on LC *n-3* PUFA status in epididymal AT.

In conclusion, our results indicate that *APOE3* and *APOE4* mice have a similar ATM infiltration rate that is increased by dietary fish oil. The ATM phenotype distribution in the epididymal AT was similar between the *APOE Epsilon* genotypes and was not influenced by dietary fish oil. However, the beneficial effect of dietary fish oil on IL-10 expression in epididymal AT was confined to mice with the *APOE4* genotype. Although more research is needed to verify these results, it is tempting to speculate that the increased AT IL-10 expression in *APOE4* mice on HFD+FO represents an *APOE Epsilon* genotype-specific fish oil-induced response to control AT inflammation and maintain adipocyte insulin sensitivity. In contrary to previous reports, we observed no differential bioavailability of LC *n-3* PUFA in epididymal AT of *APOE3* and *APOE4* mice.

Chapter 5. *In vitro* studies – macrophage polarisation: The impact of *APOE Epsilon* genotype on macrophage polarisation towards pro- (M1) and anti-inflammatory (M2) phenotype.

Studies in primary murine bone marrow-derived APOE3 and APOE4 macrophages

Introduction

Macrophages govern adipose tissue inflammation

The expansion of adipose tissue (AT) with obesity is associated with structural and functional changes and alterations in cellular composition and cell phenotype [Dalmas et al., 2011, Ouchi et al., 2011]. Adipocytes increasingly store triglycerides becoming hypertrophic and start to secrete mediators that attract macrophages which infiltrate the expanding AT. The recruited macrophages (ATM) will predominantly acquire a pro-inflammatory (M1) phenotype and start to outnumber the resident ATM that have an anti-inflammatory (M2) phenotype [Weisberg et al., 2003, Wentworth et al., 2010]. The macrophage recruitment and phenotype switch towards the M1 phenotype are thought to be fundamental steps in AT inflammation resulting in AT dysfunction and subsequent systemic insulin resistance [Osborn and Olefsky, 2012]. A simplified M1/M2 phenotype model of macrophage polarisation has been introduced in Chapter 4 [Table 4.1, Morris et al., 2011]. Briefly, M1 macrophages are classically activated pro-inflammatory macrophages that the express cell surface marker CD11c (integrin alpha X chain) and produce the pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-12 and reactive oxygen species (ROS). On the other hand, M2 macrophages are alternatively activated anti-inflammatory macrophages that express the cell surface marker CD206 (mannose receptor) and MGL1 (macrophage galactose type C-type) and produce the anti-inflammatory cytokines IL-10, IL-1 receptor antagonist (IL-1RA) and arginase (Arg). In summary, the ATM number and phenotype are important determinants of AT inflammation, AT function and metabolic health in obesity [Blüher, 2010, Lumeng et al., 2007B, Ouchi et al., 2011].

APOE Epsilon genotype and macrophage inflammatory state

Besides its role in lipoprotein metabolism apolipoprotein E (apoE) has immunomodulatory properties [Jofre-Monseny et al., 2008A]. Previous research from our group has demonstrated that the *APOE4* allele is associated with an exacerbated inflammatory response upon lipopolysaccharide (LPS) stimulation in murine RAW 264.7 macrophages

stably transfected with human *APOE3* or *APOE4* gene [Jofre-Monseny et al., 2007A]. In more detail, apoE4-expressing macrophages had increased IL-1β, TNF-α, and macrophage inflammatory protein (MIP)-α mRNA levels, increased TNF-α and decreased IL-10 protein levels, with no difference in IL-6 levels compared to apoE3-expressing macrophages. Only one study to date has specifically investigated the impact of apoE on macrophage polarisation towards M1 and M2 phenotypes. In this study, Baitsch *et al*. investigated the impact of exogenous apoE on macrophage phenotype in RAW 264.7 macrophages stably expressing the human apoE receptors, VLDL-receptor (VLDL-R) and apoE receptor-2 (apoER2), and demonstrated that exogenous exposure to apoE (5 μg/mL for 24 hr) promoted a phenotypic switch from the pro-inflammatory M1 to the anti-inflammatory M2 phenotype [Baitsch et al., 2011]. Although this study showed apoE exerts anti-inflammatory effects, it remains to be elucidated whether these effects are exerted in an isoform-dependent manner. In conclusion, these findings support a potential impact of apoE on the macrophage inflammatory response and phenotype [Jofre-Monseny et al., 2007A, Baitsch et al., 2011], however future studies are needed to verify the impact of apoE Epsilon isoforms on macrophage polarisation.

Research gaps

Our understanding of how genetic determinants interact with the environment and lead to the development of metabolic disease is still limited [Osborn and Olefsky, 2012]. The macrophage-mediated AT inflammation leading to AT dysfunction has been recognised as a main pathological feature of systemic insulin resistance and systemic inflammation in obesity. However, knowledge of the regulation of AT inflammatory status is incomplete including the factors that direct the polarisation state of ATM to either a pro- or anti-inflammatory phenotype [Osborn and Olefsky, 2012].

The described effect of apoE4 on the inflammatory response and the effect of apoE on macrophage polarisation were reported in the murine RAW 264.7 cell line, which naturally produces low levels of apoE [Jofre-Monseny et al., 2007A, Baitsch et al., 2011]. Jofre-Monseny *et al.* transfected the cells with mammalian expression vectors to induce endogenous apoE3 or apoE4 expression, whereas Baitsch *et al*. coincubated cells with exogenous apoE. Since both studies have not used primary cells and cells that naturally express apoE3 and apoE4 at physiological levels, the acquired phenotype may not fully represent of what happens in the body. Furthermore many *in vitro* studies employ supra-physiological concentrations of compounds therefore their findings may not reflect a physiological response but rather an adaptation to the stimulus (i.e. hormesis). Thus, the impact of *APOE Epsilon* genotype on inflammatory status in the more habitual basal state is still unknown.

Research question

The aim of this study was to investigate the impact of *APOE Epsilon* genotype on the 'basal' pro- and anti-inflammatory phenotype in primary murine bone marrow-derived macrophages (BMM).

Hypotheses

This study will investigate the 'basal' pro- and anti-inflammatory phenotype in primary murine BMM obtained from human *APOE* targeted replacement (TR) mice that express apoE at physiological levels [Sullivan et al., 1997]. The mice will not be exposed to a dietary stressor (nutrient and fat surplus) like our previous *in vivo* studies or an inflammatory stimulus like previous *in vitro* studies. In Chapter 4 our flow cytometry data suggested that the *APOE4* allele is associated with a greater abundance of macrophages and a higher prevalence of the M2 phenotype in the stromal vascular fraction (SVF) of disaggregated epididymal AT, although mRNA expression analysis did not corroborate this. Thus we first want to understand as to whether there is a difference in macrophage polarisation according to *APOE Epsilon* genotype, i.e. is the *APOE4* allele associated with higher expression of the anti-inflammatory M2 phenotype in macrophages compared to *APOE3* allele?

Materials & Methods

Primary BMM were obtained from the human *APOE* TR strain, which we have also used for our *in vivo* studies (see Chapter 3 and 4). Flow cytometry analysis allowed characterisation of the BMM phenotype using the cell surface expression of the macrophage phenotype markers; CD11b (macrophage marker), CD11c (M1 marker) and CD206 (M2 marker).

Bone marrow-derived macrophages isolation and culture

BMM were obtained from six 11 – 20 week old male human *APOE3* and *APOE4* TR mice (n = 3 per *APOE Epsilon* genotype). The *APOE3* and *APOE4* TR mice are homozygous for the human *APOE3* or *APOE4* gene by targeted replacement of the endogenous mouse *apoe* gene with human *APOE3 or APOE4* gene, as previously described (Chapter 3). Male mice were used to match our previous *in vivo* studies (Chapter 3 and 4). Mice were fed a palletized maintenance diet (Rat and Mouse No.3 Breeding Expanded diet (RM3); SDS Diets, Essex, UK) (Table 5.1), housed in groups of two to five per cage, allowed free access to food and water and maintained under a 12 h light – 12 h dark cycle (7 am – 7 pm) in a temperature-controlled environment (21 °C, humidity 55 %) in pathogen-free facility at the University of East Anglia.

Table 5.1. Composition of the maintenance diet.

Macronutrients (kcal%)	
Protein	27
Carbohydrate	61
Fat	12
Total	100
Kcal/g	3.31
Fatty acid composition (%)	
12:0	0.05
14:0	0.17
16:0	0.37
18:0	0.11
16:1 n-7	0.09
18:1 n-9	1.01
18:2 n-6	1.26
18:3 n-3	0.17
20:4 n-6	0.12

Bone marrow-derived macrophage culture - materials

Macrophage medium composed of Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine (2 mmol/L); 1 % sodium pyruvate; 1 % non-essential amino acids; 1 % penicillin-streptomycin (10000 IU/ml); 24 µM β-mercaptoethanol; 10 % fetal calf serum; and 10 % L-conditioned medium. L-conditioned medium was source of L-929 murine fibroblast cell culture, which produce colony stimulating factor-1, also known as macrophage colony-stimulating factor (M-CSF), that controls the survival, proliferation and differentiation of monocytes, macrophages and its progenitor cells from bone marrow [Manzanero, 2012]. L-

929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (4500 mg/L glucose, 1 % L-glutamine, 1 % sodium pyruvate, 1 % PS (10000 IU/ml), 10 % FCS) in 75 cm^2 flasks until confluent at 5 % CO_2, 37 °C, then lids of the flask were closed, and medium was left to condition with CSF-1 for 3 weeks. After three weeks, L-conditioned medium was aspirated and centrifuged, supernatants were pooled and sterile filtered (0.2 µm), 5 ml aliquots were stored at -20 °C until use. All culture medium ingredients were obtained from Life Technologies, Paisley, UK.

Bone marrow-derived macrophage culture - protocol

Bone marrow was removed from femurs by flushing with 20 ml of macrophage medium using a 25-G needle and syringe. Cells were counted on the Cellometer Auto T4 Cell Viability Counter (Nexcelom Bioscience, Lawrence, Massachusetts U.S.A), centrifuged for 5 min at 1000 rcf and resuspended at 1 x 10^6 cells/ml, and plated (11 ml, 1 x 10^6 cells/ml) onto bacteriological petri dishes (Falcon©, BD Biosciences, Oxford, UK) and incubated at 5 % CO_2, 37 °C. After three days, non-adherent stem cell population was removed and plated onto new bacteriological petri dishes (11 ml, 1 x 10^6 cells/ml) and incubated (5 % CO_2, 37 °C). After seven days, the non-adherent population was discarded and adherent BMM were scraped into fresh medium and immediately used for flow cytometry experiments.

Flow cytometry analysis

Flow cytometry analysis was performed as described in Chapter 4. Briefly, BMM were resuspended in 100 µl PBS with 10 mM D-glucose and 1 % bovine serum albumin (BSA) (1 x 10^6 cells/ml); incubated with the antibodies at the recommended concentrations, CD11b-PE, 0.2 mg/ml (BD Biosciences), CD11c-Alexa700, 0.2 mg/ml (eBiosciences), CD206-FITC, 0.1 mg/ml (AbD Serotec); and immediately analysed using BD Accuri™ C6 (BD Biosciences). Isotype controls were used to determine non-specific binding; single stain and fluorophore minus-one (FMO) controls were used to set gates and compensation; and a representative sample incubated with 7-AAD (BD Biosciences) was used to assess cell viability. For each sample, data of 30,000 events were collected using the gate capturing our target cell population ($CD11b^+$ cells) as follows; CD11b-PE, 488 nm laser with 585/40 BP filter; CD206-FITC, 488 nm laser with 533/30 BP filter; CD11c-Alexa700, 640 nm laser with 670/LP; 7-AAD, 488 nm laser with 675/25 BP filter. Forward and backward-gating strategies were used to 1.) validate our gate capturing our target cell population ($CD11b^+$ cells); 2.) verify our target cell population consisted of single cells; 3.) confirm that debri and cells excluded from analysis showed no expression of the markers of interest (data not shown). Compensation was set as described in Chapter 4 using target cell populations stained for only one of the markers of interest (single stain samples) so that the median of negative cell populations were similar (Table 5.2). No compensation was needed for CD11c-Alexa700-670/LP into 533/30 or 585/40 BP filter.

Table 5.2. Spillover table.

Spillover (%)	CD206-FITC 533/30	CD11b-PE 585/40	7-AAD 675/25	CD11c-Alexa700 670/LP
CD206-FITC 533/30		3.40	N/A	0.00
CD11b-PE 585/40	1.90		N/A	0.00
7-AAD 675/25	N/A	N/A		N/A
CD11c-Alexa700 670/LP	2.50	0.00	N/A	

In this study, on average 31 ± 7 (SD) % of all events were within our target cell population. Off the cells within our target cell population 99.5 ± 0.1 % were $CD11b^{+}$. The cell viability was 89 ± 2 % (n = 2).

Statistical analysis

Two-tailed Student's *t*-test was used to test for differences between the *APOE Epsilon* genotypes and considered statistically significant when $p < 0.05$. All statistical analysis was performed using SPSS 18.0.

Results

In this study we investigated the cell surface expression of CD11b, CD11c, and CD206 in primary murine BMM obtained from human *APOE3* and *APOE4* TR mice to determine the impact of the *APOE Epsilon* genotype on the 'basal' pro- and anti-inflammatory macrophage phenotype.

Here, we report that the *APOE4* allele is associated with a higher percentage of BMM with an anti-inflammatory M2 phenotype and a non-significant trend towards a lower percentage of BMM with a pro-inflammatory M1 phenotype compared to *APOE3* allele (Table 5.3). Briefly, *APOE4* BMM showed a higher cell surface expression of the M2 marker CD206 compared to *APOE3* BMM (41.4 % vs. 30.5%, $p < 0.05$), with no significant difference in M1 marker CD11c expression (1.7 % vs. 2.2 %, $p = 0.76$) or the M1 to M2 ratio (0.04 vs. 0.08 , $p = 0.53$) (Figure 5.1). BMM with the pro-inflammatory M1 phenotype ($CD11b^+CD11c^+CD206^-$ cells) were the smallest cell population identified, consisting of approximately 2.0 ± 0.8 (SEM) %. In addition, we identified approximately 10.8 ± 3.9 % of the BMM as double positive cells ($CD11b^+CD11c^+CD206^+$). There were no differences in the percentage of BMM ($CD11b^+$ cells) obtained from the *APOE3* and *APOE4* BMM cultures, which yielded 99% $CD11b^+$ cells.

Table 5.3. Prevalence of the pro- (M1) and anti-inflammatory (M2) phenotype in primary murine bone marrow-derived macrophages according to *APOE Epsilon* genotype.

	APOE3	**APOE4**
$CD11b^+$ cells (% of total)	98.8 ± 0.1	99.0 ± 0.2
$CD11b^+CD11c^+CD206^-$ cells (M1) (% of $CD11b^+$ cells)	2.2 ± 1.4	1.7 ± 0.9
$CD11b^+CD11c^-CD206^+$ cells (M2) (% of $CD11b^+$ cells)	30.5 ± 1.8[a]	41.4 ± 3.0[b]
$CD11b^+CD11c^+CD206^+$ cells (% of $CD11b^+$ cells)	6.8 ± 3.9	14.8 ± 6.8
M1:M2 ratio	0.08 ± 0.05	0.04 ± 0.03

All values are mean ± SEM, n = 3 primary bone marrow-derived macrophage (BMM) cultures per *APOE Epsilon* genotype. $CD11b^+$ cells are $CD11b^+$ cells within our set target cell population; $CD11b^+CD11c^+CD206^-$ (M1), $CD11b^+CD11c^-CD206^+$ (M2) and $CD11b^+CD11c^+CD206^+$ cells are percentage of $CD11b^+$ cells; the M1:M2 ratio presents the ratio of $CD11b^+CD11c^+$ to $CD11b^+CD206^+$ cells. Student's *t*-test was used to determine differences between the *APOE Epsilon* genotypes, different letters indicate different means, $p < 0.05$.

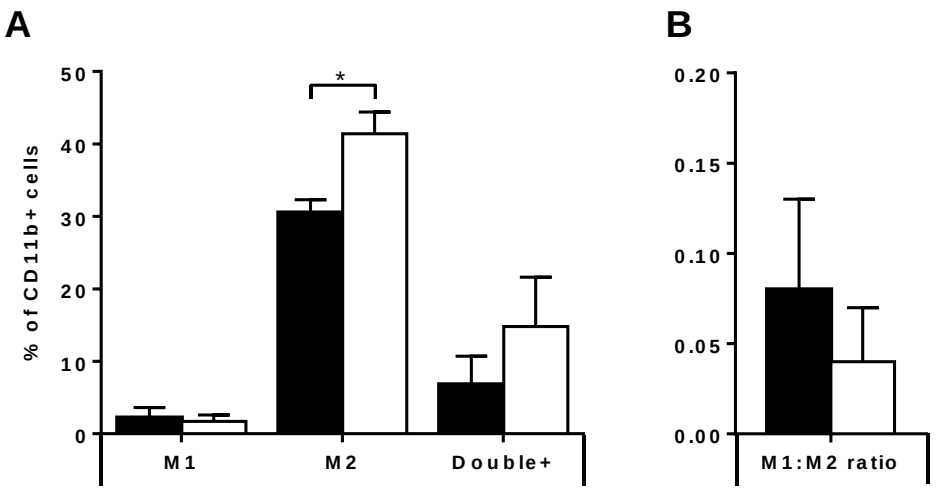

Figure 5.1. Pro- (M1) and anti-inflammatory (M2) phenotype marker expression in primary murine bone marrow-derived *APOE3* (black bars) and *APOE4* (white bars) macrophages. The cell surface expression of CD11b (macrophage marker), CD11c (M1 marker) and CD206 (M2 marker) was analysed using flow cytometry. [A] Macrophages with pro-inflammatory M1 phenotype were $CD11b^{+}CD11c^{+}CD206^{-}$; macrophages with anti-inflammatory M2 phenotype were $CD11b^{+}CD11c^{-}CD206^{+}$; Double positive (Double+) macrophages were $CD11b^{+}CD11c^{+}CD206^{+}$; data presented as percentage of $CD11b^{+}$ cells. [B] The M1:M2 ratio was ratio of $CD11b^{+}CD11c^{+}CD206^{-}$ to $CD11b^{+}CD11c^{-}CD206^{+}$ macrophages. Data is presented as mean ± SEM (n = 3). Student's *t*-test was used to determine differences between the *APOE Epsilon* genotypes, *p < 0.05. APOE, apolipoprotein E; CD11b, integrin alpha M; CD11c, Integrin alpha X; CD206, mannose receptor C type 1.

Discussion

In this study we investigated the cell surface expression of CD11b, CD11c, and CD206 in primary murine *APOE3* and *APOE4* BMM obtained from human *APOE* TR mice to determine the impact of the *APOE Epsilon* genotype on the 'basal' pro- and anti-inflammatory macrophage phenotype. Here, we report that the *APOE4* allele was associated with a higher cell surface expression of CD206, but not CD11c, in BMM compared to *APOE3* allele, indicating a higher prevalence of the anti-inflammatory M2 phenotype and no difference in the prevalence of the pro-inflammatory M1 phenotype. There was no difference in the M1 to M2 ratio between *APOE3* and *APOE4* BMM cultures indicating a similar M1:M2 phenotype distribution.

Baitsch *et al*. were the first to investigate the impact of apoE on macrophage polarisation *in vitro* using RAW 264.7 murine macrophages stably expressing human apoE receptors VLDL-R and apoeR2 and demonstrated that exogenous exposure to apoE promoted a phenotypic switch from M1 to M2 phenotype [Baitsch et al., 2011]. In more detail, macrophages exposed to apoE (5 µg/mL for 24 hr) showed increased expression of M2 markers and cytokines (arginase 1 (Arg1), found in inflammatory zone/resistin-like molecules (Fizz/Relm), suppressor of cytokine signaling 3 (Socs3), IL-1RA, granulocyte colony-stimulating factor (G-CSF), IL-13), and reduced expression of M1 cytokines (MIP-1α, IL-12). These changes were accompanied by functional characteristics of M2 macrophages, i.e. reduced migration, attenuated ROS production, and increased phagocytic activity. These findings clearly indicate a role for apoE in modulating macrophage polarisation, although a potential isoform-dependent effect was not investigated. We are the first to demonstrate that endogenous expression of apoE4 was associated with a higher prevalence of the M2 phenotype compared to apoE3 using cell surface marker expression analysis and flow cytometry. Future experiments should corroborate these findings by looking at mRNA expression levels of M1 and M2 markers, especially since we have shown that CD11b, CD11c and CD206 mRNA expression levels may not reflect their cell surface expression levels (Chapter 4). Moreover, the functional characteristics of macrophages, such as migration, ROS production, and phagocytic activity, should be investigated to confirm a differential macrophage phenotype. Our findings contradict with the previously reported immunomodulatory properties of apoE indicating that apoE4 is pro-inflammatory [Jofre-Monseny et al., 2008A]. An explanation for these conflicting results is that we investigated the impact of apoE4 in a basal unstimulated state, whereas most other studies have investigated the impact of apoE4 upon stimulation with a pro-inflammatory stimulus, such as LPS [Jofre-Monseny et al., 2008A]. This warrants further investigation into the impact of *APOE4* allele on macrophage phenotype in both basal state and upon stimulation with an inflammatory stimulus.

We identified approximately 10 % of the BMM as double positive cells for CD11c and CD206 ($CD11b^+CD11c^+CD206^+$) suggesting these macrophages have an intermediate phenotype between M1 and M2 and macrophages can potentially 'switch phenotypes', which has been implicated before [Morris et al., 2012]. Morris *et al*. proposed a new nomenclature for ATM to capture their phenotypic heterogeneity in both mice and humans describing the four best characterized types identified in mice and humans. Their classification distinguished between $CD11c^+CD206^-$, $CD11c^+CD206^+$ and $CD11c^-CD206^+$ macrophages, with $CD11c^+CD206^-$ and $CD11c^+CD206^+$ macrophages (formerly both M1, here two different subtypes) showing distinct gene expression profiles and potentially different functions. Thus, macrophages can display an intermediate phenotype and 'switch' between M1 and M2 phenotypes under the influence of their microenvironment [Morris et al., 2011]. This is relevant given that AT macrophages undergo changes in their activation state to promote AT inflammation during obesity [Lumeng et al., 2007B, Li et al., 2010] and $CD11c^+$ ATM have been associated with glucose intolerance and metabolic syndrome in humans [Wentworth et al., 2010, Wu et al., 2009]. Thus, research into potential factors, including *APOE Epsilon* genotype, to determine macrophage phenotype switching will be important for our understanding of how macrophage phenotype switching may potentially determine AT inflammation and subsequent disease risk.

Our *APOE3* and *APOE4* BMM cultures yielded approximately 99% $CD11b^+$ cells. CD11b is a cell surface marker for monocytes and macrophages, although it can also expressed by natural killer cells, activated T-cells and B-cell subsets. We used conditioned medium containing macrophage colony-stimulating factor (M-CSF), a specific growth factor inducing macrophage differentiation of myeloid cells, to selectively culture macrophages from bone marrow stem cells [Manzanero, 2012]. Therefore, we are sure that we have studied macrophages derived from bone marrow stem cells.

A strength of this study was the use of primary BMM cultures, which yield a homogeneous not-yet-activated macrophage population. An alternative method to obtain primary macrophages would be to isolate peritoneal cavity cells from mice. However, this method produces a heterogeneous cell population consisting of approximately 53 % B-cells, 40 % macrophages, and 6 % T-cells, with the potential risk of contamination with blood cells if the peritoneal lavage is not performed carefully. Thus a strength of our primary BMM model was that we were able to investigate the effect of the *APOE Epsilon* genotype on macrophage phenotype polarisation in a homogeneous macrophage cell population in a basal (not-activated) state.

The primary BMM were obtained from human *APOE3* and *APOE4* TR mice which are homozygous for the human *APOE3* or *APOE4* gene by targeted replacement of the endogenous mouse *Apoe* gene. In these mice, the expression of the human apoE3 or apoE4 isoform is under the control of the murine *Apoe* regulatory sequence and as a result

apoE3 and apoE4 are produced at physiological levels. Therefore, another strength of our study compared to the only previous study reporting an effect of apoE on macrophage phenotype polarization [Baitsch et al., 2011] is that we used primary cells endogenously expressing human *APOE* at physiological levels, whereas Baitsch *et al*. exposed a murine cell line to exogenous human apoE.

We used methods and primary BMM from male mice similar to those used in our *in vivo* studies (Chapter 3 and 4), which allowed us to directly compare the findings from both studies. Compared to Chapter 4, we used in this study modified instrument settings for the detection of Cd11c-Alexa700 with 640 nm laser and 670/LP filter using flow cytometry, which provided more reliable information on CD11c cell surface expression of BMM.

The main limitation of this study is that the data on the impact of apoE4 on the macrophage phenotype is limited to the basal unstimulated state only. Most studies have investigated the impact of apoE4 upon stimulation with a pro-inflammatory stimulus [Jofre-Monseny et al., 2008A]. Therefore, conclusions can only be drawn after comparison with stressor-induced responses has been made.

Another generic limitation of our study in that we evaluated the impact of *APOE Epsilon* genotype on macrophage phenotype in an *in vitro* primary murine cell model, which may not fully represent the macrophage cell behavior *in vivo* in humans.

A potential limitation of our cell culture protocol was the use of M-CSF to selectively grow macrophages from bone marrow stem cells, as M-CSF is a growth factor known to induce a M2 phenotype in macrophages [Jaguin et al., 2013]. This might have directed the phenotype of the BMM towards the M2 phenotype in our study. If there was any effect of M-CSF on macrophage polarisation in this study, it would indicate that *APOE4* BMM might be more responsive to M-CSF-induced M2 polarisation than *APOE3* BMM.

In conclusion, we demonstrated that the *APOE4* allele is associated with a higher prevalence of the M2 phenotype in BMM in the basal state which may represent a natural protective response to counterbalance the previously reported pro-inflammatory properties of apoE4 compared to apoE3. Future studies investigating the impact of the *APOE4* allele on the macrophage phenotype in both basal state and upon stimulation with inflammatory stimulus are needed to determine if this protective effects of the *APOE4* allele on macrophage phenotype polarisation also persists in the presence of an inflammatory stimulus.

Chapter 6. General discussion: Obesity, adipose tissue inflammation, *APOE* genotype and long chain *n-3* polyunsaturated fatty acids

Summary of results and general discussion

Main findings

In this PhD project, we investigated the relationships between *apolipoprotein E* (*APOE) Epsilon* genotype, adiposity, long chain (LC) *n-3* polyunsaturated fatty acids (PUFA) status and inflammation. In line with the small amount of recent emerging literature, we observed that *APOE3* is an energy-thrifty allele associated with the development of obesity and its associated metabolic complications, whereas *APOE4* is an energy-dissipative allele that appears to protect against the development of obesity. In addition, we observed a trend that *APOE4* allele is associated with altered LC *n-3* PUFA metabolism and bioavailability. Novel observations have been made that show no effect of the *APOE Epsilon* genotype on high fat diet (HFD)-induced macrophage infiltration and macrophage phenotype distribution in visceral AT. Finally, we are the first to show that *APOE Epsilon* genotype interacted with a single nucleotide polymorphism (SNP) in the *APOE* promoter region to determine plasma inflammatory marker concentrations in humans, which may have implications for disease risk. This demonstrates the importance of considering common genotypes in combination with often subtle effects of individual SNPs in either the same gene region or in genes in related metabolic pathways that have additive or even synergistic effects, and result in a highly significant combined impact on the phenotype of interest. Altogether this PhD project contributes to our understanding of the potential mechanisms through which *APOE Epsilon* genotype modulates disease risk and is an important lead for future nutrigenetics research.

General discussion

Single SNPs and diet-gene interactions

It has become clear that the effects of diet on health may be dependent on an individual's genetic background. A full understanding of the interplay between diet and genes to determine human health will have important implications for public health. It will not only tell us what a person's nutrient needs are based on their genetic predisposition, but also how this person will respond to dietary intervention and intake, which forms the basis of personalised nutrition.

SNPs in the coding and non-coding region of genes can influence the function and expression of a gene that in turn can predispose an individual to certain diseases or nutrient deficiencies. For example, a SNP may influence the uptake, metabolism and/or bioavailability of ingested nutrients or bioactive food compounds. This could influence the individual's response to dietary intake. The association between SNPs (i.e. genetic variants) and disease phenotypes are studied using two different approaches. The first approach is a candidate gene or single SNP study that investigate the impact of a single SNP on a disease phenotype. Candidate gene studies focus on genes that are selected based on an *a priori* hypothesis about the role of that gene in the disease aetiology or underlying biological pathways. The second approach is a genome-wide association study (GWAS) which investigates the impact of many SNPs (over 10,000) on a disease phenotype. GWAS do not require *a priori* hypothesis but require thousands of subjects. GWAS are often useful for screening the genome for candidate genes for complex traits and diseases, but are typically unable to establish disease causality or differentiate between the effects of variants in linkage disequilibrium (LD). Initially most studies were candidate gene studies, but the advances in technology have allowed the analysis of many SNPs (up to 3,000,000) on a single chip opening the door to larger GWAS. A great advantage of GWAS compared to candidate gene studies is that it allows the investigation of the impact of single SNPs in the context of other SNPs (i.e. its genetic context). The effect of an individual SNP may be modest but collectively they may have a significant larger effect on specific traits. Also the incorporation of the knowledge about the relationship between individual SNPs can help to identify clusters of genes influencing underlying molecular mechanisms of complex traits. GWAS have been shown to be useful for nominating candidate genes for traits that have high heritability and low experimental variability, such as the *APOE* gene for Alzheimer's Disease [Bertram et al., 2007]. However, the identification of genetic loci involved in more complex disease phenotypes, such as obesity [Hebebrand et al., 2010] and insulin resistance [Harrington and Phillips, 2014], have been proven to be more difficult. Although single genes, such as fat mass and obesity-associated (FTO) gene, have been identified for the risk of obesity and BMI, more than 95 % of the genetic variation in BMI remains unaccounted for [Hebebrand et al., 2010]. The failure to identify the genetic variants that determine complex traits such as BMI may be caused by the small effect size of SNPs or rare SNPs may play a more important role than initially anticipated [Frazier-Wood, 2015]. In addition, SNPs may interact with environmental factors, such as diet and exercise, to determine BMI. For example, the genetic variant -1131T/C (rs622799) in the apolipoprotein A-V (APOA5) gene has been shown to modulate the effect of dietary fat intake on BMI and obesity risk [Corella et al., 2007]. Thus the identification of SNPs with small effect size that potentially interact with diet have been proven to be difficult for complex traits. However, complex diseases are often highly heritable and human health results from complex interactions between genetic predisposition and the environment in which the genes manifest. Furthermore, genetic variants may account for the large inter-individual variation in

the response to dietary intervention. Therefore, large GWAS including tens of thousands individuals that take into account important environmental factors including diet are needed to unravel the genetic heritability of complex traits and indentify diet-gene interactions to determine complex disease phenotypes such as obesity.

Candidate gene studies and GWAS look for an association between genetic variants and a disease phenotype, but tell us nothing about the cause-effect relationship. Therefore, subsequent animal and human studies are needed to elucidate the mechanistic link between a genetic variant and disease phenotype.

Biomarkers of inflammation

Many metabolic diseases including atherosclerosis, Type 2 Diabetes Mellitus (T2DM) and obesity have an inflammatory component [Calder et al., 2011A, Hansson, 2005]. Dietary intervention studies investigating the effect of genetic and/or dietary factors on inflammation should ideally investigate the inflammatory process in the tissue of interest, i.e. brain, liver or adipose tissue (AT). However, many internal organs and tissues are not easily if not impossible or unethical to collect in human volunteers. This asks for the identification of sensitive biomarkers of inflammation. The biomarkers must reflect the inflammatory process that is studied and be predictive of future health or disease status [Biomarkers Definition Working Group, 2001].

In human studies blood samples are in general readily available and inflammation is often determined using soluble or cellular markers in blood. Common soluble plasma markers of inflammation include cytokines, chemokines, lipids, acute-phase proteins and adhesion molecules, whereas common blood cellular markers of inflammation include various subclasses of leukocytes [Calder et al., 2013]. Plasma markers of inflammation are the sum of inflammatory mediators produced by blood cells and those leaking into the circulation from the inflammatory site (i.e. AT, brain, gastrointestinal tract, vascular wall and more) [Calder et al., 2013]. They provide a measure of systemic inflammatory status, but may not fully reflect tissue inflammatory status, which is a important limitation. Moreover, there is a wide inter-individual variation in plasma markers of inflammation which are affected by various factors including age, body fatness, physical (in)activity, gender, genetic variants, smoking, gut microbiota, diet, use of medications and other factors such as emotional stress, pollution, viral infection and sleep behaviour [Calder et al., 2013]. Single circulating biomarkers of inflammation have been shown to be often not informative but rather patterns and clusters may be important as robust biomarkers of inflammation [Calder et al., 2013].

Whole blood samples are also a source of human peripheral blood mononuclear cells (PBMC), which include monocytes and lymphocytes. PBMC circulate through the body and are exposed to metabolic tissues and may therefore reflect systemic health. The immune response capacity of PBMCs *in vitro* and the PBMC inflammatory gene expression *in vivo* have been used as biomarkers of inflammation in human dietary intervention trials [Afman et al., 2014]. The omics technologies have allowed the large scale analysis of genes

(genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics). Whole genome transcriptomics of PBMC have provided a powerful tool to study the effect of dietary bioactive compounds on the expression of genes involved in metabolism and/or inflammatory processes [Afman et al., 2014, Bouwens et al., 2009]. Whole genome transcriptomics of PBMC have been proven to be a more sensitive tool than plasma biomarkers of inflammation for the detection of the effects of LC *n-3* PUFA on inflammatory marker expression [Afman et al., 2014, Bakker et al., 2010, Myhrstad et al., 2011 B]. Furthermore, the PBMC transcriptome has been shown to reflect white AT inflammation but not lipid metabolism, and thus may partly reflect AT biology [O'Grada et al., 2014]. Finally whole genome transcriptomics of PBMC enables annotation and pathways analysis that will provide more insight into underlying molecular mechanisms and have the benefit of integrating diverse biological processes such as metabolism and inflammation. In summary, whole genome transcriptomics of PBMC are a more sensitive biomarker of inflammation than circulating markers, provide a sensitive tool to investigate the effects of LC *n-3* PUFA on inflammatory processes, and could potentially explain why some individuals seem to respond to an intervention but others do not [Afman et al., 2014, Ulven et al, 2014].

It has been suggested that the dynamic response to a challenge (e.g. inflammatory) will provide a more relevant, sensitive and better interpretable indicator of health and the impact on inflammatory homeostasis and inflammatory resilience (i.e the control of an acute inflammatory response) [Calder et al., 2013, Huber et al., 2011, Van Ommen et al., 2009]. Finally, the integration of data from various omics technologies including those from the genome, epigenome, metabolome, proteome, microbiome with the use of the computational methods and predictive mathematical models may allow the identification of even more sensitive and early biomarkers for pre-disease states.

In conclusion, PBMC are an accessible tissue and whole genome transcriptomics of PBMC provide at present a sensitive tool to investigate the effects of genetic variants and/or dietary factors on inflammatory and metabolic processes. Future studies integrating data from omics technologies may lead to the discovery of even more sensitive biomarkers of inflammation.

Extrapolation of findings from animal models to humans

The mouse (*Mus musculus*) is the most commonly used laboratory animal. Many inbred and outbred, knock-in and knock-out, and genetically modified strains are available, which provide useful models for human diseases, such as atherosclerosis, diabetes mellitus, and cancer. Although human and mouse anatomy, physiology and genetics show many similarities there are also considerable differences between the two species. For example, mice are smaller and thus have a higher metabolic rate and accelerated lifespan (one mouse year equals about 30 human years).

There are some important differences between mice and humans when it comes to the development of obesity. Unlike humans, mice are generally resistant to obesity (with exception of several genetically modified strains) and require high fat diet (45 to 60 kcal%

from fat) feeding to develop obesity. The AT depots show great similarity between mice and humans. Most of white subcutaneous and visceral fat depots found in humans have an equivalent in mice. For example, the periovarian fat depot is found both in humans and mice. The epididymal fat depot that we studied in the mice in Chapter 3 and 4, however knows no human equivalent. The amount of brown fat differs between mice and humans. Mice have relatively more brown fat, because this tissue plays an important role in the regulation of body temperature in small rodents.

Human and mouse metabolism show many similarities, but there are also some differences. As mentioned earlier mice have a much higher metabolic rate. In addition, the cholesterol metabolism is somewhat different between the species. Humans carry cholesterol in their blood mainly in low-density lipoprotein (LDL) and to lesser extent in high-density lipoprotein (HDL). Whereas mice carry cholesterol mainly in HDL and to lesser extent in LDL in their blood. In humans, LDL cholesterol levels are considered the 'bad cholesterol' because it increases the risk of cardiovascular disease, but this may not apply to mice.

Mice are popular laboratory animals to create genetically modified strains. Mouse genes can be replaced with human genes to study gene function and to produce a more human-like model system in the mouse. A humanised mouse model provides a promising tool to study the effect of human genetic variants on gene and protein function. In Chapter 3 and 4, we used human *APOE* targeted replacement mice as a model system to study the function of the common human *APOE Epsilon* allelic variants. A strength of humanised mouse models is that one can control for many genetic and environmental factors such as diet which are difficult to control in human studies. However, the human gene is expressed in the genetic context of the mouse genome and although murine genes and proteins show high-degree of homology with human genes and proteins, slight differences between mouse and human proteins may influence the function of the human protein in the mouse model system. This potential limitation of a mouse model system has been illustrated in an *in vitro* study which showed that human apoE required the expression of human apoE receptors for its full effect in the murine RAW 264.7 cell line [Baitsch et al., 2012]. Therefore the findings of a human-like model system in the mouse may not directly be extrapolated to humans.

In summary, mouse models provide a promising tool to study the impact of human genetic variants and/or dietary bioactive food compounds on health outcomes and the underlying molecular mechanism. In doing so mouse models provide an important lead for future human studies, as findings from animal studies often cannot be directly extrapolated to humans.

Fish consumption versus long chain *n-3* PUFA supplementation

An important point of discussion is whether dietary recommendations for LC *n-3* PUFA should include recommendations for (oily) fish consumption and/or LC *n-3* PUFA supplements. The bioavailability, efficacy and health benefits of eicosapentaenoic acid

(EPA) and docosahexaenoic acid (DHA) derived from supplements compared that of (oily) fish consumption are namely still under debate.

The bioavailability of EPA and DHA may be affected by the food matrix and chemical form of EPA and DHA present in supplements and fish, as they appear to influence the digestion and absorption of EPA and DHA in humans. Furthermore, the dietary fat content of a meal is known to impact on the digestion and absorption of EPA and DHA [Schuchardt and Hahn, 2013], which may be specially an important determinant of the bioavailability of EPA and DHA from supplements. Supplements are namely often taken in the morning with breakfast which is usually a low-fat meal. The chemical structure of EPA and DHA in supplements, which can be either as free fatty acids (FFA), ethylesters (EE), phospholipids (PL) or triglycerides (TG), affects the bioavailability of EPA and DHA in humans [Schuchardt and Hahn, 2013]. In fish, EPA and DHA are mainly present as TG and to a lesser extent present as FFA. The naturally-occuring TG in fish and chemically-modified TG in supplements appear to have the highest bioavailability in humans.

Although an LC *n-3* PUFA intake of > 1 g/d corresponding to consumption of > 4 portions of fish a week may be difficult to achieve by diet alone, an intake of about 0.5 g/d can in general easily be achieved through fish consumption (2 portions per week) or supplements. However, the overall health benefits of LC *n-3* PUFA derived from fish consumption may differ from those derived from supplements. Fish is namely a source of other nutrients such as vitamin D, selenium, iodine and antioxidants (e.g. carotenoids), that are often missing in supplements. Also the protein present in fish may have specific beneficial effects [Rudkowska et al., 2010]. On the other hand, fish in particular oily fish and species high on the food chain (e.g. shark and swordfish) are a source of pollutants, such as heavy metals (mercury, cadmium, arsenic and lead), dioxins and polychlorinated biphenyls (PCBs). The effects of these pollutants fail to negate the beneficial effects of LC *n-3* PUFA present in fish [Mozaffarian and Rimm, 2006]. For example, the modestly increased risk of myocardial infarction (MI) associated with mercury could not negate the risk reducing effect of LC *n-3* PUFA on MI [Wennberg et al., 2012]. Furthermore, the levels of dioxins and PCBs in fish are low and similar to those in several other foods [Mozaffarian and Rimm, 2006]. Likewise, dioxins and PCBs are often not entirely eliminated from fish oil products in particular those made from farmed fish and may thus also be present in fish oil supplements. Overall, the potential risk mercury, dioxins and PCBs in adults are greatly exceeded by the benefit of fish intake [Mozaffarian and Rimm, 2006]. However, mercury may pose a risk to pregnant women who are recommended to avoid species such as shark, marlin and swordfish [SACN, 2004].

LC *n-3* PUFA intake from fish consumption or supplements will likely have differential effects on habitual diet. Dietary supplements are namely often taken on top of the normal diet and therefore people often do not account for the additional calories provided by LC *n-3* PUFA in the supplement. On the other hand, a portion of fish is in most cases consumed with a meal where it often replaces a portion of meat, thereby accounting for the calories provided by

fish. In addition, the regular consumption of fish will likely influence the consumption of other meats such as pork and beef that are rich in saturated fat, thereby having additional beneficial effects on habitual diet.

Finally, LC n-3 PUFA are very prone to oxidation because of their highly desaturated nature and therefore need to be protected from oxidation by for example antioxidants. Though it also applies to LC *n-3* PUFA in fish, it is specially of concern for LC *n-3* PUFA supplements that undergo more elaborate processing and are often stored longer. Oxidized LC *n-3* PUFA may have potential unfavourable effects, for example if present in LDL lipoproteins they may increase oxidized LDL which is known risk factor for atherosclerosis [Maiolino et al., 2013]. However, in a low quantitiy they may exert beneficial effects by activating Nrf2 an important transcription factor activating the anti-oxidant response [De Roos and Duthie, 2015].

In summary, the health benefits of LC *n-3* PUFA derived from fish are likely superior to that of supplements. This is supported by the beneficial effects of fish consumption on T2DM disease risk in populations with high habitual fish intake compared to those with a low fish intake, which consistently fail to be reproduced in randomised controlled trials (RCTs) that often use fish oil supplements [Jafari et al., 2013]. Dietary recommendations should encourage the consumption of fish, but specifying both recommended (oily) fish consumption and LC *n-3* PUFA intake. Vegans, individuals who are allergic, do not like fish or choose to not include it in their diet, or fail to meet the recommended EPA and DHA levels by diet alone, would namely depend on supplements to meet their recommended LC *n-3* PUFA intake.

Short term strategies targeting structural changes in obesity

In obesity several structural and cellular changes take place in AT. Adipocytes become hypertrophic by intracellular triglyceride (TG) accumulation and start to secrete mediators that attracts macrophages which increasingly infiltrate the AT. The recruited AT macrophages (ATM) will predominantly acquire a pro-inflammatory M1 phenotype and start to outnumber the resident ATM that have a more anti-inflammatory M2 phenotype. This is associates with increased pro-inflammatory mediator production resulting in local and systemic inflammation, which in turn increases the risk for insulin resistance (IR), metabolic disease and cardiovascular disease (CVD) [Osborn and Olefsky, 2012, Van Gaal et al., 2006]. It is believed that ATM infiltration and the ATM phenotype switch towards the pro-inflammatory M1 phenotype are the underlying cause of AT inflammation and dysfunction [Hotamisligil, 2006, Lumeng et al., 2007A, Osborn and Olefsky, 2012]. Therefore reducing ATM infiltration and switching the ATM phenotype from the M1 towards M2 phenotype may prove a strategy to reverse AT inflammation and dysfunction and disease risk in obesity.

Non-pharmacological strategies will likely be most effective in reducing AT inflammation and dysfunction if they include a combination of a healthy diet, exercise and/or caloric restriction. Diet plays an important role in the development of obesity, its metabolic complications as well as disease risk. The amount and type of dietary fat influence AT function and whole

body metabolism. Saturated fatty acids have pro-inflammatory properties, whereas LC *n-3* PUFA, EPA and DHA, have anti-inflammatory properties. Likewise the intake of saturated fat is associated with an increased risk for CVD, whereas the intake of LC *n-3* PUFA is associated with decreased risk of CVD [Willet, 2012]. Polyphenols are bioactive compounds found in plants, fruits and vegetables that have a range of valuable bioactivities including anti-oxidative, anti-obesity and anti-inflammatory properties [Bakker et al., 2010], and exert beneficial effects on AT inflammation and IR [Siriwardhana et al., 2013]. Therefore strategies should aim at a healthy diet rich in bioactive food compounds including LC *n-3* PUFA and polyphenols. Physical activity stimulates energy expenditure and exerts beneficial effects on IR. Furthermore, physical activity improves AT blood flow which in turn reduces AT hypoxia and improves AT function. Caloric restriction is an important component of long and short term strategies, as it stimulates weight loss and is associated with a reduction in ATM infiltration and systemic low grade-inflammation [Cancello et al., 2005]. Furthermore, weight loss is associated with reduction in AT mass and adipocyte size (hyperthrophy). Thereby a healthy diet, caloric restriction and/or exercise can improve and may even reverse adipocyte hyperthrophy and AT inflammation, which in turn may improve metabolic derangements and lower disease risk in obesity.

Statistical power analysis for the animal study

As discussed in Chapter 4, our study was most likely underpowered for the outcomes described in that chapter. A power calculation was employed to calculate the number of n = 10 mice per *APOE Epsilon* genotype * diet group that enabled us to detect a standardised mean difference (SMD) of 1.45 for a two sample comparison of means with power of 90 % at significance level of $\alpha = 0.05$ (Chapter 3). Our study had the power to detect a 4.4 gram difference in body weight between the diets and 6.5 gram difference in body weight between the *APOE Epsilon* genotypes. The difference in body weight between the *APOE Epsilon* genotypes at the end of the dietary intervention was about 4.0 g for the HFD group, and hence our study was underpowered to detect a potential true effect of *APOE Epsilon* genotype on body weight in human *APOE3* and *APOE4* TR mice on HFD background. Furthermore, our study was underpowered to detect a potential true effect of *APOE Epsilon* genotype on ATM phenotype in flow cytometry and mRNA expression analysis, and LC *n-3* PUFA status in epididymal AT (see discussion Chapter 4). Thus, although we did not detect a significant difference between the *APOE Epsilon* genotypes for body weight, ATM infiltration and phenotype, and AT LC *n-3* PUFA status, our study was most likely underpowered. Therefore there may have been a true difference between the *APOE Epsilon* genotypes for these outcomes, but our study was most just too small to detect it. In summary, there is insufficient data to conclude that there is no effect of *APOE Epsilon* genotype on ATM infiltration and phenotype, and LC *n-3* PUFA status. Future studies in human *APOE* TR mice, in particular those investigating ATM infiltration and phenotype and/or LC *n-3* PUFA status, are recommended to use a larger and adequate sample size.

Conclusion

This PhD project showed in line with the small amount of recent emerging literature that *APOE3* is an energy-thrifty allele, whereas *APOE4* is an energy-dissipative allele that is associated with an altered LC *n-3* PUFA metabolism and bioavailability. Novel observations have been made that show no effect of the *APOE Epsilon* genotype on HFD-induced macrophage infiltration and macrophage phenotype distribution in visceral AT. Besides, we are the first to show that *APOE Epsilon* genotype interacted with SNPs in the *APOE* promoter region to determine plasma inflammatory marker concentrations in humans, which may have implications for disease risk. This demonstrates the importance of considering common genotypes in combination with often subtle effects of individual SNPs in either the same gene region or in genes in related metabolic pathways that have additive or even synergistic effects, and result in a highly significant combined impact on the phenotype of interest. Altogether this PhD project contributes to the current understanding of the potential mechanisms through which *APOE Epsilon* genotype modulates disease risk and is an important lead for future nutrigenetics research.

Future directions

With plenty of new data we would like to discuss questions resulting from this PhD project. There are still many gaps in the knowledge on the relationships between *APOE Epsilon* genotype, adiposity and inflammation to determine disease risk and still many scientific and social relevant questions have to be addressed. We will discuss some of these research gaps and questions with a suggested approach towards their answer.

What is the impact of APOE Epsilon genotype on the basal macrophage phenotype?

Most *in vitro* studies investigating the impact of *APOE Epsilo*n genotype on the inflammatory response in macrophages reported a pro-inflammatory effect of *APOE4* allele compared to *APOE3* allele upon stimulation with a pro-inflammatory stimulus. We have used HFD feeding as a stressor to evaluate the effect of *APOE Epsilon* genotype on AT macrophage phenotype in human *APOE* TR mice. Second, we performed a pilot study using bone marrow-derived macrophages which suggested that the *APOE4* allele is associated with higher expression of the M2 phenotype in macrophages in a basal un-stimulated state compared to *APOE3* allele. Altogether, these findings suggest that *APOE4* allele may be associated with more anti-inflammatory phenotype in the basal state, yet with a more pro-inflammatory phenotype upon stimulation (i.e. activated state), compared to *APOE3* allele. Future studies should elucidate the effect of *APOE4* allele on the pro- and anti-inflammatory phenotype in macrophages in both basal and activated state. *In vitro* studies using primary macrophages from human *APOE* TR mice could provide insight into potential impact on tissue macrophages as well as underlying mechanism, whereas studies using primary human macrophages are warranted to verify these results to humans. Primary human macrophages can be obtained from whole blood human peripheral blood mononuclear cells (PBMC) which include macrophages, monocytes and lymphocytes, by isolating the monocyte fraction from the PBMC first using a magnetic selection step and then culture with M-CSF to differentiate the monocytes into macrophages. Flow cytometry could be used to determine the effect of *APOE Epsilon* genotype on the phenotype of murine and human primary macrophages in the basal and activated state and the macrophage phenotype response to LC *n-3* PUFA supplementation using cell surface markers including CD11b, CD11c and CD206. The involvement of inflammatory signalling pathways such as nuclear factor kappa-B (NF-κB) and peroxisome proliferator activated receptor (PPAR)-γ in mediating the *APOE Epsilon* genotype effect on macrophage polarisation could be investigated using western-blot, which can be used to quantify the levels of key enzymes and proteins involved in the activation of these signalling pathways such as phospho-NF-kappaB p65.

Do LC n-3 PUFA increase the number of macrophages in AT and what are the health consequences?

We showed that LC *n-3* PUFA increase the number of macrophages in AT without changing their phenotype distribution in human *APOE* TR mice. Also, we found insufficient evidence to indicate that this was associated with an increased AT inflammation, suggesting that the increase in ATM may not be detrimental. In fact, macrophages may exert beneficial effects in AT, such as limit adipocyte hypertrophy, regulating extra-cellular matrix remodelling and fibrosis, restore local lipid homeostasis with their phagocytic activity against lipids [Dalmas et al., 2011]. Future studies using immunohistochemistry techniques should investigate the impact of different doses of LC *n-3* PUFA on the number, phenotype and localisation of macrophages in AT in mouse models and humans to give more insight into dose-dependent effect of LC *n-3* PUFA on AT inflammation and function. Human RCTs could prospective recruit individual according to their baseline level of AT inflammation (i.e. low versus high) and use different dosages of LC *n-3* PUFA (e.g. 0.5 g/d, 1 g/d and 3 g/d) to determine the optimal dosage of LC *n-3* PUFA on AT inflammation response in humans according to baseline AT inflammation level.

Could the altered LC n-3 PUFA metabolism and bioavailability in APOE4 carriers explain their higher CHD risk?

The *APOE4* allele is associated with 6 – 42% increased coronary heart disease (CHD) risk [Bennet et al., 2007, Song et al., 2004, Wilson et al., 1996] and an altered LC *n-3* PUFA metabolism and bioavailability [Chouinard-Watkins et al., 2013, Conway et al., 2014, Vandal et al., 2014]. CHD patients have lower levels of LC *n-3* PUFA compared to healthy controls [Shearer et al., 2009] and LC *n-3* PUFA have been shown to reduce cardiac mortality in humans [Harris et al., 2008]. This raises the possibility that the lower LC *n-3* PUFA bioavailability in *APOE4* carriers could be involved in their increased CHD risk, something that remains to be investigated in future prospective studies and trials. One approach would be to retrospective genotype for *APOE Epsilon* genotype in an existing prospective cohort with data on LC *n-3* PUFA intake, plasma or tissue fatty acid status, cardiovascular events and mortality, and a follow-up period that was sufficient to observe a significant number of cardiovascular events. The inclusion of tissue fatty acid status is recommended because this is where LC *n-3* PUFA are bioactive. Circulating fatty acid status in different plasma lipid fractions (i.e. TG, PL, CE and total lipids) and red blood cells should be investigated as it is likely that the plasma lipid fraction, cell type or tissue studied will influence the impact of *APOE Epsilon* genotype on circulating fatty acid status in humans. If these studies provide supporting evidence for a relationship between *APOE4* genotype, a low LC *n-3* PUFA status and increased risk of cardiovascular events and mortality, RCT should be conducted to investigate first if LC *n-3* PUFA supplementation and what dosage can restore plasma and/or tissue LC *n-3* PUFA levels in *APOE4* carriers. Kinetics studies using isotopically labelled [^{13}C]EPA or [^{13}C]DHA can provide insight into mechanisms underlying the lower LC

n-3 PUFA status in *APOE4* carriers by investigating fatty acid absorption, incorporation in plasma lipid fractions and tissues (if biopsy sampling is possible), and fatty acid utilisation as fuel substrate. Next, RCT should investigate if restoring LC *n-3* PUFA levels via LC *n-3* PUFA supplementation in *APOE4* carriers can result in a risk reduction for CHD.

What is the recommended intake of EPA and DHA for optimal health? And do we have to make recommendations according to APOE Epsilon genotype?

Many organisations and expert committees have acknowledged the important role of LC *n-3* PUFA in human nutrition, but to date no dietary reference intakes (DRI) have been set for EPA and DHA [Flock et al., 2013]. The first challenge to making DRI recommendations is the selection of a health outcome of interest, such as prevention of obesity, CHD or mortality, on which DRI will be based. Flock *et al*. reviewed the extensive data on the effect of LC *n-3* PUFA on CVD outcomes to make DRI recommendations for EPA and DHA and proposed an adequate intake (AI) of 566 mg/d for EPA and DHA which was associated with the greatest reduction (37 %) in risk for CHD mortality [Flock et al., 2013, Harris et al., 2008]. However, more dose-response data is needed to determine optimal intake of EPA and DHA in various populations, specially for the *APOE Epsilon* genotypes. Genetic variants influencing the synthesis, absorption and/or metabolism of LC *n-3* PUFA will likely be an important determinant of the response to LC *n-3* PUFA supplementation. First, prospective and cross-sectional cohort studies should investigate the impact of *APOE Epsilon* genotype on tissue LC *n-3* PUFA status and it correlation with CHD disease risk in humans, thereby distinguishing between individuals consuming no LC *n-3* PUFA and individuals with a high LC *n-3* PUFA intake. This would provide us more insight into the impact of *APOE Epsilon* genotype on LC *n-3* PUFA status and its relation with risk of CHD in humans and if dietary LC *n-3* PUFA intake can potentially overcome the genotype effect. Next, RCT and dose-response studies will have to verify the optimal intake of EPA and DHA for the different *APOE Epsilon* genotypes to obtain optimal LC *n-3* PUFA status in humans and its potential implications for the risk of CHD. Thereby addressing the question if in the future a stratified approach to dietary recommendations for EPA and DHA should be taken, with higher intakes suggested for carriers of the *APOE4* allele. The previously described recommendations for DRI for EPA and DHA were aimed at reducing the risk of CHD. More dose-response data is needed to determine optimal intake of EPA and DHA on other outcomes including inflammatory disease and body weight control.

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Appendices

Appendix 2-1. Baseline plasma long chain *n-3* PUFA status

There was no significant effect of *APOE Epsilon* genotype, -219G/T genotype, -219G/T * *APOE Epsilon* haplotype, gender, smoking, alcohol intake and habitual diet other than oily fish intake on the plasma long chain (LC) *n-3* PUFA concentrations at baseline (Table A2-1.1). The mean baseline plasma LC *n-3* PUFA concentrations according to *APOE Epsilon* genotype are presented in Table A2-1.2.

Table A2-1.1. Impact of subject characteristics and habitual diet on plasma long chain *n-3* PUFA status.

Variable	Outcome measure	Model 1	Model 2	Direction of effect
APOE Epsilon genotype	EPA	p=0.29	p=0.50	
	DPA	p=0.53	p=0.35	
	DHA	p=0.27	p=0.42	
	LC n-3 PUFA	p=0.17	p=0.43	
	EPA:AA ratio	p=0.50	p=0.79	
	SFA:PUFA ratio	p<0.05	p<0.05	E3 > E2 and E4
-219G/T genotype	EPA	p=0.32	p=0.22	
	DPA	p=0.20	p=0.10	
	DHA	p=0.10	p=0.20	
	LC n-3 PUFA	p=0.38	p=0.27	
	EPA:AA ratio	p=0.34	p=0.20	
	SFA:PUFA ratio	p=0.45	p=0.48	
-219G/T * *APOE Epsilon* haplotype	EPA	p=0.78	p=0.83	
	DPA	p=0.75	p=0.37	
	DHA	p=0.23	p=0.49	
	LC n-3 PUFA	p=0.63	p=0.81	
	EPA:AA ratio	p=0.80	p=0.80	
	SFA:PUFA ratio	p=0.13	p=0.18	
Age (years)	EPA	p<0.001	p<0.001	β = 0.01
	DPA	p<0.001	p<0.001	β = 0.01
	DHA	p<0.001	p<0.001	β = 0.02
	LC n-3 PUFA	p<0.001	p<0.001	β = 0.04
	EPA:AA ratio	p<0.001	p=0.001	β = 0.001
	SFA:PUFA ratio	p=0.018	p=0.042	β = 0.001
Gender	EPA	p=0.56	p=0.84	
	DPA	p<0.01	p<0.001	women < men
	DHA	p=0.09	p=0.60	
	LC n-3 PUFA	p=0.18	p=0.93	
	EPA:AA ratio	p=0.57	p=0.77	
	SFA:PUFA ratio	p=0.51	p=0.77	
BMI (kg/m2)	EPA	p=0.78	p=0.12	
	DPA	p=0.28	p=0.27	
	DHA	p=0.11	p<0.001	β = -0.06
	LC n-3 PUFA	p=0.24	p<0.01	β = -0.09
	EPA:AA ratio	p=0.79	p=0.10	
	SFA:PUFA ratio	p=0.13	p=0.29	
Waist circumference (cm)	EPA	p=0.73	p<0.05	β = -0.15
	DPA	p=0.22	p=0.93	
	DHA	p=0.18	p=0.78	
	LC n-3 PUFA	p=0.33	p=0.35	
	EPA:AA ratio	p=0.86	p=0.10	
	SFA:PUFA ratio	p=0.10	p=0.21	

Table A2-1.1. continued.

Variable	Outcome measure	Model 1	Model 2	Direction of effect
Smoking	EPA	p=0.18	p=0.52	
	DPA	p=0.13	p=0.12	
	DHA	p<0.05	p=0.09	
	LC n-3 PUFA	p<0.01	p<0.05	smoker < non-smoker
	EPA:AA ratio	p=0.17	p=0.76	
	SFA:PUFA ratio	p=0.75	p=0.37	
Alcohol intake (units of alcohol / wk)*	EPA	p=0.16	p=0.52	
	DPA	p=0.97	p=0.69	
	DHA	p=0.40	p=0.90	
	LC n-3 PUFA	p=0.83	p=0.26	
	EPA:AA ratio	p=0.51	p=0.12	
	SFA:PUFA ratio	p=0.39	p=0.18	
Total energy from alcohol (%)	EPA	p=0.73	p=0.45	
	DPA	p=0.80	p=0.59	
	DHA	p=0.29	p=0.80	
	LC n-3 PUFA	p=0.45	p=0.93	
	EPA:AA ratio	p=0.78	p=0.92	
	SFA:PUFA ratio	p=0.08	p=0.20	
Habitual oily fish intake (portions / week)	EPA	p<0.001	p<0.001	β = 0.16
	DPA	p=0.19	p=0.54	
	DHA	p<0.001	p<0.001	β = 0.29
	LC n-3 PUFA	p<0.001	p<0.001	β = 0.64
	EPA:AA ratio	p<0.001	p<0.001	β = 0.03
	SFA:PUFA ratio	p=0.83	p=0.71	
Fruit and vegetable intake (portions / day	EPA	p<0.05	p=0.44	
	DPA	p=0.23	p=0.19	
	DHA	p=0.25	p=0.13	
	LC n-3 PUFA	p=0.005	p=0.90	
	EPA:AA ratio	p<0.05	p=0.76	
	SFA:PUFA ratio	p=0.58	p=0.31	
Total energy intake (MJ)	EPA	p=0.44	p<0.05	β = 0.25
	DPA	p=0.44	p=0.80	
	DHA	p=0.55	p<0.05	β = -0.06
	LC n-3 PUFA	p=0.59	p<0.01	β = -0.09
	EPA:AA ratio	p=0.37	p<0.01	β = -0.004
	SFA:PUFA ratio	p=0.40	p=0.41	
Total energy from fat (%)	EPA	p<0.01	p<0.01	β = -0.01
	DPA	p=0.80	p=0.73	
	DHA	p=0.56	p=0.42	
	LC n-3 PUFA	p<0.05	p<0.05	β = -0.05
	EPA:AA ratio	p<0.05	p<0.05	β = -0.002
	SFA:PUFA ratio	p<0.05	p<0.05	β = -0.002
SFA intake (proportion of total fat)	EPA	p=0.12	p<0.05	β = -0.01
	DPA	p=0.49	p=0.70	
	DHA	p=0.37	p=0.13	
	LC n-3 PUFA	p=0.09	p=0.006	β = -0.02
	EPA:AA ratio	p=0.25	p=0.045	β = -0.001
	SFA:PUFA ratio	p=0.72	p=0.68	
MUFA intake (proportion of total fat)	EPA	p=0.15	p<0.05	β = -0.01
	DPA	p=0.97	p=0.75	
	DHA	p=0.24	p<0.05	β = -0.01
	LC n-3 PUFA	p=0.17	p<0.01	β = -0.03
	EPA:AA ratio	p=0.24	p<0.05	β = -0.0002
	SFA:PUFA ratio	p=0.50	p=0.55	

Table A2-1.1. continued.

Variable	Outcome measure	Model 1	Model 2	Direction of effect
PUFA intake (proportion of total fat)	EPA	p=0.16	p<0.01	β = -0.02
	DPA	p=0.84	p=0.52	
	DHA	p=0.16	p<0.01	β = -0.03
	LC n-3 PUFA	p=0.35	p<0.01	β = -0.05
	EPA:AA ratio	p=0.20	p<0.01	β = -0.002
	SFA:PUFA ratio	p=0.38	p=0.38	
Dietary P:S ratio	EPA	p=0.56	p=0.90	
	DPA	p=0.33	p=0.26	
	DHA	p=0.56	p=0.10	
	LC n-3 PUFA	p=0.24	p=0.91	
	EPA:AA ratio	p=0.40	p=0.83	
	SFA:PUFA ratio	p=0.38	p=0.37	

Model 1, ANOVA main-effects model (unadjusted) including only the explanatory variable. Model 2, ANOVA main-effects model (adjusted) including covariates age, gender, BMI, centre, smoking, habitual oily fish intake. The direction of effect is the regression coefficient (β) for continues variables and the trend for categorical variables for variables with a significant main effect in Model 2. * 1 UK unit = 10 mL or 8 g pure alcohol. APOE, apolipoprotein E; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid ; EPA:AA ratio, ratio of EPA to arachidonic acid; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid; SFA:PUFA ratio, ratio of saturated fatty acids to PUFA.

Table A2-1.2. Baseline plasma long chain *n-3* PUFA status according to *APOE* Epsilon genotype.

	APOE2	APOE3	APOE4
EPA	1.71 ± 0.10	1.54 ± 0.07	1.65 ± 0.07
DPA	1.08 ± 0.03	1.12 ± 0.03	1.12 ± 0.03
DHA	4.37 ± 0.14	4.10 ± 0.11	4.27 ± 0.10
LC n-3 PUFA	7.29 ± 0.22	6.79 ± 0.18	7.15 ± 0.17
EPA:AA ratio	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
SFA:PUFA ratio	1.02 ± 0.01	1.04 ± 0.01	1.02 ± 0.01

All values are mean ± SEM. *APOE Epsilon* groups, APOE2 (E2/E2, E2/E3, n = 87), APOE3 (E3/E3, n = 111), APOE4 (E3/E4, E3/E4, n = 114). Student's *t*-test using Bonferroni correction revealed no difference between the *APOE Epsilon* genotypes, p > 0.05. APOE, apolipoprotein E; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid ; EPA:AA ratio, ratio of EPA to arachidonic acid; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid; SFA:PUFA ratio, ratio of saturated fatty acids to PUFA.

Appendix 2-2. Plasma oxLDL-C to apoB ratio

There was no effect of BMI on the plasma oxidized LDL-C (oxLDL-C) to apolipoprotein B (apoB) ratio at baseline adjusting for age, gender, centre, smoking and habitual oily fish intake (p = 0.80) (Figure A2-2.1).

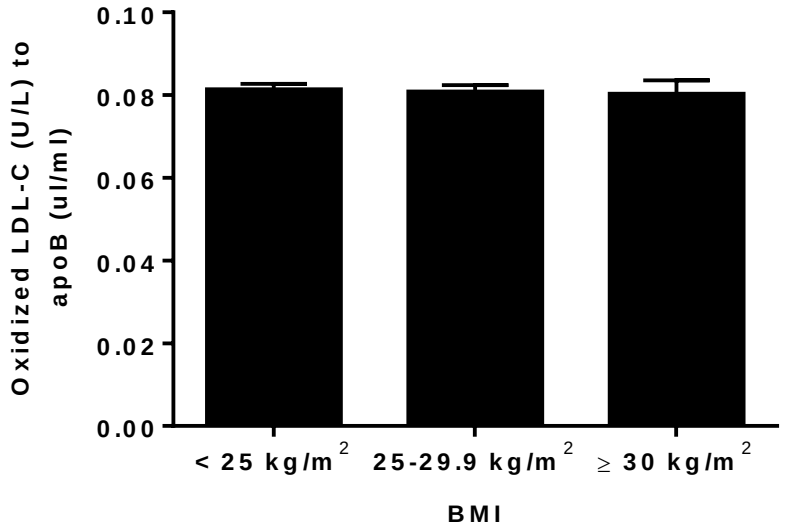


Figure A2-2.1. Baseline plasma oxLDL-C to apoB ratio according to BMI. Data is presented as mean ± SEM for BMI groups < 25 kg/m2 (n = 155), 25 – 29.9 kg/m2 (n = 128) and ≥ 30 kg/m2 (n = 29). One-way ANOVA including covariates age, gender, centre, smoking and habitual oily fish intake revealed no significant effect of BMI. apoB, apolipoprotein B; BMI, body mass index; oxLDL-C, oxidized low-density cholesterol.

Appendix 2-3. Age, gender, BMI and baseline plasma inflammatory marker concentrations

There was a significant effect of gender ($p < 0.05$; Table A2-3.1), BMI ($p < 0.05$; Table A2-3.2), but not of age ($p \geq 0.07$; Table A2-3.3), on plasma inflammatory marker concentrations at baseline.

Table A2-3.1. Baseline plasma inflammatory marker concentrations according to gender.

	Men	Women	Model 1	Model 2
CRP (mg/l)	1.36 ± 0.13	1.54 ± 0.13	p=0.31	p=0.07
TNF-α (pg/ml)	1.69 ± 0.07[a]	1.42 ± 0.05 [b]	p<0.01	p<0.05
IL-6 (pg/ml)	1.32 ± 0.10	1.25 ± 0.10	p=0.64	p=0.69
IL-10 (pg/ml)	1.04 ± 0.08	1.02 ± 0.09	p=0.92	p=0.83
ICAM-1 (ng/ml)	303 ± 10	301 ± 10	p=0.90	p=0.89
VCAM-1(ng/ml)	1877 ± 70	1906 ± 74	p=0.77	p=0.74
E-sel (ng/ml)	80.3 ± 3.1[a]	66.7 ± 2.6[b]	p<0.001	p<0.05
P-sel (ng/ml)	63.8 ± 4.8	64.3 ± 4.9	p=0.95	p=0.98

All values are mean ± SEM for men (n = 149) and women (n = 163). Model 1, ANOVA main-effects model (unadjusted) including only gender as the explanatory variable. Model 2, ANOVA main-effects model (adjusted) including covariates age, BMI, centre, smoking, habitual oily fish intake. CRP, C-reactive protein; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; IL-10, interleukin 10; ICAM, intercellular adhesion molecule; VCAM, vascular cellular adhesion molecule; E-sel, E-selectin; P-sel, P-selectin.

Table A2-3.2. Baseline plasma inflammatory marker concentrations according to BMI.

	<25 kg/m^2	25-29.9 kg/m^2	≥30 kg/m^2	Model 1	Model 2
CRP (mg/l)	1.28 ± 0.12[a]	1.50 ± 0.15[ab]	2.12 ± 0.31[b]	p<0.01	p<0.01
TNF-α (pg/ml)	1.45 ± 0.06[a]	1.60 ± 0.07[b]	1.86 ± 0.20[b]	p<0.05	p=0.22
IL-6 (pg/ml)	1.27 ± 0.11	1.29 ± 0.11	1.32 ± 0.17	p=0.73	p=0.74
IL-10 (pg/ml)	1.02 ± 0.09	1.04 ± 0.09	1.06 ± 0.24	p=0.83	p=0.72
ICAM-1 (ng/ml)	296 ± 10	315 ± 10	278 ± 29	p=0.78	p=0.85
VCAM-1(ng/ml)	1967 ± 78	1840 ± 72	1729 ± 162	p<0.05	p<0.05
E-sel (ng/ml)	65.3 ± 2.7[a]	80.4 ± 3.1[b]	83.2 ± 8.4[b]	p<0.001	p<0.01
P-sel (ng/ml)	63.4 ± 5.0	62.6 ± 5.1	74.1 ± 12	p=0.56	p=0.88

All values are mean ± SEM for BMI groups <25 kg/m2 (n=155), 25-29.9 kg/m2 (n=128) and ≥30 kg/m2 (n=29). Model 1, ANOVA main-effects model (unadjusted) including only gender as the explanatory variable. Model 2, ANOVA main-effects model (adjusted) including covariates age, gender, centre, smoking, habitual oily fish intake. CRP, C-reactive protein; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; IL-10, interleukin 10; ICAM, intercellular adhesion molecule; VCAM, vascular cellular adhesion molecule; E-sel, E-selectin; P-sel, P-selectin.

Table A2-3.3. Baseline plasma inflammatory marker concentrations according to age.

	20-29 yr	30-39 yr	40-49 yr	50-59 yr	60-70 yr	Model 1	Model 2
CRP (mg/l)	1.52 ± 0.25	1.40 ± 0.24	1.18 ± 0.13	1.52 ± 0.19	1.72 ± 0.23	p=0.50	p=0.97
TNF-α (pg/ml)	1.43 ± 0.11	1.55 ± 0.09	1.42 ± 0.10	1.53 ± 0.08	0.86 ± 0.14	p=0.05	p=0.16
IL-6 (pg/ml)	0.98 ± 0.13	1.46 ± 0.23	1.28 ± 0.18	1.32 ± 0.12	1.32 ± 0.12	p=0.28	p=0.18
IL-10 (pg/ml)	0.99 ± 0.13	1.11 ± 0.15	1.06 ± 0.14	1.08 ± 0.14	0.84 ± 0.10	p=0.70	p=0.41
ICAM-1 (ng/ml)	310 ± 17	279 ± 16	297 ± 16	308 ± 13	321 ± 19	p=0.23	p=0.15
VCAM-1(ng/ml)	2036 ± 118	1898 ± 121	1739 ± 107	1906 ± 97	1926 ± 134	p=0.72	p=0.90
E-sel (ng/ml)	61.1 ± 4.3	74.9 ± 4.3	71.4 ± 3.8	81.0 ± 4.3	73.4 ± 6.1	p=0.06	p=0.20
P-sel (ng/ml)	58.8 ± 7.4	52.0 ± 6.6	64.3 ± 6.9	76.4 ± 8.0	65.4 ± 8.9	p=0.09	

All values are mean ± SEM for BMI groups <25 kg/m2 (n = 155), 25-29.9 kg/m2 (n = 128) and ≥30 kg/m2 (n = 29). Model 1, ANOVA main-effects model (unadjusted) including only gender as the explanatory variable. Model 2, ANOVA main-effects model (adjusted) including covariates gender, BMI, centre, smoking, habitual oily fish intake. CRP, C-reactive protein; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; IL-10, interleukin 10; ICAM, intercellular adhesion molecule; VCAM, vascular cellular adhesion molecule; E-sel, E-selectin; P-sel, P-selectin.

Appendix 2-4. Habitual oily fish intake and baseline plasma inflammatory marker concentrations

There was a significant effect of habitual oily fish intake (portions / week) on plasma IL-10 concentrations adjusting for age, gender, BMI, centre and smoking ($p < 0.01$), but not on any of the other inflammatory markers (Table A2-4.1).

Table A2-4.1. Baseline plasma inflammatory marker concentrations according to habitual oily fish intake.

	Q1	Q2	Q3	Q4	Q5	Model 1	Model 2
CRP (mg/l)	1.59 ± 0.22	1.16 ± 0.11	1.16 ± 0.15	1.65 ± 0.32	1.78 ± 0.22	p=0.92	p=0.98
TNF-α (pg/ml)	1.57 ± 0.10	1.42 ± 0.10	1.51 ± 0.09	1.73 ± 0.15	1.55 ± 0.08	p=0.98	p=0.48
IL-6 (pg/ml)	1.49 ± 0.20	1.01 ± 0.08	1.19 ± 0.15	1.33 ± 0.20	1.43 ± 0.19	p=0.99	p=0.97
IL-10 (pg/ml)	0.84 ± 0.09	0.89 ± 0.10	1.08 ± 0.16	1.00 ± 0.15	1.35 ± 0.16	p<0.01	p<0.05
ICAM-1 (ng/ml)	284 ± 13	314 ± 16	305 ± 18	300 ± 17	307 ± 16	p=0.74	p=0.38
VCAM-1(ng/ml)	1901 ± 116	1824 ± 108	1739 ± 114	1980 ± 112	2045 ± 119	p=0.21	p=0.14
E-sel (ng/ml)	73.3 ± 4.2	73.8 ± 4.7	73.6 ± 4.4	69.7 ± 4.3	74.8 ± 5.2	p=0.59	p=0.28
P-sel (ng/ml)	75.8 ± 8.6	60.6 ± 7.6	64.5 ± 8.1	52.4 ± 6.4	65.1 ± 6.9	p=0.86	

All values are mean ± SEM for quintiles of oily fish intake; Q1, 0; Q2, 0.2 – 0.5; 0.7 – 0.9; Q4, 1.0 – 1.6; Q5, 1.7 – 7.7 portions / week. Model 1, ANOVA main-effects model (unadjusted) including only habitual oily fish intake as the explanatory variable. Model 2, ANOVA main-effects model (adjusted) including covariates age, gender, BMI, centre, smoking. Student's t-test using Bonferroni correction revealed no difference in plasma IL-10 concentrations between quintiles of oily fish intake, $p > 0.05$. CRP, C-reactive protein; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; IL-10, interleukin 10; ICAM, intercellular adhesion molecule; VCAM, vascular cellular adhesion molecule; E-sel, E-selectin; P-sel, P-selectin.

Appendix 2-5. Plasma long chain *n-3* PUFA status and plasma IL-10 concentrations

There was no association between the quintiles of plasma LC *n-3* PUFA status and plasma IL-10 concentrations (p = 0.17; Table A2-5.1).

Table A2-5.1. Plasma IL-10 concentrations across quintiles of plasma LC *n-3* PUFA status.

Q1	Q2	Q3	Q4	Q5	Model 1	Model 2
4.87 ± 0.07	5.96 ± 0.03	6.68 ± 0.03	7.71 ± 0.07	10.16 ± 0.18	p=0.24	p=0.17

All values (pg/ml) are mean ± SEM for quintiles of plasma LC *n-3* PUFA status; Q1, 3.6 – 5.5; Q2, 5.5 – 6.3; 6.3 – 7.0; Q4, 7.0 – 8.6; Q5, 8.6 – 15.2 % of total fatty acids. Model 1, ANOVA main-effects model (unadjusted) including only plasma LC *n-3* PUFA status as the explanatory variable. Model 2, ANOVA main-effects model (adjusted) including covariates age, gender, BMI, centre and smoking. IL-10, interleukin 10; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid.

Appendix 2-6. Multiple linear regression analysis

To determine what factors, i.e. *APOE Epsilon* genotype, age, gender, BMI and habitual oily fish intake, were the strongest determinant of the baseline plasma LC *n-3* PUFA concentrations, we performed multiple linear regression analysis (Table A2-6.1).

Table A2-6.1. Multiple linear regression analysis plasma LC *n-3* PUFA status.

EPA			**Overall model fit** R^2 = 0.11		
Predictor variable	**B**	**SEM B**	**t**	**P**	**95% CI**
APOE2	0.140	0.109	1.29	0.20	-0.074, 0.354
APOE4	0.075	0.101	0.74	0.46	-0.125, 0.275
AGE	0.011**	0.003	3.29	<0.01	0.005, 0.018
FEM	-0.029	0.090	-0.32	0.75	-0.207, 0.149
BMI	-0.025	0.014	-1.77	0.08	-0.052, 0.003
SMOKE	-0.084	0.133	-0.64	0.53	-0.346, 0.177
FISH	0.151***	0.036	4.18	<0.001	0.080, 0.222
CON	1.534***	0.360	4.26	<0.001	0.825, 2.243
DHA			**Overall model fit** R^2 = 0.17		
Predictor variable	**B**	**SEM B**	**t**	**P**	**95% CI**
APOE2	0.204	0.158	1.29	0.20	-0.107, 0.514
APOE4	0.105	0.147	0.71	0.48	-0.185, 0.395
AGE	0.019***	0.005	3.68	<0.001	0.009, 0.029
FEM	0.049	0.131	0.37	0.71	-0.210, 0.307
BMI	-0.057**	0.020	-2.81	<0.01	-0.097, 0.088
SMOKE	-0.292	0.193	-1.51	0.13	-0.672, 0.088
FISH	0.291***	0.053	5.54	<0.001	0.188, 0.395
CON	4.443***	0.523	8.49	<0.001	3.412, 5.473
LC *n-3* PUFA			**Overall model fit** R^2 = 0.26		
Predictor variable	**B**	**SEM B**	**t**	**P**	**95% CI**
APOE2	0.359	0.243	1.48	0.14	-0.119, 0.838
APOE4	0.217	0.227	0.96	0.34	-0.229, 0.664
AGE	0.035***	0.008	4.43	<0.001	0.019, 0.049
FEM	-0.027	0.202	-0.13	0.89	-0.424, 0.371
BMI	-0.093**	0.031	-2.98	<0.01	-0.154, -0.031
SMOKE	-0.570	0.297	-1.92	0.06	-1.154, 0.015
FISH	0.632***	0.081	7.81	<0.001	0.473, 0.791
CON	7.108***	0.805	8.82	<0.001	5.523, 8.693

Multiple linear regression model containing *APOE Epsilon* genotype as explanatory variable including covariates age, gender, BMI, smoking and habitual oily fish intake. Predictor variables, APOE2, APOE2 genotype (APOE3 is reference); APOE4, APOE4 genotype (APOE3 is reference); AGE, age (continuous); FEM, female gender (male is reference); BMI, BMI (continuous); SMOKE, smoker (non-smoker is reference); FISH, habitual oily fish intake (continuous); CON, constant. B, beta coefficient; SEM B, standard error B; t, t-test statistic; P, p-value; 95% CI, 95% confidence interval. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. APOE, apolipoprotein E; BMI, body mass index; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid ; EPA:AA ratio, ratio of EPA to arachidonic acid; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid; n/a, not applicable; SFA:PUFA ratio, ratio of saturated fatty acids to PUFA.

To determine what factors, i.e. -219G/T genotype, *APOE Epsilon* genotype, age, gender, BMI and habitual oily fish intake, were the strongest determinant of the baseline plasma inflammatory marker concentrations, we performed similar multiple linear regression analysis (Table A2-6.2).

Table A2-6.2. Multiple linear regression analysis plasma inflammatory marker concentrations.

CRP (mg/l)			**Overall model fit R^2 = 0.06**		
Predictor variable	**B**	**SEM B**	**t**	**P**	**95% CI**
APOE2	0.010	0.245	0.04	0.97	-0.472, 0.492
APOE4	-0.032	0.216	-0.15	0.88	-0.456, 0.392
-219GT	-0.340	0.240	-1.42	0.16	-0.813, 0.132
-219TT	-0.374	0.286	-1.31	0.19	-0.935, 0.188
AGE	-0.001	0.007	-0.15	0.88	-0.016, 0.013
FEM	0.340	0.191	1.78	0.08	-0.035, 0.716
BMI	0.090**	0.029	3.07	<0.01	0.032, 0.148
SMOKE	0.531	0.298	1.79	0.08	-0.053, 1.116
FISH	-0.010	0.076	-0.14	0.89	-0.156, 0.138
CON	-0.716	0.798	-0.90	0.37	-2.286, 0.855
TNF-α (pg/ml)			**Overall model fit R^2 = 0.06**		
Predictor variable	**B**	**SEM B**	**t**	**P**	**95% CI**
APOE2	0.042	0.119	0.36	0.72	-0.192, 0.276
APOE4	0.112	0.106	1.05	0.29	-0.097, 0.321
-219GT	-0.157	0.115	-1.37	0.17	-0.384, 0.069
-219TT	-0.009	0.138	-0.06	0.95	-0.280, 0.263
AGE	0.006	0.004	1.70	0.09	-0.001, 0.013
FEM	-0.257**	0.094	-2.72	<0.01	-0.442, -0.071
BMI	0.013	0.014	0.89	0.38	-0.016, 0.041
SMOKE	-0.013	0.138	-0.09	0.93	-0.285, 0.259
FISH	-0.015	0.037	-0.40	0.69	-0.089, 0.059
CON	1.129**	0.391	2.89	<0.01	0.359, 1.897
IL-6 (pg/ml)			**Overall model fit R^2 = 0.03**		
Predictor variable	**B**	**SEM B**	**t**	**P**	**95% CI**
APOE2	0.107	0.199	0.54	0.59	-0.285, 0.499
APOE4	0.077	0.178	0.43	0.67	-0.273, 0.427
-219GT	0.029	0.193	0.15	0.88	-0.351, 0.409
-219TT	0.198	0.231	0.86	0.39	-0.256, 0.652
AGE	0.008	0.006	1.31	0.19	-0.004, 0.020
FEM	-0.051	0.158	-0.32	0.75	-0.362, 0.260
BMI	-0.004	0.024	-0.14	0.89	-0.051, 0.044
SMOKE	0.608*	0.234	2.60	<0.05	0.148, 1.0677
FISH	0.001	0.063	0.01	0.99	-0.122, 0.124
CON	0.848	0.658	1.29	0.20	-0.446, 2.142

Table A2-6.2. continued.

IL-10 (pg/ml)			**Overall model fit R^2 = 0.05**		
Predictor variable	**B**	**SEM B**	**t**	**P**	**95% CI**
APOE2	-0.154	0.160	-0.97	0.33	-0.469, 0.160
APOE4	-0.192	0.143	-1.35	0.18	-0.473, 0.089
-219GT	0.187	0.155	1.21	0.23	-0.117, 0.491
-219TT	0.105	0.185	0.57	0.57	-0.259. 0.469
AGE	-0.003	0.005	-0.69	0.49	-0.013, 0.006
FEM	-0.023	0.127	-0.18	0.86	-0.272, 0.226
BMI	-0.003	0.019	-0.15	0.88	-0.041, 0.035
SMOKE	0.146	0.187	0.78	0.44	-0.223, 0.514
FISH	0.181***	0.050	3.61	<0.001	0.082, 0.280
CON	1.055	0.526	2.00	0.05	0.019, 2.090
ICAM-1 (ng/ml)			**Overall model fit R^2 = 0.03**		
Predictor variable	**B**	**SEM B**	**t**	**P**	**95% CI**
APOE2	3.824	19.344	0.20	0.84	-34.243, 41.891
APOE4	5.572	17.239	0.32	0.75	-28.353, 39.497
-219GT	-35.644	18.725	-1.90	0.06	-72.493, 1.206
-219TT	-3.209	22.388	-0.14	0.89	-47.266, 40.848
AGE	0.749	0.587	1.28	0.20	-0.406, 1.904
FEM	-6.090	15.338	-0.40	0.69	-36.273, 24.093
BMI	-1.372	2.362	-0.58	0.56	-6.020, 3.277
SMOKE	34.510	22.496	1.53	0.13	-9.759, 78.779
FISH	0.993	6.080	0.16	0.87	-10.973, 12.959
CON	316.406***	63.717	4.97	<0.001	1991.018, 441.793
VCAM-1 (ng/ml)			**Overall model fit R^2 = 0.06**		
Predictor variable	**B**	**SEM B**	**t**	**P**	**95% CI**
APOE2	307.609*	134.765	2.28	<0.05	42.400, 572.818
APOE4	334.072**	120.551	2.85	<0.01	106.837, 581.307
-219GT	-186.325	130.556	-1.43	0.16	-443.250, 70.601
-219TT	52.842	156.824	0.34	0.74	-255.777, 361.461
AGE	-0.316	4.094	-0.08	0.94	-8.372, 7.740
FEM	-73.420	106.853	-0.69	0.49	-283.699, 136.860
BMI	-35.826*	16.413	-2.18	<0.05	-68.126, 3.527
SMOKE	-68.962	156.983	-0.44	0.66	-377.892, 239.969
FISH	44.839	42.740	1.06	0.29	-38.679, 128.357
CON	2680.64***	44.666	6.03	<0.001	1805.568, 3556.712
E-sel (ng/ml)			**Overall model fit R^2 = 0.10**		
Predictor variable	**B**	**SEM B**	**t**	**P**	**95% CI**
APOE2	-9.609	5.280	-1.82	0.07	-20.000, 0.781
APOE4	-12.030*	4.705	-2.56	<0.05	21.288, 2.771
-219GT	5.411	5.116	1.06	0.29	-4.657, 15.478
-219TT	-1.977	6.127	-0.32	0.75	-14.034, 10.080
AGE	0.219	0.160	1.37	0.17	-0.096, 0.534
FEM	-8.645*	4.180	-.207	<0.05	-16.871, -0.420
BMI	1.851**	0.643	2.88	<0.01	0.586, 3.116
SMOKE	4.820	6.152	0.78	0.43	-7.286, 16.927
FISH	-0.865	1.664	-0.52	0.60	-4.139, 2.410
CON	26.463	17.401	1.52	0.13	-7.780, 60.705

Table A2-6.2. continued.

P-sel (ng/ml)			Overall model fit R^2 = 0.07		
Predictor variable	**B**	**SEM B**	**t**	**P**	**95% CI**
APOE2	-36.536***	9.059	-4.03	<0.001	-54.363, 18.709
APOE4	-26.772	8.032	-3.33	<0.01	-42.579, 10.965
-219GT	-6.593	8.774	-0.75	0.453	-23.860, 10.673
-219TT	-16.169	10.477	-1.54	0.12	-36.787, 4.450
AGE	0.461	0.274	1.68	0.09	-0.079, 1.001
FEM	2.644	7.187	0.37	0.72	-11.500, 2.605
BMI	0.410	1.115	0.37	0.71	-1.785, 2.605
SMOKE	7.166	10.510	0.68	0.50	-13.518, 27.849
FISH	-0.419	2.849	-0.15	0.88	-6.024, 5.187
CON	58.485	30.148	1.94	0.05	-0.844, 117.815

Multiple linear regression model containing *APOE Epsilon* genotype and -219G/T genotype as explanatory variable including covariates age, gender, BMI, smoking and habitual oily fish intake. Predictor variables, APOE2, APOE2 genotype (APOE3 is reference); APOE4, APOE4 genotype (APOE3 is reference); -219GT, -219GT genotype (-219GG is reference); -219TT, -219TT genotype (-219GG is reference); AGE, age (continuous); FEM, female gender (male is reference); BMI, BMI (continuous); SMOKE, smoker (non-smoker is reference); FISH, habitual oily fish intake (continuous); CON, constant. B, beta coefficient; SEM B, standard error B; t, t-test statistic; P, p-value; 95% CI, 95% confidence interval. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. APOE, apolipoprotein E; BMI, body mass index; CRP, C-reactive protein; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; IL-10, interleukin 10; ICAM, intercellular adhesion molecule; VCAM, vascular cellular adhesion molecule; E-sel, E-selectin; P-sel, P-selectin.

Appendix 3-1. Fatty acid composition of the diets

Animals had free access to food and water, with fresh food being provided every 2nd/3rd day. The stability of the fatty acids in the experimental diets over the course of three days was investigated to confirm that the fatty acid composition did not change over time. The results are displayed in Table A3-1.1 presenting the fatty acid composition of the experimental diets after 24, 48 and 72 hr at room temperature and exposed to air. Briefly, the results confirm that the fatty acid composition, in particular that of EPA and DHA, of the experimental diets did not significantly change after 3 days at room temperature.

Table A3-1.1. Fatty acid composition of the experimental diets over the course of three days.

		HFD			HFD+FO		
% of total fatty acids		day 1	day 2	day 3	day 1	day 2	day 3
saturated							
14:0	Myristic acid	5.6	6.0	5.1	25.6	33.1	36.9
15:0	Pentadecanoic acid	ND	ND	ND	1.8	2.3	2.4
16:0	Palmitic acid	107.7	116.3	97.3	99.6	130.4	137.2
17:0	Margaric acid	1.9	2.1	1.7	5.5	7.0	8.0
18:0	Stearic acid	58.9	63.3	52.5	42.6	55.7	57.6
20:0	Arachidic acid	1.2	1.3	1.1	3.2	13.8	2.6
22:0	Behenic acid	ND	ND	ND	0.6	0.9	0.8
24:0	Lignoceric acid	ND	ND	ND	0.6	0.7	0.7
monounsaturated							
14:1	Myristoleic acid	ND	ND	ND	ND	ND	ND
16:1 n-7	Palmitoleic acid	7.5	8.2	6.8	30.8	38.2	43.1
18:1 trans		1.4	1.5	1.0	0.9	1.8	1.4
18:1 n-7	Vaccenic acid	10.8	11.7	9.8	12.4	15.4	17.0
18:1 n-9	Oleic acid	177.0	193.6	161.9	125.2	155.7	172.7
20:1 n-9	Gondoic acid	3.0	3.3	2.7	2.6	5.7	3.9
24:1 n−9	Nervonic acid	0	0	0	4.0	5.1	5.7
polyunsaturated							
18:2 n-6	Linoleic acid	124.6	133.6	112.5	88.6	107.9	122.5
18:3 n-3	α-Linolenic acid	8.2	8.9	7.6	10.0	13.9	13.8
18:4 n-3	Parinaric acid	ND	ND	ND	4.7	6.0	6.7
20:2 n-6	Eicosadienoic acid	3.6	3.8	3.2	2.3	2.6	3.2
20:3 n-6	Dihomo-gamma-linolenic acid	ND	ND	ND	0.8	1.0	1.1
20:3 n-3	Eicosatrienoic acid	0.6	0.6	0.5	0.6	0.8	0.9
20:4 n-3	Eicosatetraenoic acid	ND	ND	ND	2.1	3.1	3.1
20:4 n-6	Arachidonic acid	1.2	1.1	1.0	3.2	3.8	4.5
20:5 n-3	Eicosapentaenoic acid	ND	ND	ND	20.7	26.2	29.6
22:5 n-3	Docosapentaenoic acid	ND	ND	ND	0.6	0.7	0.9
22:6 n-3	Docosahexaenoic acid	ND	ND	ND	31.1	40.1	44.4

HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA / kg of diet.

Appendix 3-2. mRNA expression analysis by RT-QPCR

Reference gene selection

The six reference candidate genes *18S rRNA gene* (*18S*), *actin, beta, cytoplasmic* (*Actb*), *ATP synthase subunit* (*Atp5b*), *eukaryotic translation initiation factor 4A2* (*Eif4a2*), *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*), *ribosomal protein L13a* (*Rpl13a*), were screened for gene expression stability using 3 – 4 random samples from each *APOE Epsilon* genotype * diet group. The average expression stability of the six reference candidate genes, expressed as geNorm M-values, is presented in Figure A3-2.1. The analysis of the optimal number of reference genes, indicated by geNorm Vn/n+1 values, is depicted in Figure A3-2.2.

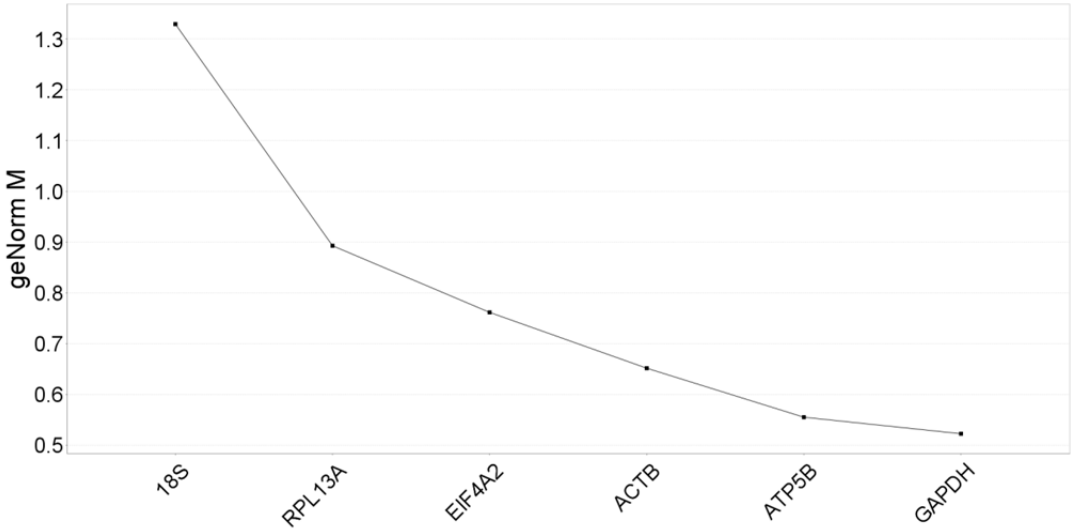


Figure A3-2.1. GeNorm M graph presenting the average expression stability of the reference candidate genes. The geNorm M graph shows the ranking of the reference candidate genes according to their stability, expressed in geNorm M values, from most unstable genes at the left (high M value) to the best reference genes at the right (low M value). The threshold value for M is 0.5, with values ≤ 0.5 indicating a high expression stability. The six reference candidate genes screened all had M-values greater than 0.5, indicating a suboptimal to poor average expression stability. *18S rRNA gene* (*18S*), *actin, beta, cytoplasmic* (*Actb*), *ATP synthase subunit* (*Atp5b*), *eukaryotic translation initiation factor 4A2* (*Eif4a2*), *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*), *ribosomal protein L13a* (*Rpl13a*).

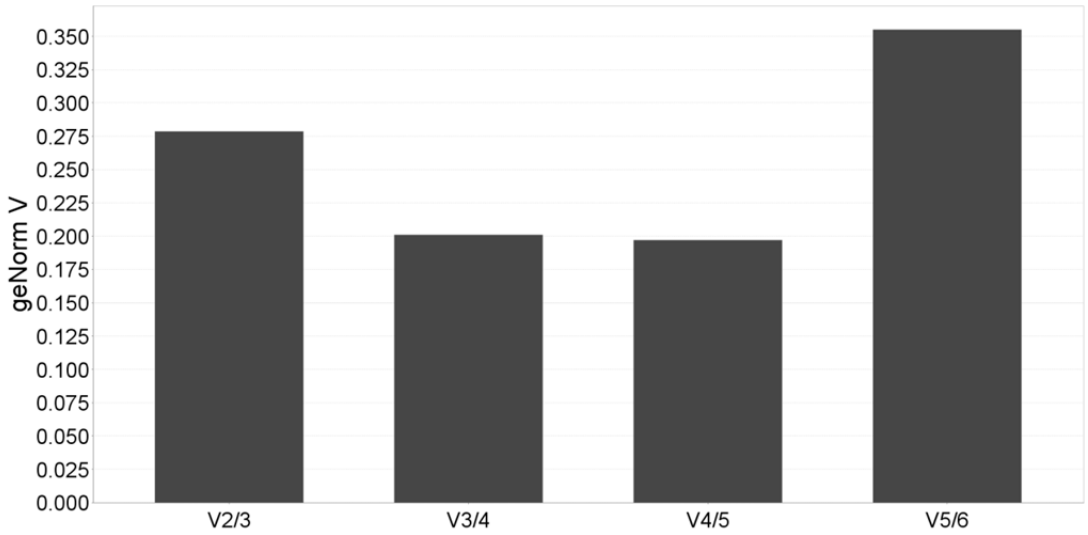


Figure A3-2.2. GeNorm V graph indicating the optimal number of reference genes. The GeNorm V graph presents the Vn/n+1 value for every comparison between two consecutive number (n and n+1) of candidate reference genes. The benefit of using an extra (n+1)th reference gene is limited as soon as the Vn/n+1 value drops below the 0.15 threshold (green horizontal line) [Hellemans et al., Genome Biology, 2007.]. The lowest Vn/n+1 value was obtained for V3/4 and V4/5, indicating that the optimal number of reference genes to use was 3 – 4 target reference genes with the lowest M value (i.e. highest expression stability). The Vn/n+1 value did not drop below the 0.15 threshold indicating that the variability between sequential normalization factors (based on the n and n+1 least variable reference target genes) was relatively high.

In summary, the six reference candidate genes had a low expression stability (geNorm M > 0.5). In addition, the variability between sequential normalization factors (based on the n and n+1 least variable reference genes) was relatively high (geNorm V > 0.15), which made it difficult to determine the optimal number of reference genes. Due to high variability in normalisation factors and the low reference gene stability, it was recommended to use 3 – 4 reference genes with the lowest M value, as the use of multiple reference genes results in more accurate normalization compared to the use of a single non-validated reference gene.

The reference candidate genes *Act*), *Atp5b* and *Gapdh* had the most stable gene expression levels between the mice and different treatment groups. Therefore, data was normalized against the reference genes *actin, beta* (*Actb*), *ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit* (*Atp5b*) and *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*).

RT-QPCR data control, modification and exclusion

Prior to the analysis of the relative expression levels, the RT-QPCR raw data was investigated to verify an exponential amplification efficiency and correct for the sensitivity limit of the assay. In more detail, the samples with a non-exponential amplification, indicating poor reaction efficiency, were omitted from analysis (missing values). Samples with cycle threshold (C_T) values below the sensitivity limit of the assay but 5 or more C_T's greater than the NTC for that assay (indication of target-specific amplification), were assigned the C_T value corresponding to the detection limit of that assay, in order to minimise the bias

resulting from the exclusion of these samples on the end results (Table A3-2.1). Sample 4 from the APOE3-HFD group was excluded from all analysis due to too many missing values.

Table A3-2.1. Overview of samples with C_T-values below the detection limit or missing values.

Gene	Sample (*APOE Epsilon* genotype* diet group, sample no.)	C_T change (original -> assigned value)
Apoe	APOE3-HFD #4 APOE3-HFD #1 APOE4-HFD #8	Missing values Missing values Missing values
Arg1	APOE3-HFD #4 APOE3-HFD+FO #3, 4, 5, 6, 7 APOE4-HFD #2, 3, 4, 9 APOE4-HFD+FO #3, 4, 7, 8 APOE4-HFD+FO #9, 10	Missing values 26.8, 26.8, 26.2, 28.0, 27.2 -> 26 26.2, 27.8, 27.5, 28.3 -> 26 26.4, 26.4, 27.3, 26.4 -> 26 Missing values
Cd11b (*Itgam*)	APOE3-HFD #4 APOE4-HFD #8	Missing values Missing values
Cd11c (*Itgax*)	APOE3-HFD #4 APOE3-HFD+FO #6 APOE3-HFD+FO #10 APOE4-HFD+FO #1	Missing values 32.4 -> 31 Missing values Missing values
Cd206 (*Mrc1*)	APOE3-HFD #8 APOE3-HFD+FO #10 APOE4-HFD #8 APOE4-HFD+FO #7	Missing values Missing values Missing values Missing values
Il6	APOE4-HFD #3, 4, 6, 9 APOE4-HFD+FO #9,10	31.5, 32.5, 31.5, 31.7 -> 31 Missing values
Il10	APOE3-HFD #4 APOE4-HFD #6	Missing values Missing values
Mgl1/2	APOE3-HFD #4 APOE4-HFD #6	37.0 -> 31 Missing values
Nos2	n/a	n/a
Tnfa	n/a	n/a

Apoe, apolipoprotein E; *Arg1*, arginase 1; *Cd11b*, integrin alpha M (*Itgam*); *Cd11c*, integrin alpha X (*Itgax*); *Cd206*, mannose receptor, C type 1 (*Mrc1*); HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA / kg of diet; *Il6*, interleukin 6; *Il10*, interleukin 10; *Mgl1/2*, C-type lectin domain family 10, member A (*Clec10a*, also known as *Mgl1*) and macrophage galactose N-acetyl-galactosamine specific lectin 2 (*Mgl2*); n/a, not applicable; *Nos2*, nitric oxide synthase 2, inducible; *Tnfa*, tumor necrosis factor alpha.

Appendix 3-3. Food intake results

Average food intake per week

The average food intake (gram / mouse / day) of the human *APOE3* and *APOE4* targeted replacement mice fed a high-fat diet (HFD) without or with 30 g EPA+DHA / kg of diet (HFD+FO) per week is presented in Figure A3-3.1. Briefly, *APOE3* mice on the HFD had a consistent higher average food intake (g / mouse / day) compared to the *APOE3* mice on HFD+FO and the *APOE4* mice on either of the two diets, which was most pronounced in the first three weeks.

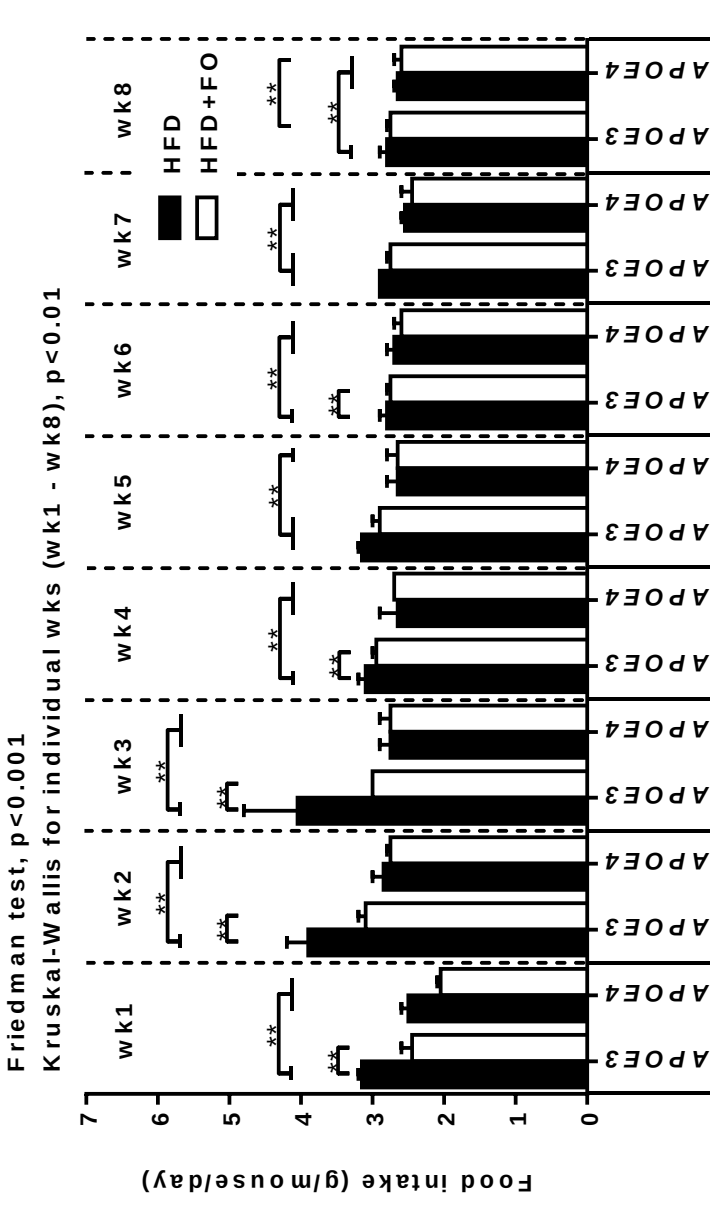


Figure A3-3.1. Average food intake of human *APOE3* and *APOE4* targeted replacement mice fed a high-fat diet without (HFD) or with 30 g EPA+DHA / kg of diet (HFD+FO). Data is presented as mean ± SEM average food intake (gram / mouse / day) per week, n = 10 mice / group. The average food intake (g/day) per mouse was calculated by dividing the weekly joint food intake (g) of 5 mice in a single cage by 5 (accounting for the 5 mice) and then by 7 days. Statistical analysis within subjects was done by Friedman test and revealed there were differences in the food intake between the weeks ($X_2(7) = 223$, $p < 0.001$). Statistical analysis between subjects was done by Kruskal-Wallis test and considered statistically significant when $p < 0.05$. Significant effects are displayed on top of the figure. Differences in the average food intake between the groups for the individual weeks were tested using Mann-Whitney U test with Bonferroni correction; *$p < 0.05$, **$p < 0.01$. APOE, apolipoprotein E genotype; HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA / kg of diet.

Food efficiency per week

There were no major differences in the average weekly food efficiency of the human *APOE3* and *APOE4* targeted replacement mice fed a high-fat diet (HFD) without or with 30 g EPA+DHA / kg of diet (HFD+FO) (Figure A3-3.2). Overall, mice gained on average 0.07 g of body weight for each gram of food consumed (Figure 3.6B).

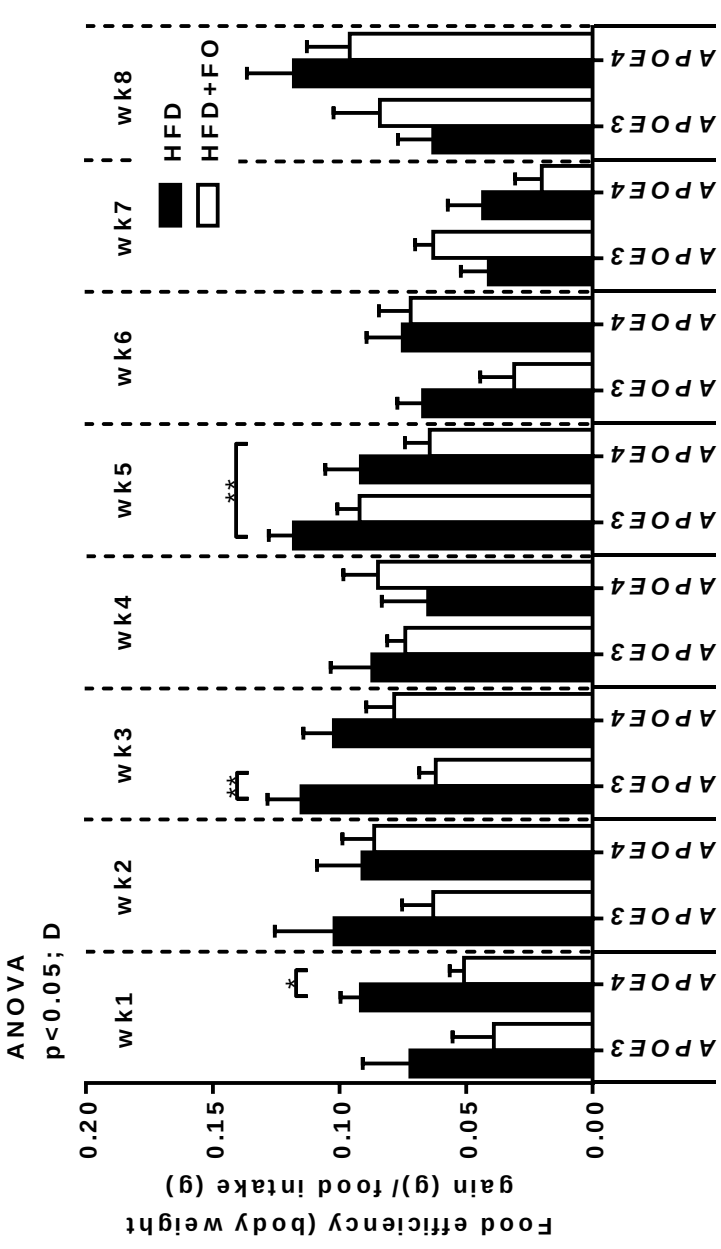


Figure A3-3.2. Food efficiency of human *APOE3* and *APOE4* targeted replacement mice fed a high-fat diet (HFD) without or with 30 g EPA+DHA / kg of diet (HFD+FO). Data is presented as mean ± SEM food efficiency (food intake (g) / body weight gain (g)) per week, n = 10 mice / group. The weekly food efficiency was calculated for each mouse by dividing the weekly estimated total food intake (g/mouse) by the weekly mouse-specific body weight gain (g). The weekly total food intake (g/day) per mouse was estimated by dividing the weekly joint food intake (gram) of 5 mice in a single cage by 5 (accounting for the 5 mice). Statistical analysis within subjects was done by repeated measures ANOVA with Greenhouse-Geisser correction; time $p < 0.001$, time* genotype $p < 0.05$, time*diet $p = 0.12$, time*genotype*diet $p = 0.14$. Statistical analysis between subjects was done by repeated measures ANOVA with genotype (G) and diet (D) as independent factors; genotype $p = 0.61$, diet $p < 0.05$, genotype*diet $p = 0.77$. Differences between the groups for the individual weeks were tested using Student's *t*-test or Mann-Whitney U test with Bonferroni correction; *p < 0.05, **p < 0.01. APOE, apolipoprotein E genotype; HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA / kg of diet.

Appendix 3-4. Adipose tissue long chain *n-3* PUFA status and IL-10 mRNA expression

There was no relationship between between adipose tissue long chain (LC) *n-3* PUFA, EPA or DHA content and IL-10 expression levels (Table A3-4.1).

Table A3-4.1. Correlation LC *n-3* PUFA status and measures related to insulin resistance.

Dependent variable	Independent variable	Pearson r	p-value
IL-10 mRNA	LC n-3 PUFA	0.287	p=0.08
	EPA	0.276	p=0.09
	DHA	0.282	p=0.09

Pearson's correlation efficient (r) was used to determine the correlation between the adipose tissue LC *n-3* PUFA, EPA and DHA concentrations and adipose tissue IL-10 mRNA expression levels. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IL-10, interleukin 10; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid.

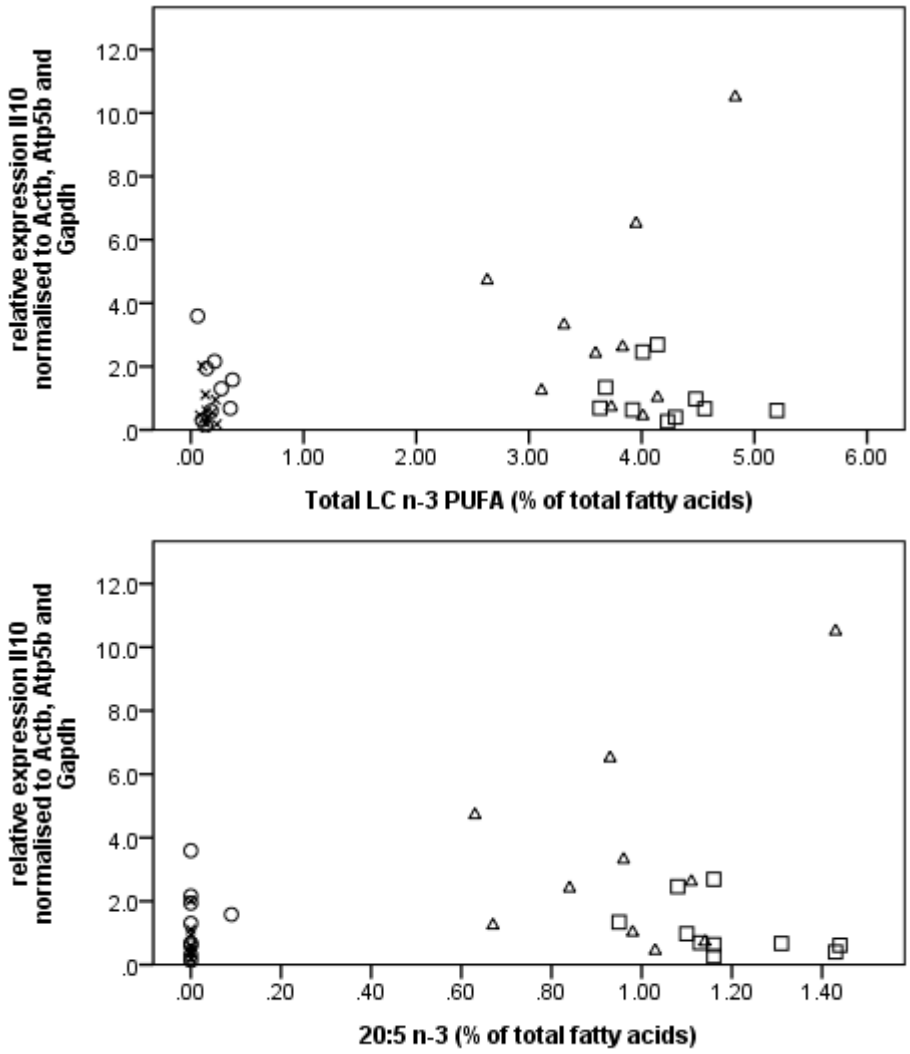


Figure A3-4.1. continues on next page...

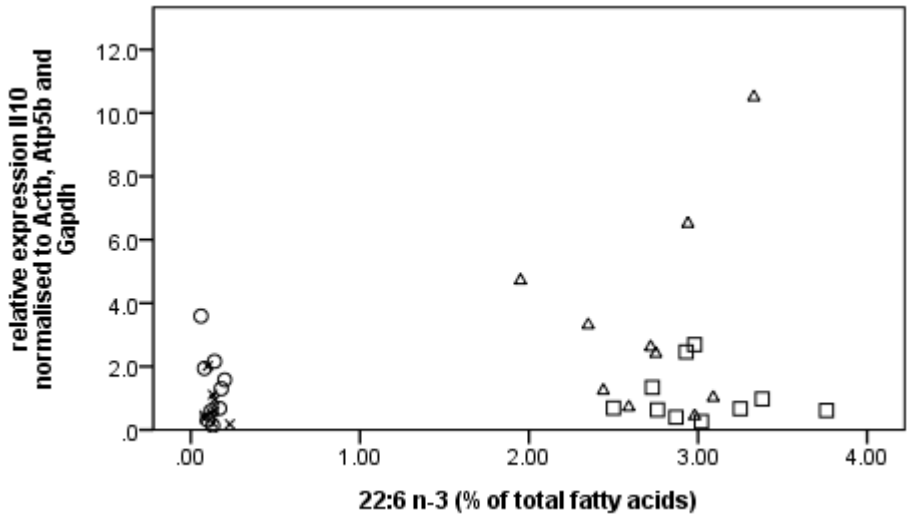


Figure A3-4.1. Correlation between adipose tissue IL-10 mRNA expression levels and adipose tissue LC *n-3* PUFA (r = 0.287), EPA (r = 0.276) and DHA (r = 0.282) concentrations. Crosses, APOE3-HFD; squares, APOE3-HFD+FO; circles, APOE4-HFD; triangles, APOE4-HFD+FO.

Appendix 4-1. Flow cytometry analysis

Flow cytometry data collection

For each sample, data of 30,000 events was collected using a gate excluding debri (gate P1, Figure A4-1.1).

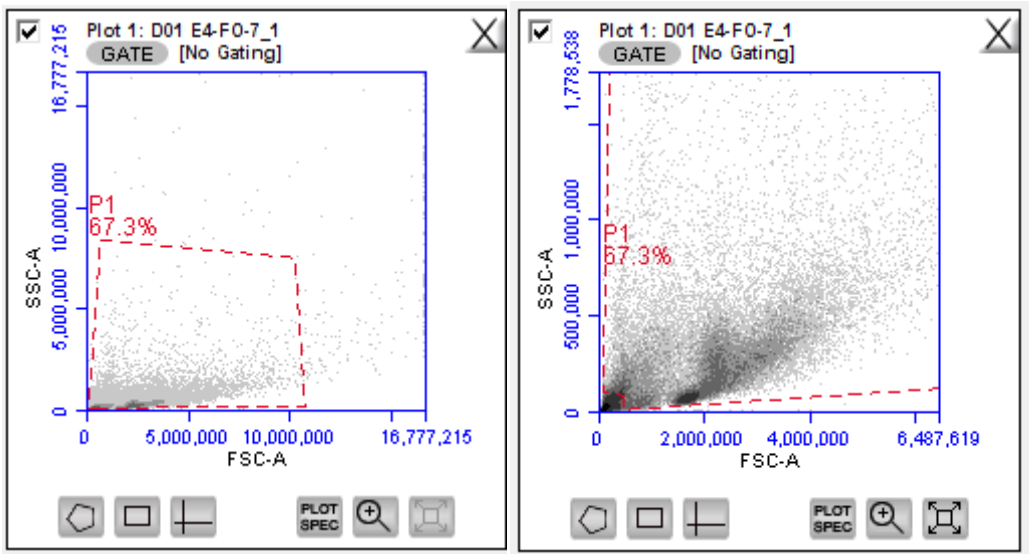


Figure A4-1.1. The gate P1 that was used to collect 30,000 events for each sample. On the left the forward (FSC-A) and sideward (SSC-A) scatter plot with gate P1, and on the right the same graph but then zoomed in showing the target cell population is presented.

Gate setup

The setup of the gate E including the adipose tissue macrophages (ATM) and excluding debris, small cell (i.e. red blood cells) and cell clumps, which was applied to the original data of 30,000 collected events, is presented in Figure A4-1.2. The setup of gate E was based on the methodology described by Danett and Smith (*Methods in Molecular Biology, Vol. 456: Adipose Tissue Protocols, Second Edition*.). The setup of the thresholds to define the positive and negative cell populations for the cell surface markers CD11b, CD11c and CD206 using fluorophore minus-one control and single-stained samples is presented in Figure A4-1.3.

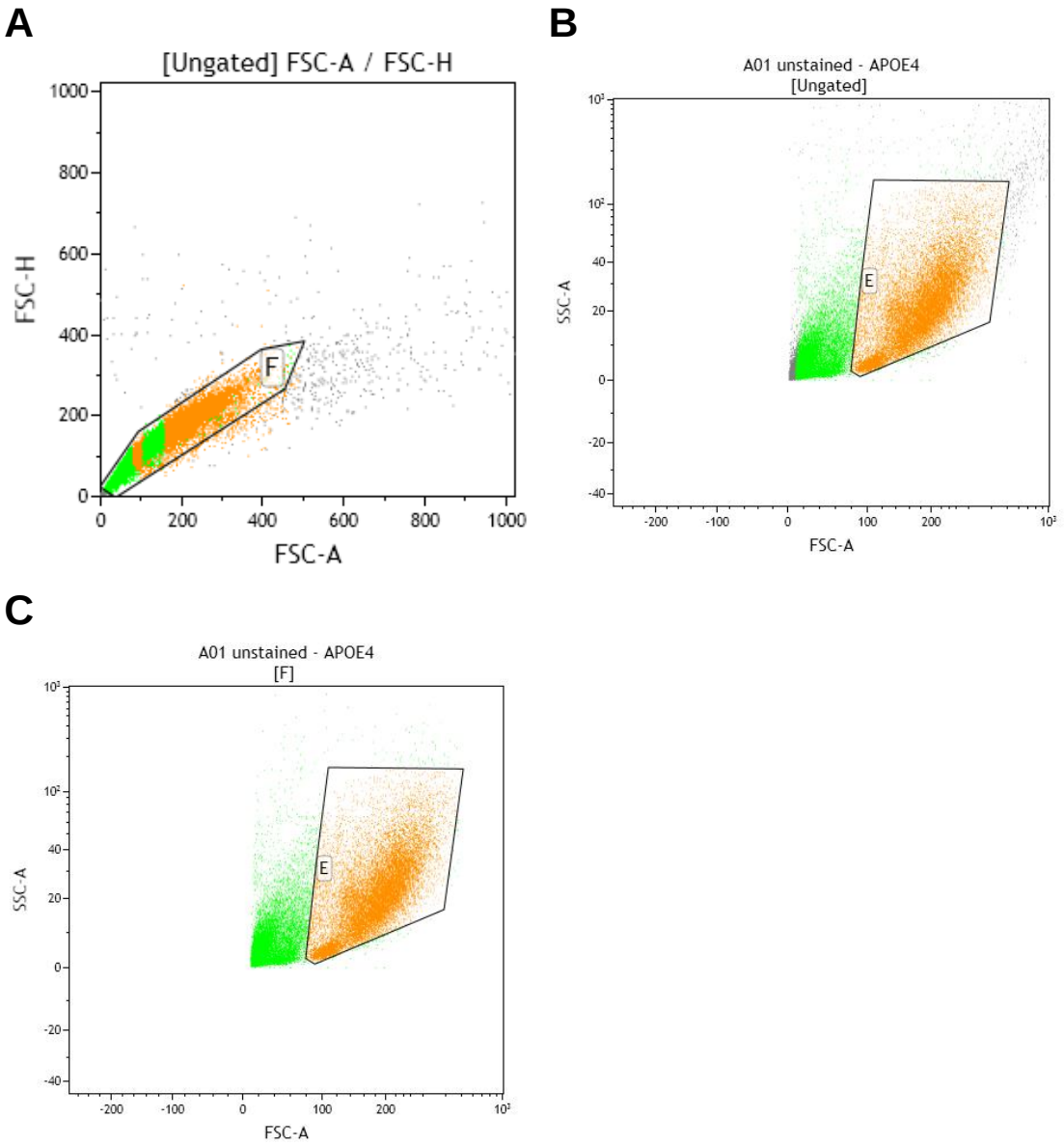
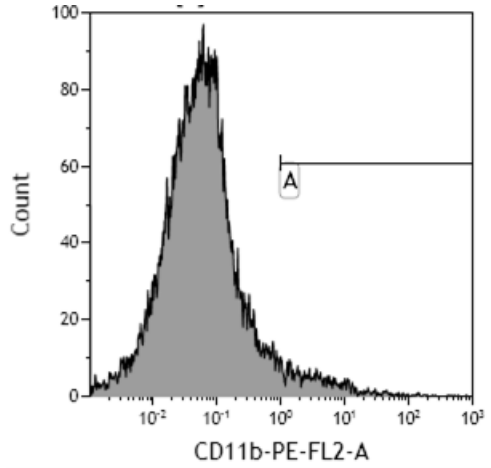


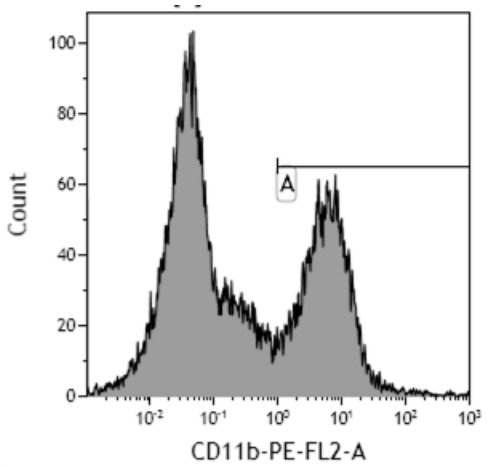
Figure A4-1.2. The setup of gate E to exclude cell clumps using the correlation between forward scatter height (FSC-H) and area (FSC-A). The left graph (A) presenting the forward scatter height (FSC-H) versus area (FCS-A) plot of all 30,000 events (ungated), shows the single cell population in gate F. Each cell, singlet or doublet, generates upon passing the laser a pulse that has a height (H), width (W) and area (A) underneath the peak. For single cells, there is a linear correlation between the height (FSC-H) and the area under the peak (FSC-A). However, this correlation will be violated by cell clumps, which have the same FSC-H but a greater FSC-A, and would be to the right of the linear correlation between FSC-A and FSC-H (indicated as gate F). The two other graphs presenting the sideward scatter height (FSC-H) versus forward scatter area (FCS-A) plot of all 30,000 events (ungated) (B) and of events in gate F (C), show that doublets and cell clumps (grey dots) are mainly located outside on the top right of gate E.Most single cells (coloured dots, graph A) are located within the gate E (orange dots), whereas very small cells (i.e. red blood cells) and debris are located to the left of gate E (green dots). The data was obtained from an unstained stromal vascular fraction (SVF) sample of the epididymal adipose tissue from a male *APOE4* mouse.

A



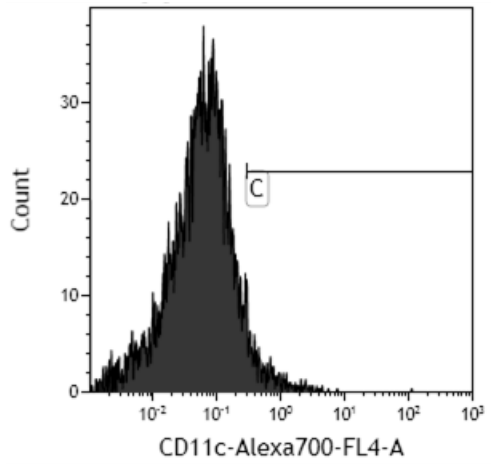
Gate	%Total	%Gated
All	35.87	100.00
A	1.79	4.99

B



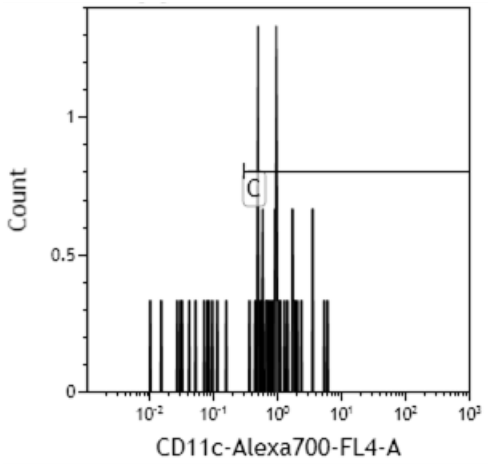
Gate	%Total	%Gated
All	39.06	100.00
A	13.96	35.75

C



Gate	%Total	%Gated
All	17.22	100.00
C	0.68	3.96

D



Gate	%Total	%Gated
All	0.09	100.00
C	0.06	70.00

Figure A4-1.3. continues on next page.

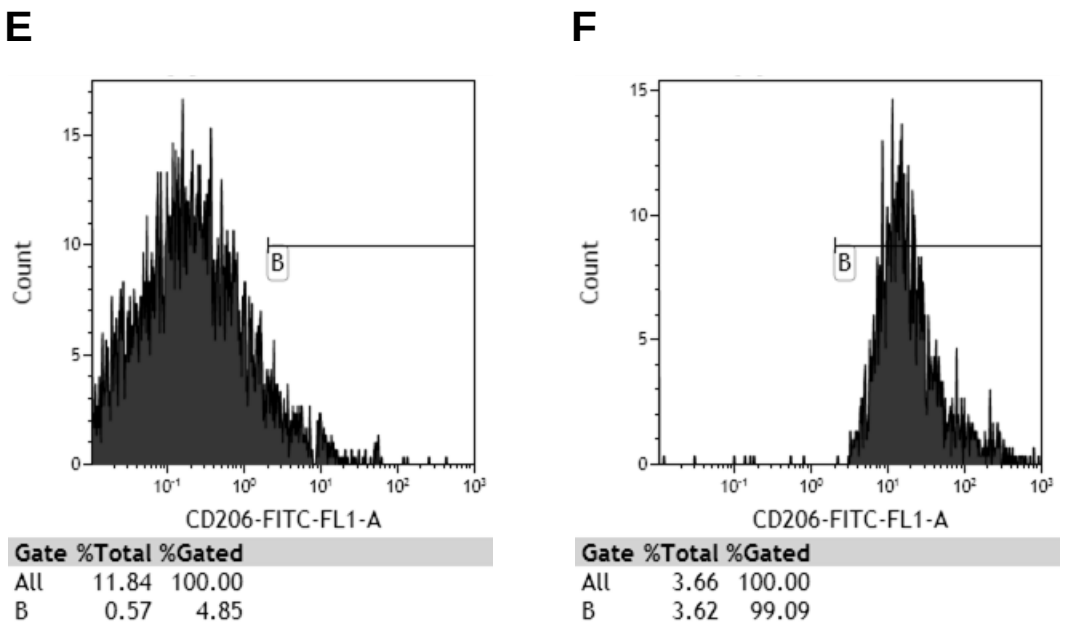


Figure A4-1.3. The setup of the thresholds to define positive and negative cell populations for the cell surface markers CD11b, CD11c and CD206 using fluorophore minus-one control and single-stained samples. The fluorophore minus-one control sample was used to define the negative cell population. The single stained sample was used to confirm that the positive cell population can be distinguished using this threshold. The threshold was set at the point to exclude the fluorescent signal (peak) of the negative cell populations (graphs A, C and E) and to include the fluorescent signal (peak) of the positive cell population (graphs B, D and F). The threshold for CD11b-PE-FL2-A with positive cell population in gate A, classified 4.52 % of cells from fluorophore minus-one sample (graph A) and 35.75 % of cells in the single stained sample (graph B) as cells expressing CD11b on their cell surface. The threshold for CD11c-Alexa700-FL4-A with positive cell population in gate C, classified 3.96 % of cells from fluorophore minus-one sample (graph C) and 70.00 % of cells in the single stained sample (graph D) as cells expressing CD11c on their cell surface. A limitation of the CD11b single stained sample was that it contained very few CD11b positive cells. The threshold for CD206-FITC-FL1-A with positive cell population in gate B, classified 4.85 % of cells from fluorophore minus-one sample (graph E) and 99.09 % of cells in the single stained sample (graph F) as cells expressing CD206 on their cell surface. Data was obtained from stromal vascular fraction (SVF) cell samples from various epididymal adipose tissues from male *APOE3*, *EFAD*, *APOE4* mice. The *EFAD* mouse strain expresses the human gene associated with the familial form of Alzheimer's disease (FAD) and the human *APOE4* gene, a known risk factor for Alzheimer's disease.

Cell yield

The cell yield in gate E (% of the total collected events) was between 27.6 ± 1.48 (SEM) % and 41.1 ± 2.43 % for the *APOE Epsilon* genotype*diet groups and was significantly higher for the APOE4-HFD group compared to the APOE3-HFD+FO group ($p < 0.05$).

Table A4-1.1. Cell yield in gate E (% of total events) for the *APOE Epsilon* genotype*diet groups.

Group	Mean (% of total)*	SD	n	SEM
APOE3-HFD	32.8	13.610	10	4.304
APOE3-HFD+FO	27.6	4.433	9	1.478
APOE4-HFD	41.1	7.685	10	2.430
APOE4-HFD+FO	29.1	9.172	10	2.901

*Kruskal-Wallis test revealed a significant difference between the *APOE Epsilon* genotype*diet groups ($p<0.05$), with a higher cell yield for APOE4-HFD group compared to APOE3-HFD+FO group ($p<0.05$). HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA/kg of diet.

Compensation set up

Single stain control samples were used to determine spill over of CD206-FITC into 585/40 BP and 670/LP filter; and CD11b-PE into 533/30 BP filter and 670/LP filter, to apply appropriate compensation (Table A4-1.2).

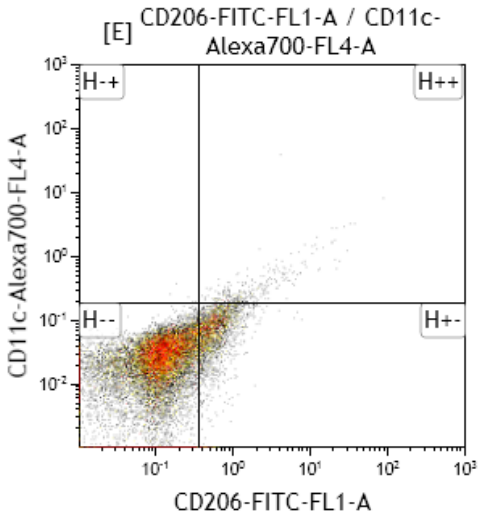
Table A4-1.2. Compensation table - Spill over (%) matrix

Spill over (%)	**FL1**	**FL2**	**FL3**	**FL4**
FL1		2.70	N/A	1.30
FL2	1.90		N/A	0.00
FL3	N/A	N/A		N/A
FL4	0.80	0.00	N/A	

The compensation was setup using the target cell population (gate E), which was also used for final analysis of experimental samples. This target population contained besides our adipose tissue macrophages of interest also eosinophils, neutrophils and granulocytes. These last three cell types have a high autofluoresence resulting in a population with a tail in the CD11c-Alexa700-FL4 versus CD206-FITC-FL1 plot (Figure A4-1.4A and A4-1.4B). This posses some challenge to setting compensation, since the tail is fairly impossible to bring down and compensation aims to lower the fluorescence median of this population to a value similar to the negative cell population. Future experiments are recommended to include in the panel one or more markers for these cells, so that the cells positive for CD11b+, but negative for the eosinophil/neutrophil/granulocyte marker(s) can be selected (negative selection).

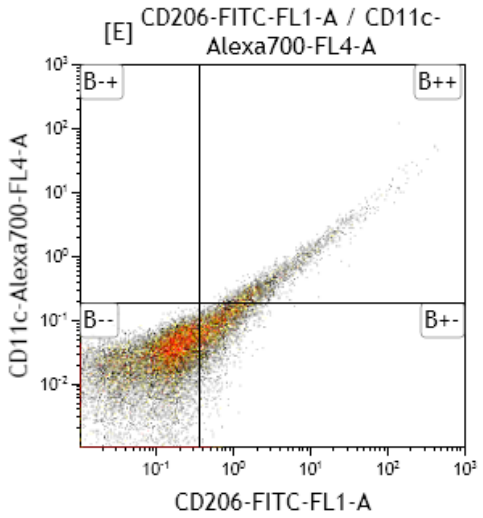
Using the single stain CD11b-PE sample spill over into CD206-FITC-FL1 and CD11c-Alexa700-FL4 was determined; a compensation of 2.70 % was needed to correct for spill over of CD11b-PE into CD206-FITC-FL1 (Figure A4-1.5A), whereas there was no spill over into CD11c-Alexa700-FL4 (Figure A4-1.5B). Using the single stain CD11c-Alexa700 sample spill over into CD11b-PE-FL2 and CD206-FITC-FL1 was determined; a compensation of 1.30 % was needed to correct for spill over of CD11c-Alexa700 into CD206-FITC-FL1 (Figure A4-1.6B). However, no compensation could be set for spill over of CD11c-Alexa700 into CD11b-PE-FL2 (Figure A4-1.6A) due to lack of CD11c positive cells in the sample. Using the single stain CD206-FITC sample spill over into CD11b-PE-FL2 and CD11c-Alexa700-FL4 was determined; a compensation of 1.90 % and 0.80 % was needed to correct for spill over of CD206-FITC into CD11b-PE-FL2 (Figure A4-1.7A) and CD11c-Alexa700-FL4 (Figure A4-1.7B).

A



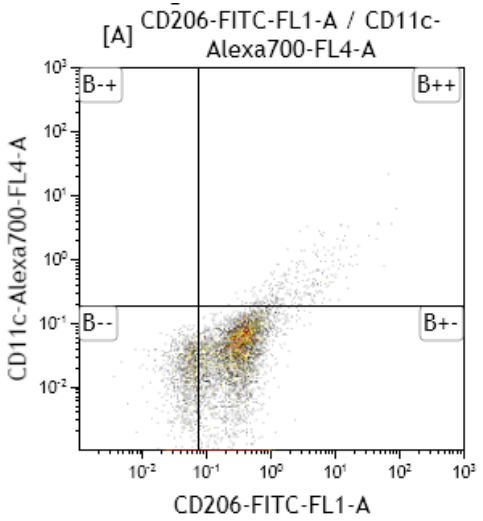
Gate	%Total	%Gated	X-Med	Y-Med
All	39.91	100.00	0.13	0.02
H--	33.80	84.67	0.11	0.02
H-+	0.01	0.02	0.27	0.23
H+-	5.52	13.84	0.56	0.07
H++	0.58	1.46	1.40	0.27

B



Gate	%Total	%Gated	X-Med	Y-Med
All	49.62	100.00	0.17	0.03
B--	36.19	72.94	0.12	0.02
B-+	0.00	0.00	0.37	0.19
B+-	9.20	18.54	0.63	0.08
B++	4.22	8.51	2.86	0.37

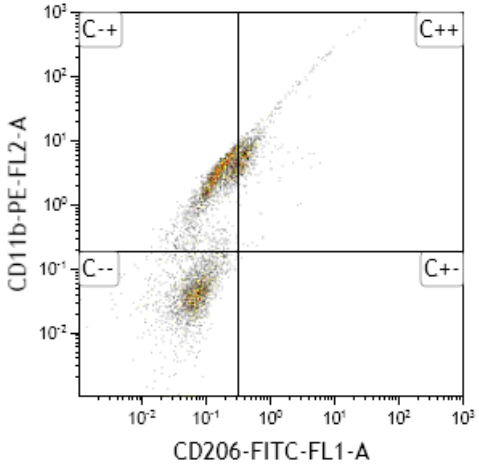
C



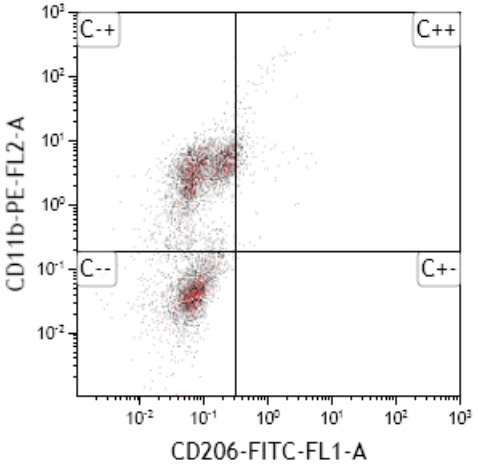
Gate	%Total	%Gated	X-Med	Y-Med
All	13.56	100.00	0.25	0.02
B--	2.90	21.36	0.05	0.01
B-+	N/A	N/A	N/A	N/A
B+-	10.19	75.19	0.30	0.03
B++	0.47	3.45	2.43	0.36

Figure A4-1.4. Plots illustrating the presence of highly autofluorescent cells (eosinophils, neutrophils and granulocytes) in the stromal vascular fraction as indicated by the tail (upper right corner graph A and B). The percentage of autofluorescent eosinophils and granulocytes - the cells in the upper right corner (plot area H++, graph A; B++ graph B) in the CD11c-Alexa700-FL4 versus CD206-FITC-FL1 plot - is approximately 5.0 % (range 1.46 – 8.51 %) of total cells in gate E. Among the CD11b-positive cells the percentage of autofluorescent cells is 3.45 % (plot area B++ graph C). Data was obtained from stromal vascular fraction (SVF) cell samples from various epididymal adipose tissues from male *APOE3*, *EFAD*, *APOE4* mice. The *EFAD* mouse strain expresses the human gene associated with the familial form of Alzheimer's disease (FAD) and the human *APOE4* gene, a known risk factor for Alzheimer's disease.

A

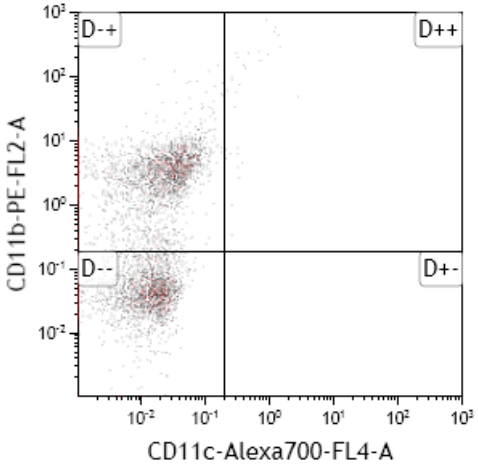


Gate	%Total	%Gated	X-Med	Y-Med
All	8.73	100.00	0.11	1.12
C--	3.62	41.45	0.07	0.04
C-+	3.83	43.83	0.15	2.68
C+-	0.00	0.02	0.35	0.15
C++	1.28	14.70	0.43	6.43



Gate	%Total	%Gated	X-Med	Y-Med
All	8.73	100.00	0.07	1.12
C--	3.62	41.45	0.07	0.04
C-+	4.77	54.63	0.08	3.24
C+-	0.00	0.02	0.35	0.15
C++	0.34	3.90	0.43	7.83

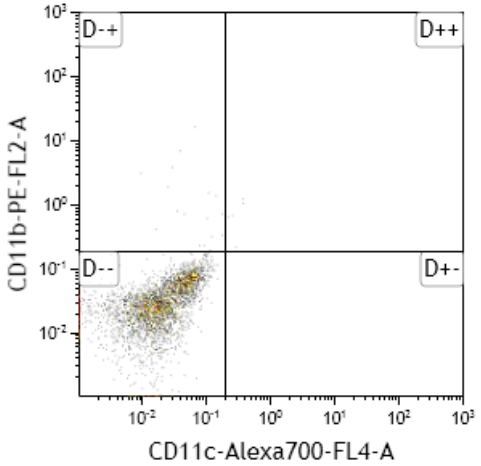
B



Gate	%Total	%Gated	X-Med	Y-Med
All	8.73	100.00	0.01	1.12
D--	3.62	41.46	0.01	0.04
D-+	5.06	57.96	0.01	3.36
D+-	N/A	N/A	N/A	N/A
D++	0.05	0.57	0.33	76.27

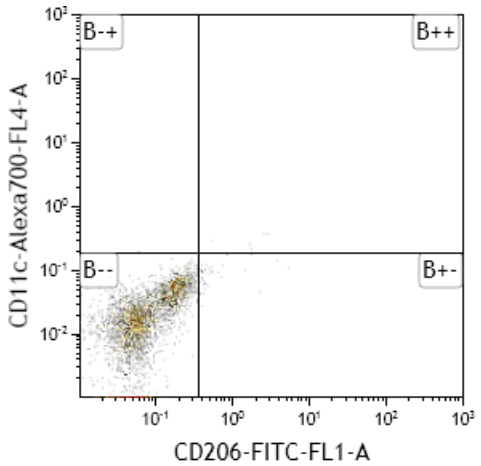
Figure A4-1.5. Plots demonstrating a compensation of 2.70 % was needed to correct for spill over of CD11b-PE into CD206-FITC-FL1 (graph A), whereas there was no spill over of CD11b-PE into CD11c-Alexa700-FL4 (graph B). The presence of autofluorescent cells indicated by the tail in the upper right corner (graph A) made it difficult to set compensation, although Y-Median was fairly similar after compensation (0.08 vs. 0.07 (C-+ vs. C--, graph A). Data was obtained from CD11b-PE single stained stromal vascular fraction (SVF) sample from epididymal adipose tissue from male *EFAD* mouse. The *EFAD* mouse strain expresses the human gene associated with the familial form of Alzheimer's disease (FAD) and the human *APOE4* gene, a known risk factor for Alzheimer's disease.

A

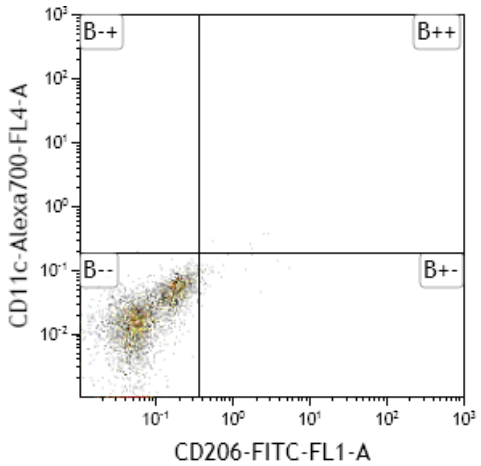


Gate	%Total	%Gated	X-Med	Y-Med
All	7.89	100.00	0.02	0.03
D--	7.84	99.44	0.02	0.03
D-+	0.03	0.44	0.07	0.74
D+-	N/A	N/A	N/A	N/A
D++	0.01	0.12	0.28	0.68

B



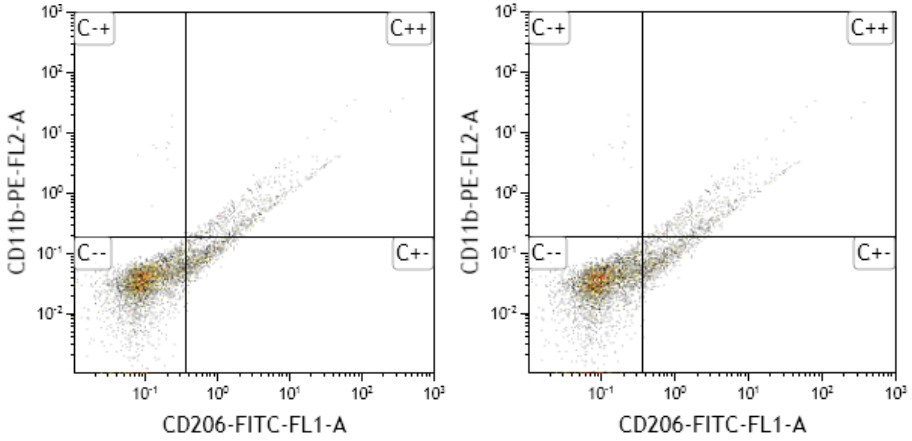
Gate	%Total	%Gated	X-Med	Y-Med
All	7.89	100.00	0.07	0.02
B--	7.80	98.95	0.07	0.02
B-+	0.00	0.02	0.06	0.19
B+-	0.07	0.91	0.44	0.09
B++	0.01	0.12	1.79	0.28



Gate	%Total	%Gated	X-Med	Y-Med
All	7.89	100.00	0.07	0.02
B--	7.80	98.95	0.06	0.02
B-+	0.00	0.02	0.06	0.19
B+-	0.07	0.91	0.44	0.09
B++	0.01	0.12	1.79	0.28

Figure A4-1.6. Plots demonstrating no compensation could be set for spill over of CD11c-Alexa700 into CD11b-PE-FL2 (graph A) and a compensation of 1.30 % was needed to correct for spill over of CD11c-Alexa700 into CD206-FITC-FL1 (graph B). The number of CD11c positive cells was so low that compensation was fairly impossible. Data was obtained from CD11c-Alexa700 single stained stromal vascular fraction (SVF) sample from epididymal adipose tissue from male *EFAD* mouse. The *EFAD* mouse strain expresses the human gene associated with the familial form of Alzheimer's disease (FAD) and the human *APOE4* gene, a known risk factor for Alzheimer's disease.

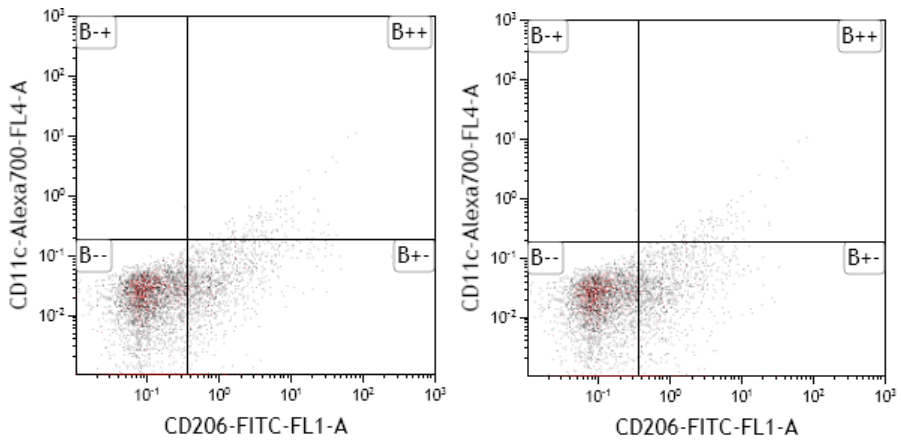
A



Gate	%Total	%Gated	X-Med	Y-Med
All	12.13	100.00	0.13	0.04
C--	9.05	74.64	0.10	0.04
C-+	0.03	0.24	0.21	5.59
C+-	1.73	14.24	0.60	0.09
C++	1.32	10.88	2.31	0.41

Gate	%Total	%Gated	X-Med	Y-Med
All	12.13	100.00	0.13	0.04
C--	9.05	74.64	0.10	0.03
C-+	0.03	0.24	0.21	5.59
C+-	1.85	15.25	0.61	0.08
C++	1.20	9.88	2.59	0.40

B



Gate	%Total	%Gated	X-Med	Y-Med
All	12.13	100.00	0.13	0.02
B--	9.08	74.86	0.10	0.02
B-+	0.00	0.02	0.33	0.66
B+-	2.77	22.80	0.79	0.03
B++	0.28	2.32	3.99	0.30

Gate	%Total	%Gated	X-Med	Y-Med
All	12.13	100.00	0.13	0.02
B--	9.08	74.86	0.10	0.02
B-+	0.00	0.02	0.33	0.66
B+-	2.83	23.32	0.81	0.02
B++	0.22	1.81	3.90	0.34

Figure A4-1.7. Plots demonstrating a compensation of 1.90 % and 0.80 % was needed to correct for spill over of CD206-FITC into CD11b-PE-FL2 (graph A) and CD11c-Alexa700-FL4 (graph B). The presence of autofluorescent cells indicated by the tail in the upper right corner, in combination with very few CD206 positive cells in the sample made it was difficult to set compensation, with the Y-Median still remaining slightly different after compensation, 0.08 vs. 0.03 (C+- vs. C--, graph A). Data was obtained from CD206-FITC single stained stromal vascular fraction (SVF) sample from epididymal adipose tissue from male *APOE4* mouse.

Non-specific binding

The non-specific binding determined using isotype controls was for CD11b 17.62 %, for CD11c 5.8 % and for CD206 8.1 % cells of gate E. Representative graphs presenting the percentage of events that are classified as positive cells expressing the cell surface markers CD11b, CD11c, and CD206 of stromal vascular fraction (SVF) sample stained with the appropriate isotype control, are presented below.

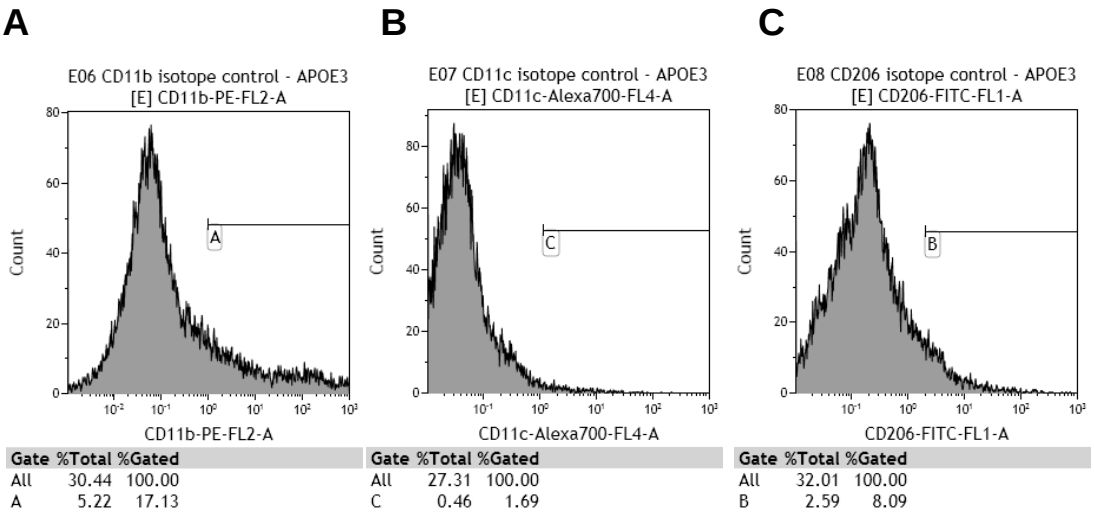


Figure A4-1.8. Plots presenting the non-specific binding for the antibodies used to stain for the surface markers CD11b (graph A), CD11c (graph B), and CD206 (graph C). The non-specific binding was for CD11b 17.62 % (events in gate A expressed as percentage of % cells gate E), for CD11c 5.8 % (events in gate C expressed as percentage of % cells gate E) and for CD206 8.1 % (events in gate B expressed as percentage of % cells gate E). The data was obtained using a representative stromal vascular fraction (SVF) cell sample from epididymal adipose tissue from a male *APOE3* mouse incubated with isotype control Rat igG2b, kappa, isotype-PE (0.2 mg/ml) for CD11b, Armenian Hamster IgG isotype-Alexa700 (0.2 mg/ml) for CD11c and Rat igG2aFITC (0.1 mg/ml) for CD206.

Cell viability

The cell viability of the stromal vascular fraction (SVF) cell samples was 83 ± 8.3 % (SD).

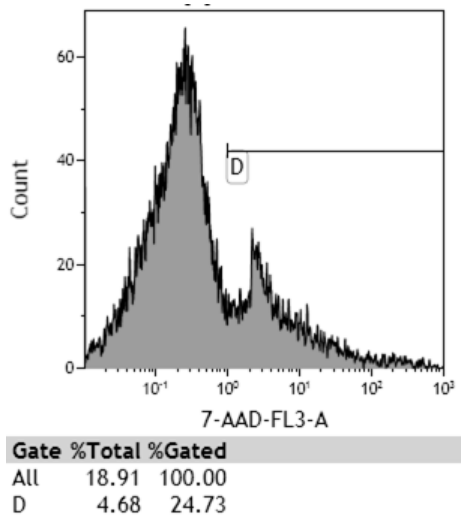


Figure A4-1.9. Plot showing the cell viability of a representative stromal vascular fraction (SVF) sample of the epididymal adipose tissue from a male *APOE3* mouse stained with cell viability stain 7-AAD presenting events within gate E. The viability of the cells within gate E was 75 % ("100 - gate D, %Gated"), whereas the cell viability of all 30,000 event was 95 % ("100 - gate D, %Total").

Appendix 4-2. Flow cytometry results

The forward (FSC-A) versus sideward scatter area (SSC-A) plot of a representative stromal vascular fraction (SVF) sample from each *APOE Epsilon* genotype*diet group is presented in Figure A4-2.1. The percentage of adipose tissue macrophages (ATM) (CD11b+ cells in target cell population [gate E]) and their M1 (CD11b+CD11c+ cells) and M2 (CD11b+CD206+ cells) phenotype in the SVF for a representative sample from each *APOE Epsilon* genotype*diet group are presented in Figure A4-2.2. Finally, a graphical representation of the expression of CD11c versus CD206 in the CD11b+ cell population is presented in Figure A4-2.3.

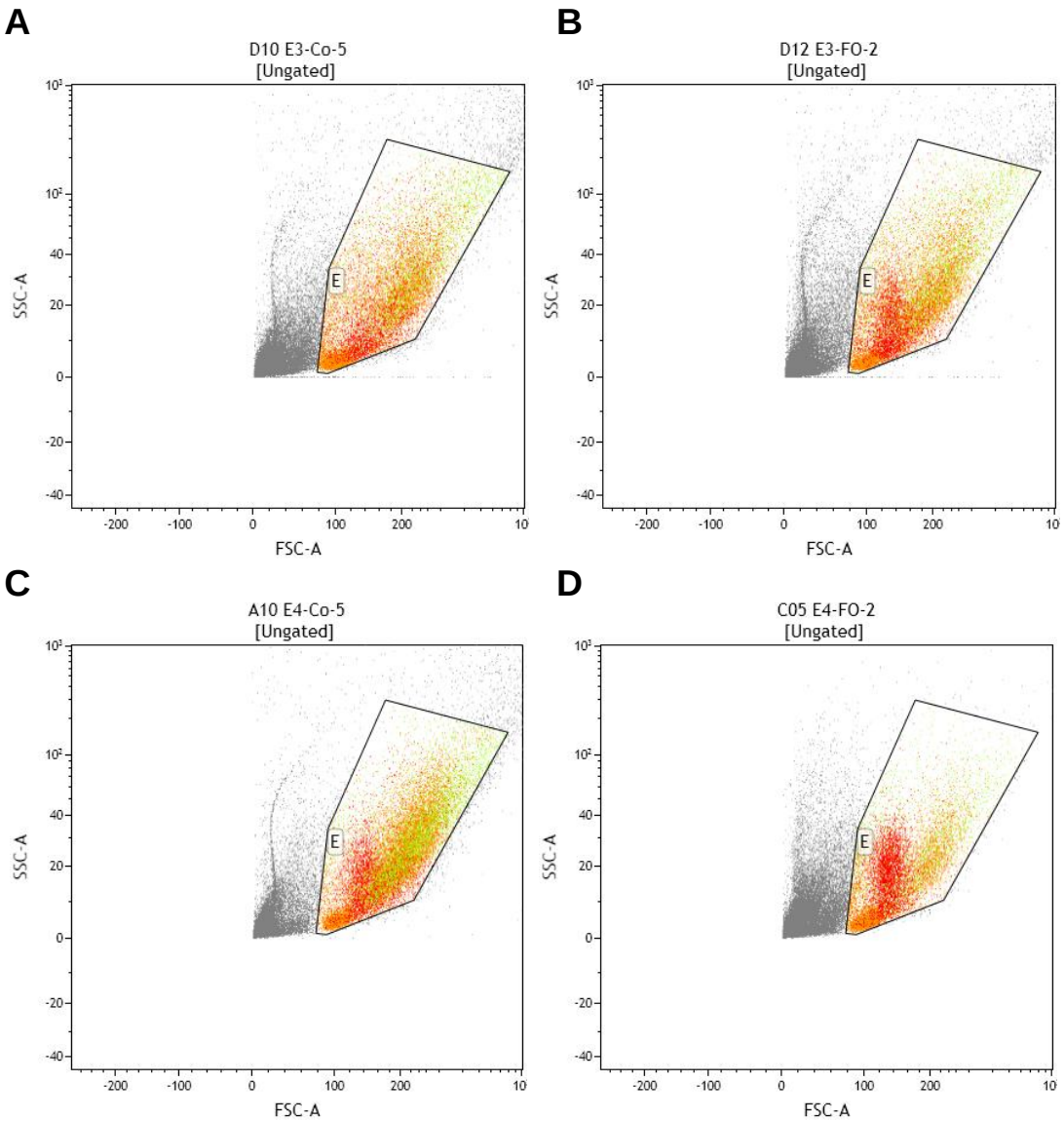
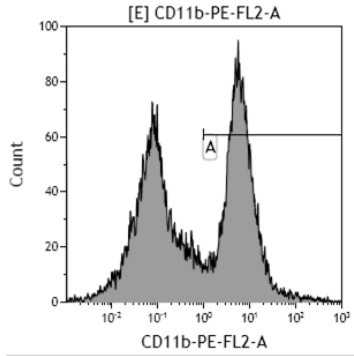
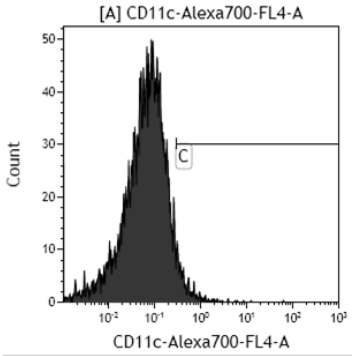


Figure A4-2.1. Forward (FSC-A) versus sideward scatter area (SSC-A) plot of a representative sample from the *APOE Epsilon* genotype*diet groups. The plot shows the different cell populations (CD11b+ (red), CD11b+CD11c+ (orange), CD11b+CD206+ (green)) within the investigated cell population (gate E) in a representative stromal vascular fraction (SVF) sample of the epididymal adipose tissue from a male mouse from the APOE3-HFD (A), APOE3-HFD+FO (B), APOE4-HFD (C) and APOE4-HFD+FO (D) group.

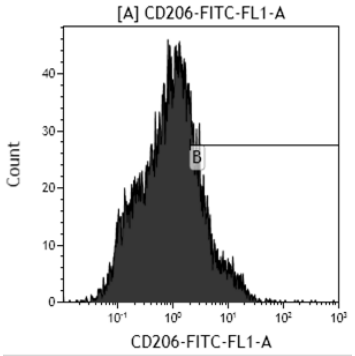
A



Gate	%Total	%Gated
All	40.60	100.00
A	19.51	48.04

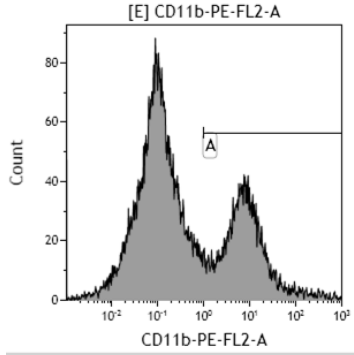


Gate	%Total	%Gated
All	19.51	100.00
C	0.71	3.65

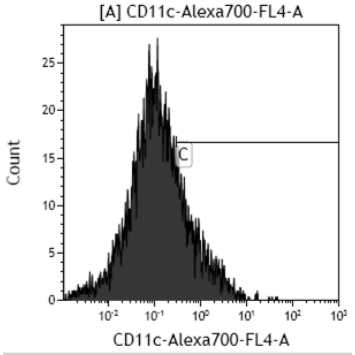


Gate	%Total	%Gated
All	19.51	100.00
B	4.81	24.67

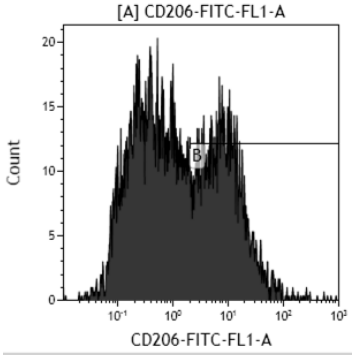
B



Gate	%Total	%Gated
All	35.89	100.00
A	11.68	32.54

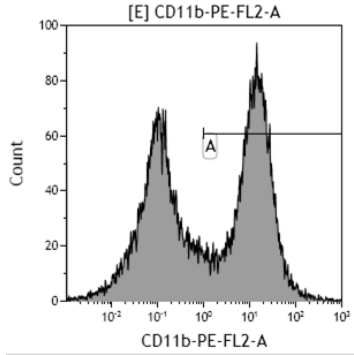


Gate	%Total	%Gated
All	11.68	100.00
C	2.25	19.29

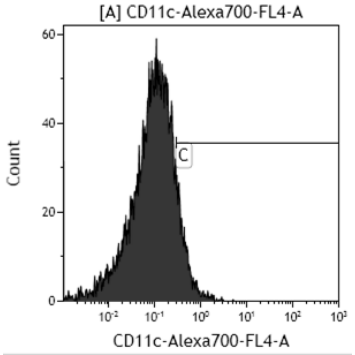


Gate	%Total	%Gated
All	11.68	100.00
B	4.85	41.50

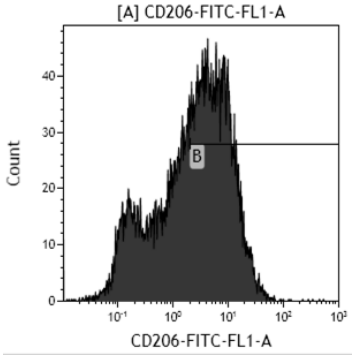
C



Gate	%Total	%Gated
All	54.39	100.00
A	27.87	51.24

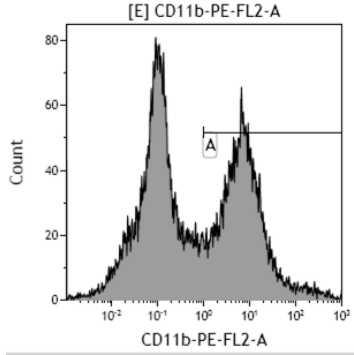


Gate	%Total	%Gated
All	27.87	100.00
C	2.40	8.63

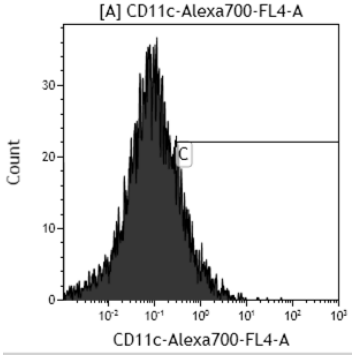


Gate	%Total	%Gated
All	27.87	100.00
B	16.37	58.73

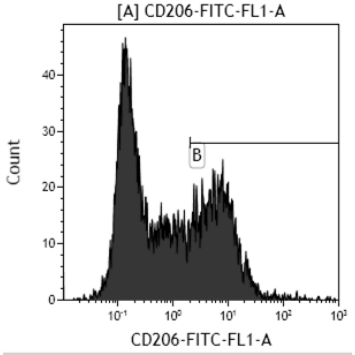
D



Gate	%Total	%Gated
All	40.24	100.00
A	17.75	44.11



Gate	%Total	%Gated
All	17.75	100.00
C	2.39	13.44



Gate	%Total	%Gated
All	17.75	100.00
B	6.56	36.95

Figure A4-2.2. Plots presenting the CD11b+ (gate A, left graph) and CD11b+CD11c+ (gate C, middle graph) and CD11b+CD206+ (gate B, right graph) cell populations in representative samples from the *APOE Epsilon* genotype*diet groups. Data is shown for a representative stromal vascular fraction (SVF) sample of the epididymal adipose tissue from a male mouse from the APOE3-HFD (A), APOE3-HFD+FO (B), APOE4-HFD (C) and APOE4-HFD+FO (D) group. The CD11b+ cells are presented as percentage of cells within investigated cell population (gate E), and the CD11b+CD11c+ and CD11b+CD206+ cells are expressed as percentage of CD11b+ cells (gate A). APOE, apolipoprotein E; HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA + DHA per kg of diet.

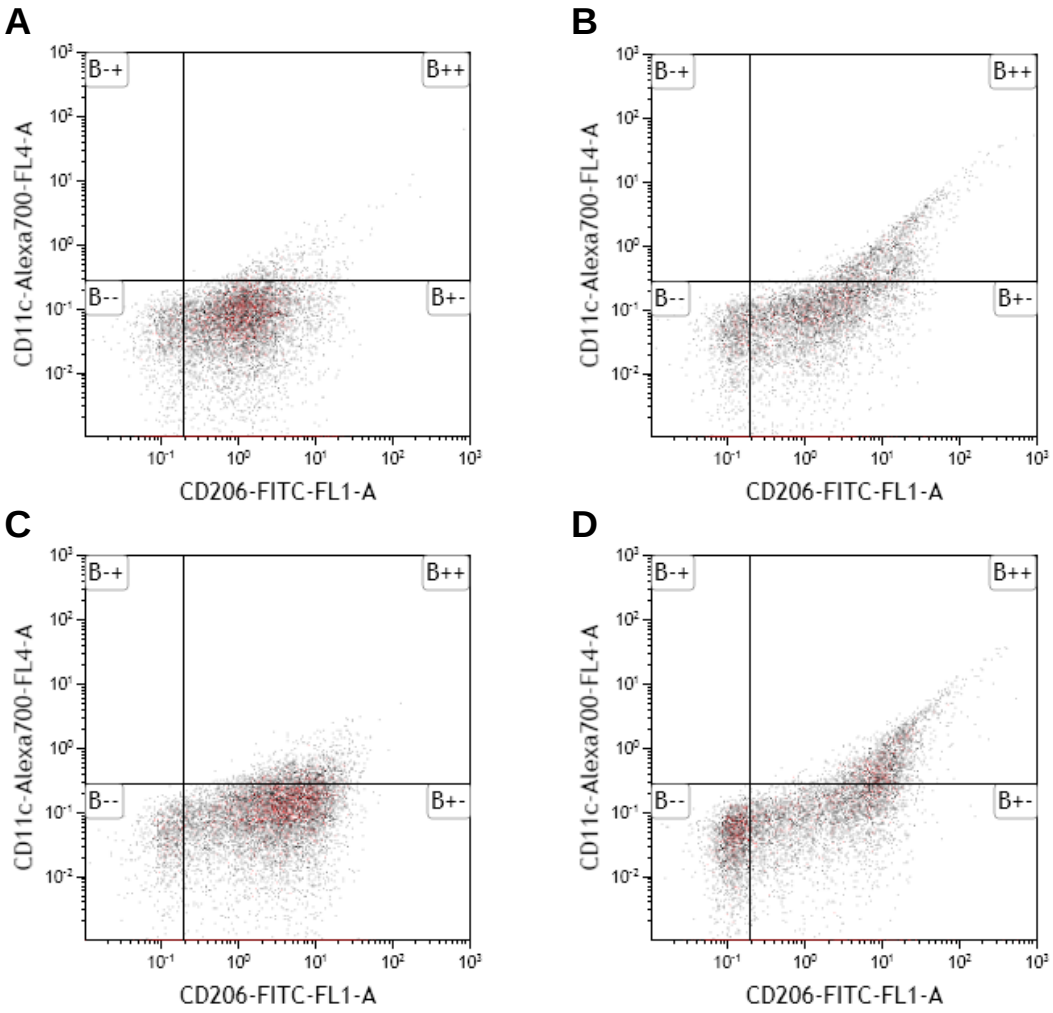


Figure A4-2.3. The expression of the M1 (CD11c) and M2 (CD206) phenotypic markers in adipose tissue macrophages (ATM) in representative samples for the different *APOE Epsilon* genotype*diet groups. Data is presented as cell surface expression of CD11b and CD206 in the ATM cell population (CD11b+ cells in gate E) of the epididymal stromal vascular fraction from *APOE3* [A, B] and *APOE4* [C, D] mice fed a high-fat diet (HFD) [A,C] or a HFD containing 30 g EPA + DHA per kg of diet (HFD+FO) [B,D]. The plots show a clear change in the appearance of the CD11b+ ATM cell population based on their expression of CD11b and CD206 for the mice on HFD+FO [B, D] compared those on the HFD [A,C]. The plots from the mice on the HFD+FO diet shows a more-spread out cell population with an upward 'tail' compared to a dense almost-single cell population ('dot') in the mice on HFD, that is associated with a greater number of CD11b+ cells that co-express CD11b and CD206. Furthermore, the plots from the mice on a HFD [A,D] reveals that the CD11b+ cell population from the *APOE4* mice [C] has a slightly greater expression of CD11b and CD206, as indicated by a very-slight shift of the cell population respectively upwards and to the right across the axes, compared to the *APOE3* mice [A] on a HFD. APOE, apolipoprotein E.

Table A4-2.1. Summary statistics for Figure A4-2.3. The mean expression of M1 (CD11c) and M2 (CD206) phenotypic markers in adipose tissue macrophages (ATM, CD11b+ cells) for the different *APOE Epsilon* genotype*diet groups.

	APOE3-HFD	APOE3-HFD+FO	APOE4-HFD	APOE4-HFD+FO
$CD11b^+CD11c^-CD206^-$ cells (B--) (% of $CD11b^+$ cells)	13.5 ± 1.0	16.3 ± 1.3	17.6 ± 2.3	27.0 ± 1.9
$CD11b^+CD11c^+CD206^-$ cells (B-+) (% of $CD11b^+$ cells)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
$CD11b^+CD11c^-CD206^+$ cells (B+-) (% of $CD11b^+$ cells)	79.3 ± 7.1	66.6 ± 1.2	72.3 ± 2.5	57.3 ± 1.9
$CD11b^+CD11c^+CD206^+$ cells (B++) (% of $CD11b^+$ cells)	7.1 ± 1.3	17.1± 0.9	10.0 ± 0.7	15.7 ± 0.6

Values are mean ± SEM of 9 – 10 animals per group. Data is presented as percentage of CD11b+ cells in gate E that confines the cell population of interest. APOE, apolipoprotein E genotype; CD11b, integrin alpha M; CD11c, Integrin alpha X; CD206, mannose receptor C type 1; HFD, high-fat diet; HFD+FO, HFD containing 30 g EPA+DHA / kg of diet.

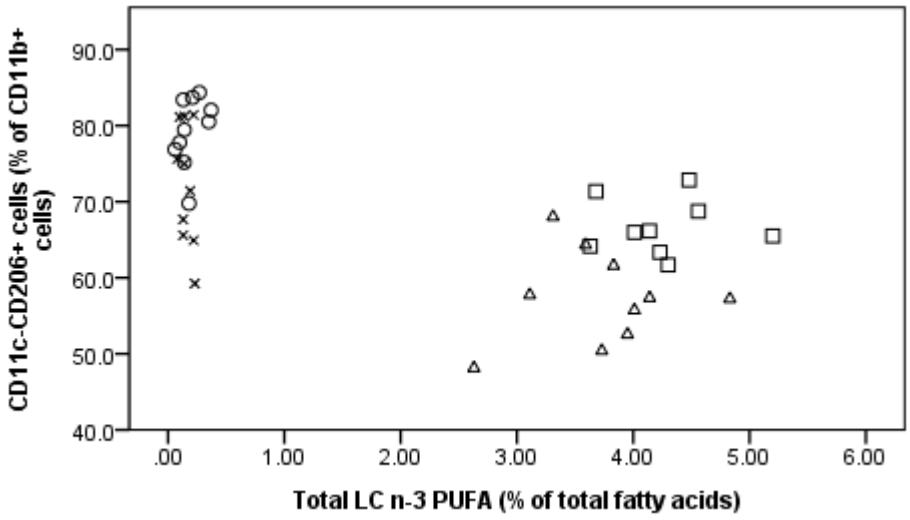
Appendix 4-3. Pearson's correlation analysis

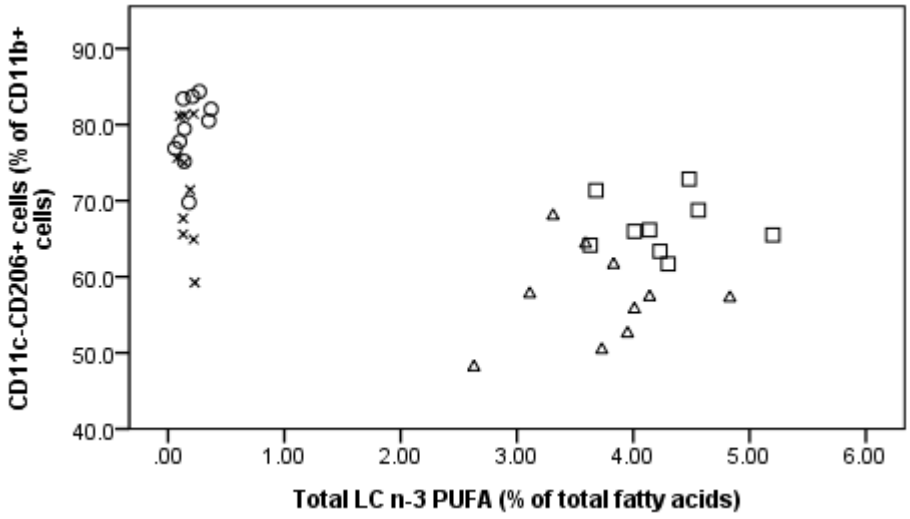
To explore if the LC *n-3* PUFA, EPA and DHA enrichment of epididymal adipose tissue (AT) determined the response to fish oil supplementation in *APOE3* and *APOE4* mice, we investigated the correlation between LC *n-3* PUFA, EPA and DHA levels and several outcome measures related to AT inflammation (Table A4-3.1; Figure A4-3.1 to A4-3.4) and insulin resistance (Table A4-3.2; Figure A4-3.5 to A4-3.8) using the Pearson's correlation coefficient.

Table A4-3.2. Correlation LC *n-3* PUFA status and measures related to insulin resistance.

Dependent variable	Independent variable	Pearson r	p-value
ATM flow	LC n-3 PUFA	-0.002	0.99
	EPA	-0.040	0.81
	DHA	0.006	0.97
M1 flow	LC n-3 PUFA	-0.221	0.17
	EPA	-0.227	0.16
	DHA	-0.220	0.18
M2 flow	LC n-3 PUFA	-0.661	p<0.001
	EPA	-0.644	p<0.001
	DHA	-0.668	p<0.001
Double positive flow	LC n-3 PUFA	0.809	p<0.001
	EPA	0.805	p<0.001
	DHA	0.810	p<0.001
CD11c:CD206 flow	LC n-3 PUFA	0.680	p<0.001
	EPA	0.685	p<0.001
	DHA	0.677	p<0.001
F4/80 mRNA	LC n-3 PUFA	0.298	0.07
	EPA	0.339	p<0.05
	DHA	0.282	0.08
CD11b mRNA	LC n-3 PUFA	0.344	p<0.05
	EPA	0.348	p<0.05
	DHA	0.337	p<0.05
CD11c mRNA	LC n-3 PUFA	0.147	0.38
	EPA	0.107	0.53
	DHA	0.162	0.33
CD206 mRNA	LC n-3 PUFA	0.045	0.79
	EPA	0.094	0.59
	DHA	0.028	0.87
IL-10 mRNA	LC n-3 PUFA	0.287	0.08
	EPA	0.094	0.59
	DHA	0.028	0.87

Pearson's correlation efficient (r) was used to determine the correlation between the adipose tissue LC *n-3* PUFA, EPA and DHA concentrations and several outcome measures related to AT inflammation. ATM, adipose tissue macrophage; CD11b, integrin alpha X (Itgax); CD11b, integrin alpha M (Itgam); CD206, mannose receptor C type 1 (Mrc1); DHA, docosahexaenoic acid; Double positive, macrophages expressing both M1 marker CD11c and M2 marker CD206; EPA, eicosapentaenoic acid; F40/80, EGF-like module containing mucin-like hormone receptor-like sequence 1; IL-10, interleukin 10; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid; M1, proinflammatory macrophage phenotype; M2, anti-inflammatory macrophage phenotype.





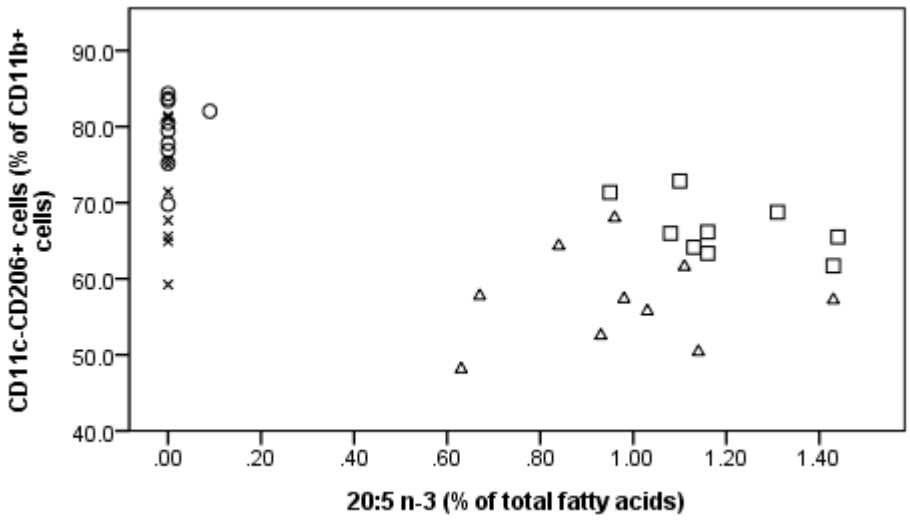


Figure A4-3.1. Correlation between percentage of M2 adipose tissue macrophages (CD11b+CD11c-CD206+ cells) in stromal vascular fraction and adipose tissue LC *n-3* PUFA (r = -0.661), EPA (r = -0.644) and DHA (r = -0.668) concentrations. Crosses, APOE3-HFD; squares, APOE3-HFD+FO; circles, APOE4-HFD; triangles, APOE4-HFD+FO.

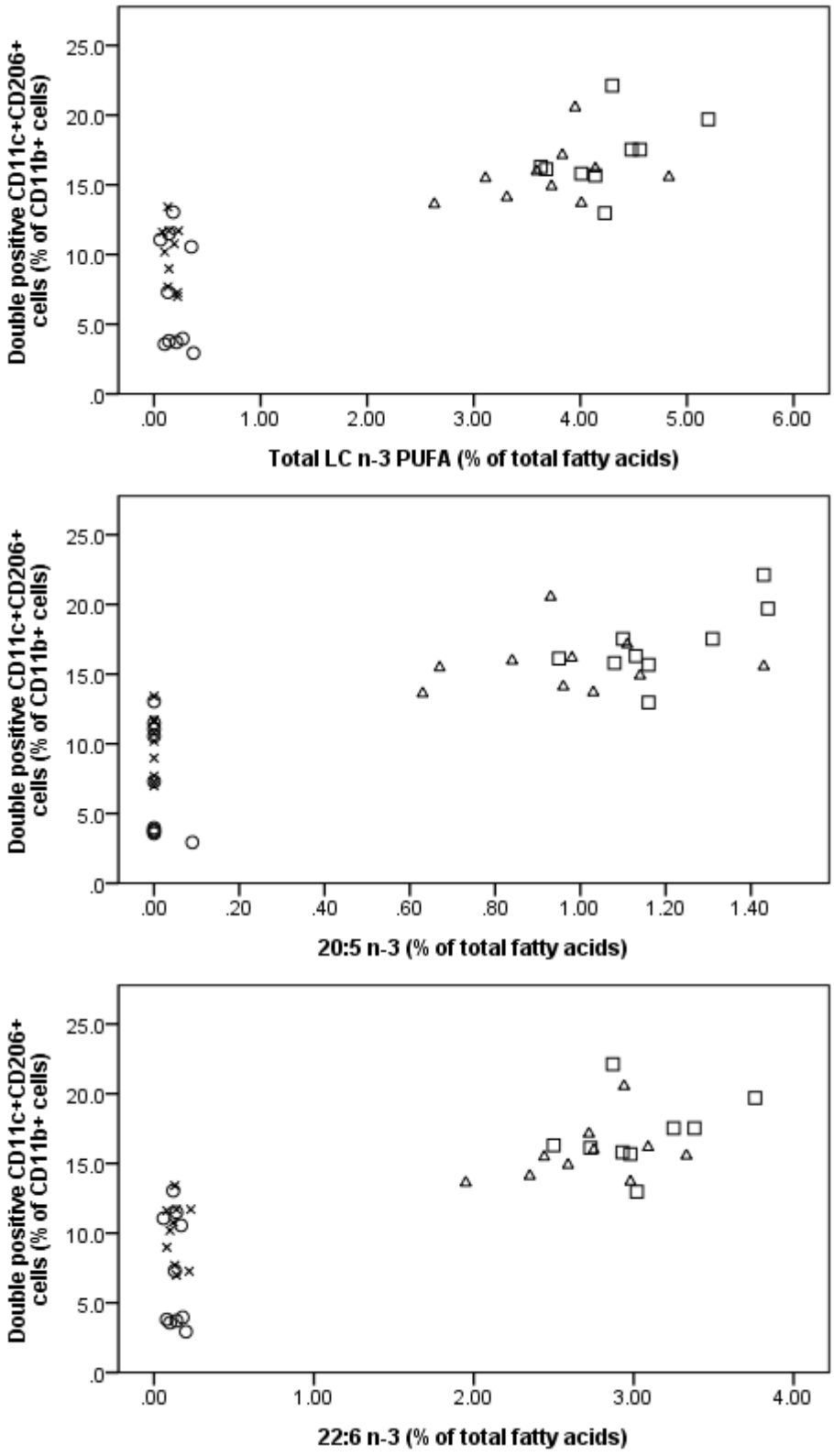


Figure A4-3.2. Correlation between percentage of double positive adipose tissue macrophages (CD11b+CD11c+CD206+ cells) in stromal vascular fraction and adipose tissue LC *n-3* PUFA (r = 0.809), EPA (r = 0.805) and DHA (r = 0.677) concentrations. Crosses, APOE3-HFD; squares, APOE3-HFD+FO; circles, APOE4-HFD; triangles, APOE4-HFD+FO.

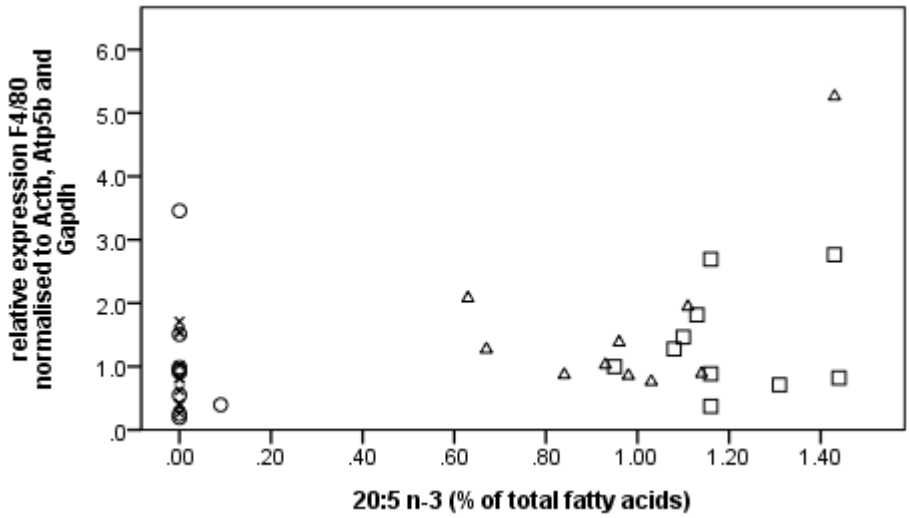


Figure A4-3.3. Correlation between adipose tissue F4/80 mRNA expression levels and adipose tissue EPA (r = 0.339) concentrations. Crosses, APOE3-HFD; squares, APOE3-HFD+FO; circles, APOE4-HFD; triangles, APOE4-HFD+FO.

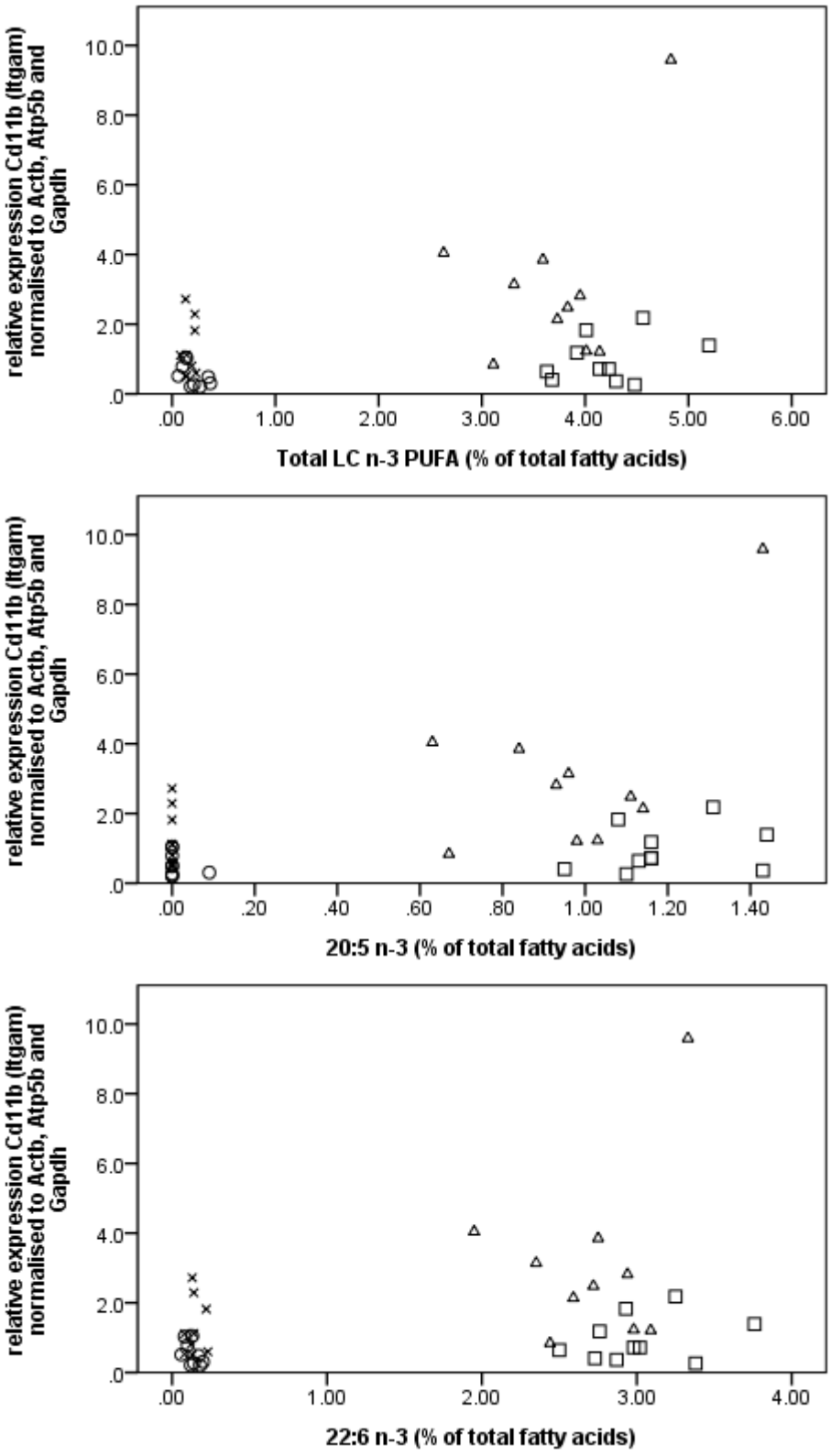


Figure A4-3.4. Correlation between adipose tissue Cd11b mRNA expression levels and adipose tissue LC *n-3* PUFA (r = 0.344), EPA (r = 0.348) and DHA (r = 0.337) concentrations. Crosses, APOE3-HFD; squares, APOE3-HFD+FO; circles, APOE4-HFD; triangles, APOE4-HFD+FO.

Table A4-3.2. Correlation LC *n-3* PUFA status and measures related to insulin resistance.

Dependent variable	Independent variable	Pearson r	p-value
Glut4 mRNA	LC n-3 PUFA	0.401	p<0.05
	EPA	0.459	p<0.01
	DHA	0.376	p<0.05
HOMA-IR	LC n-3 PUFA	-0.540	p<0.001
	EPA	-0.534	p<0.001
	DHA	-0.541	p<0.001
plasma glucose	LC n-3 PUFA	-0.416	p<0.01
	EPA	-0.420	p<0.01
	DHA	-0.410	p<0.01
plasma insulin	LC n-3 PUFA	-0.520	p<0.01
	EPA	-0.514	p<0.01
	DHA	-0.522	p<0.01

Pearson's correlation efficient (r) was used to determine the correlation between the adipose tissue LC *n-3* PUFA, EPA and DHA concentrations and several outcome measures related to AT inflammation and insulin reistance (IR). DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Glut4, solute carrier family 2 (facilitated glucose transporter) member 4 (Slc2a4); HOMA-IR, homeostatic model assessment - insulin resistance; LC *n-3* PUFA, long chain *n-3* polyunsaturated fatty acid.

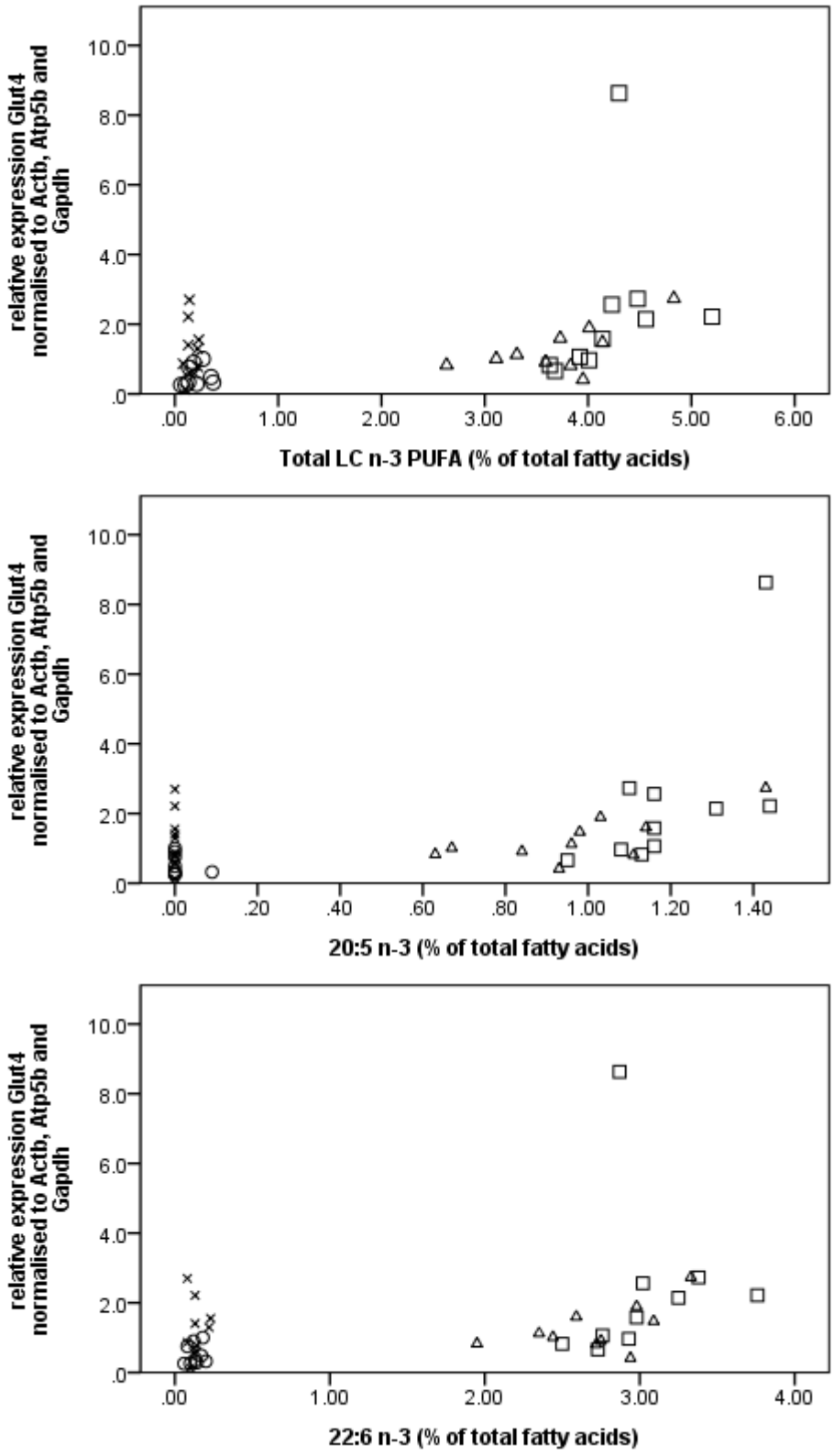


Figure A4-3.5. Correlation between adipose tissue Glut 4 mRNA expression levels and adipose tissue LC *n-3* PUFA (r = 0.401), EPA (r = 0.459) and DHA (r = 0.376) concentrations. Crosses, APOE3-HFD; squares, APOE3-HFD+FO; circles, APOE4-HFD; triangles, APOE4-HFD+FO.

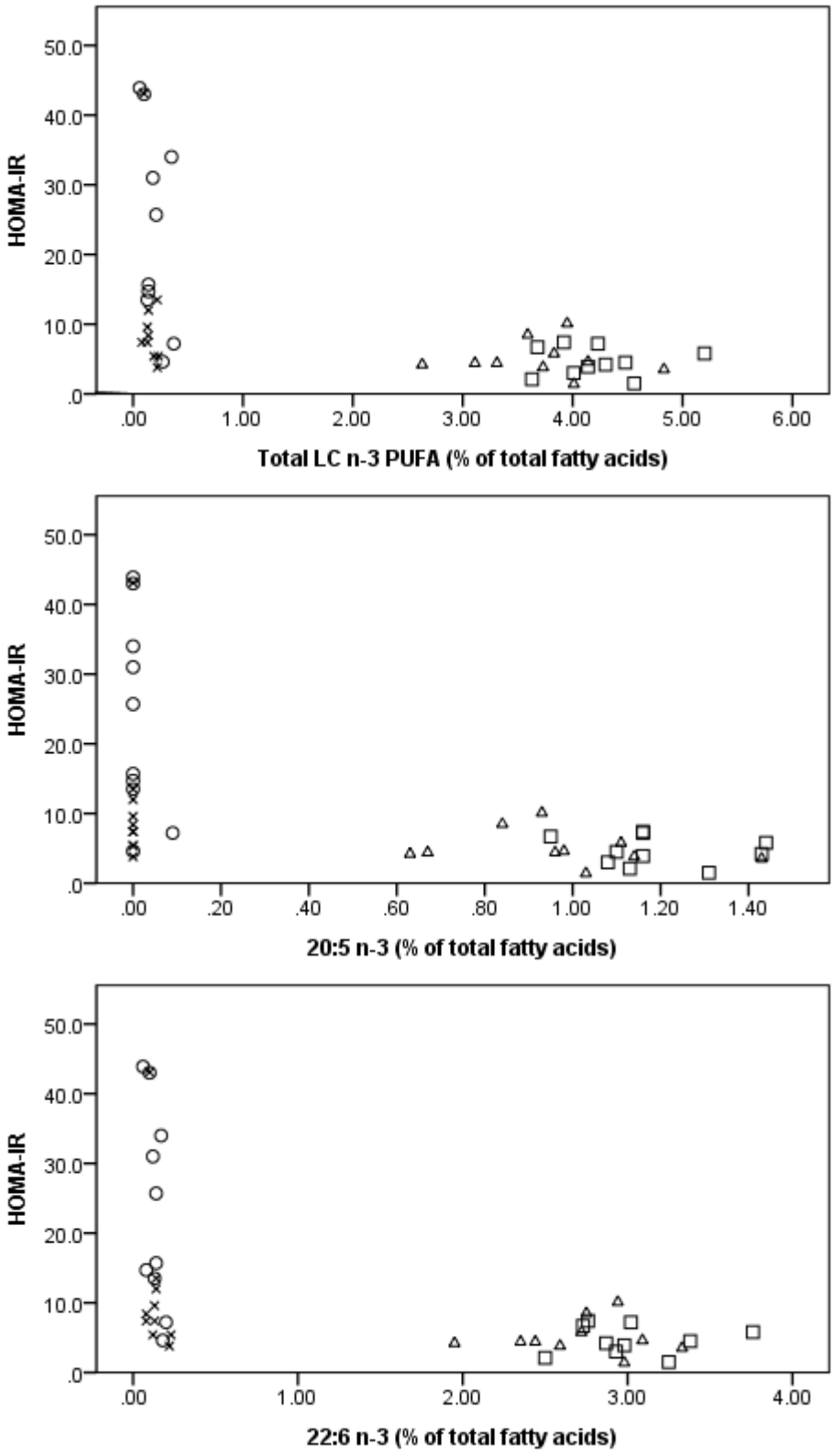


Figure A4-3.6. Correlation between HOMA-IR and adipose tissue LC *n-3* PUFA (r = -0.540), EPA (r = -0.534) and DHA (r = -0.541) concentrations. Crosses, APOE3-HFD; squares, APOE3-HFD+FO; circles, APOE4-HFD; triangles, APOE4-HFD+FO.

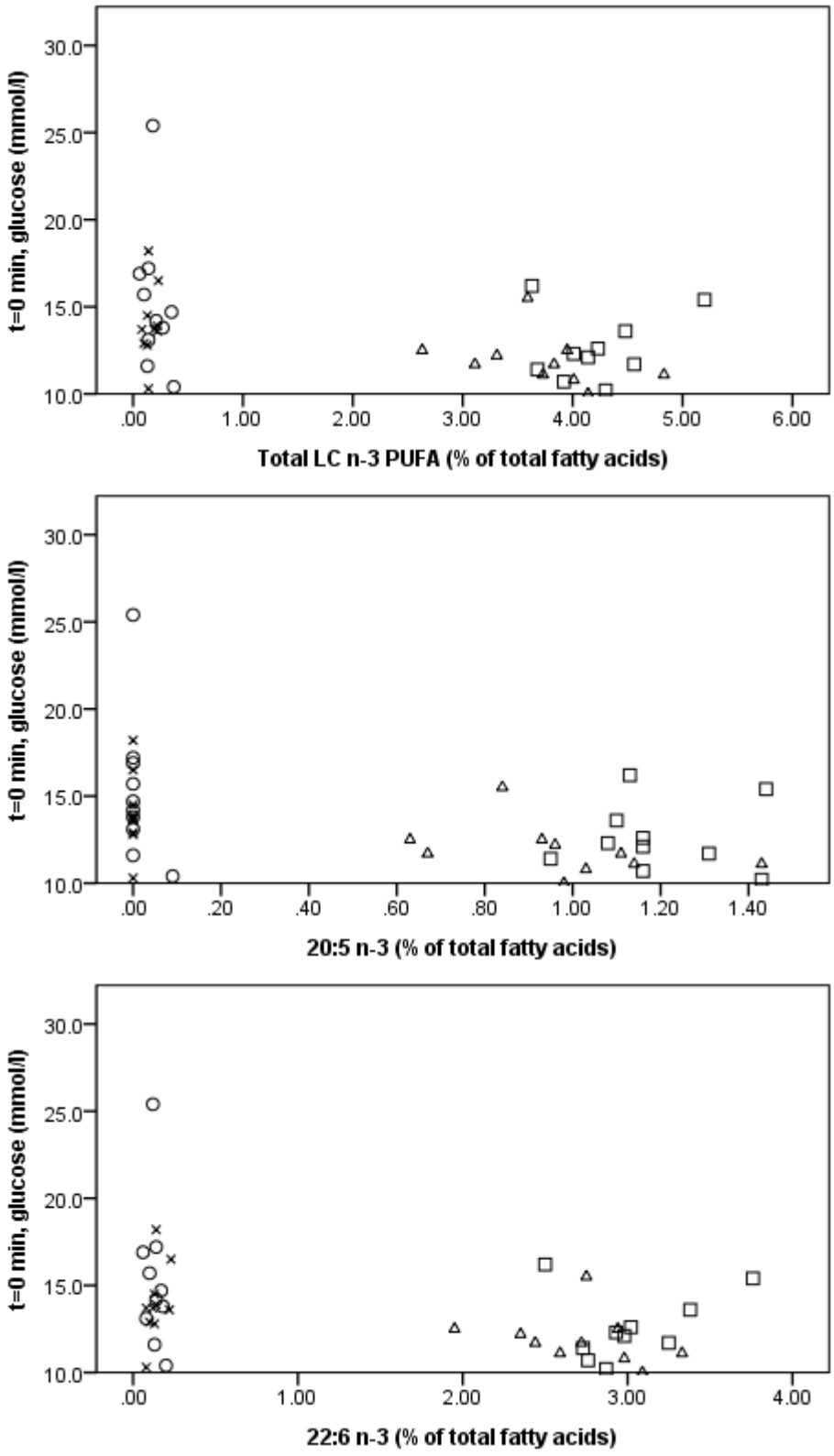


Figure A4-3.7. Correlation between fasting plasma glucose and adipose tissue LC *n-3* PUFA (r = -0.416), EPA (r = -0.420) and DHA (r = -0.410) concentrations. Crosses, APOE3-HFD; squares, APOE3-HFD+FO; circles, APOE4-HFD; triangles, APOE4-HFD+FO.

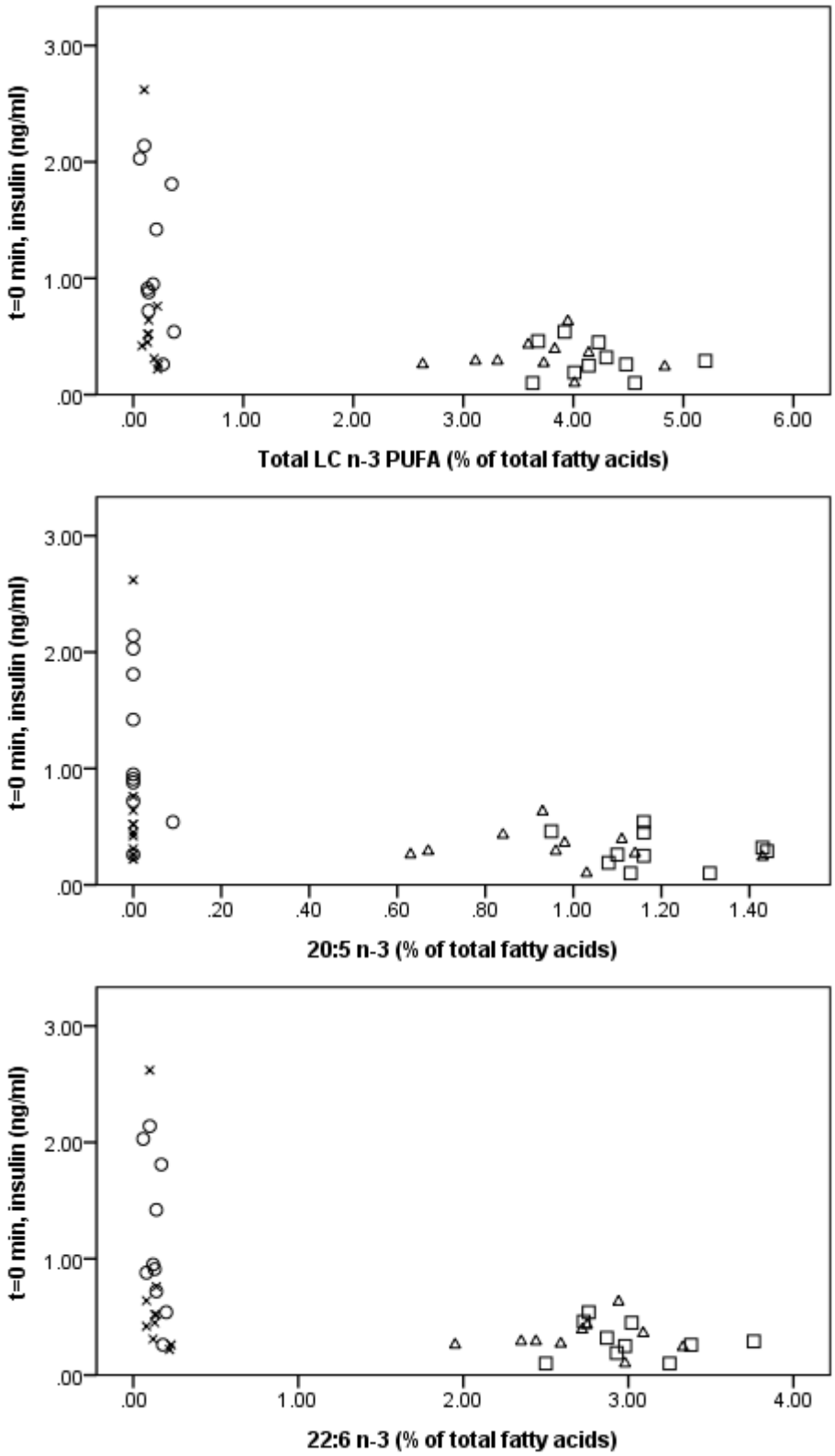


Figure A4-3.8. Correlation between fasting plasma insulin and adipose tissue LC *n-3* PUFA (r = -0.520), EPA (r = -0.514) and DHA (r = -0.522) concentrations. Crosses, APOE3-HFD; squares, APOE3-HFD+FO; circles, APOE4-HFD; triangles, APOE4-HFD+FO.