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Modulation of gene expression of GPR fatty acids sensors/receptors by dietary fatty acids influences inflammatory response in adipocytes

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Abstract

The fate of dietary fatty acids is finely regulated by specific cellular receptors capable of sensing the quantity and the quality of fatty acids reaching different organs via lipoproteins or as free fatty acids (FFAs). These receptors named GPR fatty acids receptors/sensors seem to play a fundamental role in governing the fate of dietary fatty acids in adipocytes.

Based on these features, in this work we evaluated the effect of free fatty acids on inflammatory markers and the involvement of GPR120 and GPR84 in modulating the inflammatory response in obesity as assessed in human adipocytes.

Cells were differentiated and incubated at 12 days post-induction of differentiation with inflammatory cytokines, hormones or fatty acids for 4 and 24 h. Both TNFα (tumor necrosis factor-α) and IL-1β induced a reduction in GPR120 expression at 4h and 24 h. In marked contrast, GPR84 mRNA level was dramatically increased by treatment with the pro-inflammatory cytokines. The PPARγ agonist rosiglitazone had no effect on GPR84 expression, but there was a stimulation of GPR120 expression which was most marked at 24 h. Dexamethasone and insulin had little or no effect on GPR120 and GPR84 expression. Docosahexaenoic acid (DHA) was able to increase GPR120 gene expression only after 24h of incubation while arachidonic acid (ARA) strongly decreased GPR120 gene expression at the same time point; both had no effect on GPR84 expression.

The GPR FFA receptors are sensitive to circulating levels of FFAs. In order to evaluate whether concentration of free DHA present in human plasma changes based on its dietary intake, we carried out a pilot study. Normalweight, overweight and obese subjects were treated with an intake of 2 g/d of EPA/DHA supplements in two different formulations: fish oil (FO) and Krill oil (KO). Control group was treated with olive oil (OO). Plasma free fatty acids, particularly those relevant for the effects on GPRs, namely EPA (eicosapentenoic acid), DHA, POA (palmitoleic acid) and ARA and also the plasmatic level of TNFα were taken into consideration. In overweight and obese subjects FO was more efficient in increasing plasma free DHA and POA, while in the case of KO it was EPA even though not significantly. In addition, we found that free ARA decreased with FO treatment leading to an increase of DHA/ARA ratio. This increase mirrored the decrease of TNFα. Interestingly the increase of DHA and POA was associated to a decrease of circulating TNFα. The changes were only evident in the obese subjects probably because they had higher levels of circulating FFAs, and higher concentration of TNFα.

Therefore, our data suggest that KO, as opposite to FO, being mostly incorporated into PLs, decreases circulating FFAs and thereby DHA and POA, with a possible less marked effect on GPR120 and consequent less efficient ability to reduce inflammation through this pathway. These data point out the importance not only on the type of dietary fatty acids but also on the formulation which may strongly influence their activities in lipid and energy metabolism in the obese by affecting the inflammatory response by targeting GPR120.

Future studies should be carried out in larger cohorts and possible modulation of resolvins and protectins biosynthesis should be taken into consideration. In addition, we will aim to evaluate whether in experimental animals and humans the dietary fats by modifying fatty acid profile in different lipid fractions may modulate omega-3 effects, affecting metabolic dysfunction and/or inflammation.

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Abbreviations

AdipoR1-2	Adiponectin receptor 1-2
Akt	Protein kinase B or PKB
ALA	A-linolenic acid
ARA	Arachidonic Acid
AT	Adipose tissue
ATM	Adipose tissue macrophages
BAT	Brown adipose tissue
cAMP	Adenosine monophosphate cyclic
CCK	Cholecystokinin
CCL11	Chemokine (C-C motif) ligand 11
CCL19	Chemokine (C-C motif) ligand 19
CCL2	Chemokine (C-C motif) ligand 2
CCR2	C-C chemokine receptor type 2
Con	Control
COX	Cyclooxygenase
CXCL10	Chemokine (C-X-C motif) ligand 10
CXCL11	Chemokine (C-X-C motif) ligand 11
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ERK1/2	Extracellular signal-regulated kinases
FFARs	Fatty Free acid receptors
FFAs	Free fatty acids
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
GLUT4	Glucose transporter 4
GPR	G-protein coupled receptor
GSIS	Glucose-stimulated insulin secretion
HFD	High fat diet
HD	High dose
ICAM	Intercellular Adhesion Molecule
IL10	Interleukin-10
IL12	Interleukin-12
IL15	Interleukin-15
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IR	Insulin resistance
IRS-1	Insulin receptor substrate-1
JAK	Janus kinase
JNK	Jun N-terminal kinase

KO	Knockout
LA	Linoleic acid
LCFA	Long-chain fatty acids
LD	Low dose
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCFAs	Medium-chain fatty acids
MCM	Macrophage-conditioned medium
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor kappa B
OA	Osteoarthritis
PA	Palmitic Acid
PAHSA	Palmitic-acid-hydroxystearic-acid
PGE2	Prostaglandin E2
PLC	Phospholipase C
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PUFAs	Polisantured fatty acids
RXR	Retinoid x receptor
SAT	Subcutaneous adipose tissue
SCFAs	Short-chain fatty acids
SOCS3	Suppressor of cytokine signalling 3
STAT	Signal transducer and activator of transcription
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TAB1	Transforming growth factor-β activated kinase 1 (TAK1)-binding protein 1
TAG	Triacylglycerols
TAK1	Transforming growth factor beta-activated kinase 1
Th	T helper
TLR	Toll like receptor
TNFα	Tumor necrosis factor α
TZD	Thiazolidinediones
VAT	Visceral adipose tissue
VCAM	Vascular cell adhesion protein
WAT	White adipose tissue
WT	Wild type

1. Introduction

Over the past decades, obesity became one of the most critical health problems worldwide [*International Diabetes Federation, 2013; Perez-Martinez P. et al. 2011*]. Obesity develops as a result of an imbalance between energy expenditure and intake *[Greenwood H.C. et al., 2011]*. Indeed, excessive food intake together with insufficient exercise and genetic susceptibility lead to metabolic diseases, such as type 2 diabetes (T2D) and obesity *[Miyamoto J. et al., 2016]*. It has become increasingly evident that all macronutrients, including carbohydrates, proteins and lipids, play an important role in the regulation of energy metabolism.

Adipose tissue has been shown to be a major endocrine organ involved in the secretion of a number of factors, including lipid moieties such as free fatty acids and steroid hormones, and protein signals termed adipokines *[Ahima R.S. et al., 2000; Guerre-Millo M 2002; Trayhurn P. et. al., 2001]*. The production and release of these factors can be greatly altered in obesity and this may underline the development of associated diseases such as the metabolic syndrome and T2D [*Trayhurn P. et al., 2004*].

Free fatty acids (FFAs) are known to exert biological effects [*Itoh Y. et al., 2003; Briscoe C.P. Et al., 2003*] by acting directly on intracellular and cell surface receptors as precursors of various oxidised signal molecules [*Ran-Ressler R.R. et al., 2014*]. These fatty acid receptors are G protein coupled 7-transmembrane receptors activated by different groups of FFAs and have all been associated in various ways with T2D and other metabolic and inflammatory disorders. Their established biological activities propose fatty acids as potential candidates for functional ingredients responsible for dietary health effects [*Christiansen E. et. al., 2015*].

1.2 G-protein coupled fatty acid receptors (GPRs)

The GPRs, also named Free Fatty Acid Receptors (FFARs), are the largest family of cell-surface receptors which have also been identified for broader subclasses of FFAs which can act as precursors of potent messenger molecules and also act themselves directly on as receptors that function as nutrient sensors *[Ulven T. et al., 2015]* modulating their signal and gene expression *[Milligan G. et al., 2014]*.

Recently, based on tissue expression patterns of these receptors and the fact that they may elicit the production of a range of appetite and hunger-regulating peptides, GPR fatty acid receptors are attracting considerable attention due to their potential to modulate satiety, improve glucose homeostasis and suppress the production of various pro-inflammatory mediators. Indeed, they are currently being explored as therapeutic targets to regulate metabolic diseases. *[Milligan G. et al. 2014].* Specific fatty acids have been identified as ligands for each FFARs: short-chain fatty acids (SCFAs) can activate GPR41/FFAR3 and GPR43/FFAR2, while medium-chain fatty acids (MCFAs) and long-chain fatty acids (LCFAs) can activate GPR40/FFAR1 and GPR120/FFAR4 [*Brown A.J. et al., 2003; Briscoe C.P. et al., 2003; Itoh Y. et al., 2003; Kotarsky K. et al,. 2003; Le Poul E. et al., 2003; Nilsson N.E. et al., 2003; Hara T. et al., 2013*]. In 2006, GPR84 was described as an MCFA receptor and associated with pro-inflammatory action *[Wang J.H. Et al., 2006]*.

Despite the interest in these nutrient GPRs as sensors of nutritional status and targets for limiting the development of metabolic diseases, there are major challenges to their use as potential therapeutic tools. Mostly, this is due to limited characterisation and validation of

these receptors because of the scarcity of selective and high-potency/affinity pharmacological agents to define their detailed function and regulation. GPRs regulate the physiology and function of all cells and tissues as the molecular targets for a vast range of water-soluble hormones and neurotransmitters. Indeed, they have also been the most effectively targeted group of proteins for small-molecule therapeutic medicines designed to modulate or mask the pathophysiological manifestations of diseases. Because of the high affinity of many peptides and other hormones for GPRs, the first reports of their ability to be activated by relatively high concentrations of various nutrients and metabolic intermediates were proved to have limited physiological relevance *[Milligan G. et al., 2014]*.

The FFARs are widely regarded as nutrient sensors and are all implicated in the regulation of inflammatory and metabolic processes and are potential targets for both drug discovery and dietary intervention *[Dranse HJ. et al., 2013]*.

1.2.1 GPR40/FFA1

GPR40 is a receptor for MCFAs and LCFAs at micro molar concentrations and is reported to bind with Gq protein which is a heterotrimeric G protein subunit that activates phospholipase C (PLC), intracellular Ca2+ release and activates the extracellular signal-regulated kinases (ERK1/2) signalling cascade. GPR40 also increases cyclic (c)AMP levels via a Gs-coupled pathway *[Briscoe C.P. et al., 2003; Welters H.J. et al., 2006]*.

GPR40 has been identified in immune cells, taste buds, central nervous system and in the intestine *[Sykaras A.G. et al., 2012; Edfalk S. et al., 2008; Hirasawa A. et al., 2008; Cartoni C. et al.,*

2010; Liou A.P. et al., 2011; Ma D. et al., 2007]. Indeed, GPR40 is expressed in intestinal L and K cells, which produce incretin hormones, such as glucagon-like peptide (GLP)-1 and gastric inhibitory polypeptide (GIP), as well as cholecystokinin (CCK)-producing cells.

Stimulation of GPR40 by LCFAs enhances glucose-stimulated insulin secretion (GSIS) by direct secretion of insulin from pancreatic beta cells and indirectly via up-regulation of GLP-1, GIP and CCK in the intestine *[Hara T. et al., 2014; Tomita T. et al., 2014]*.

The function of GPR40 in taste buds need further studies to clarify the key role in the perception of lipid taste *[Cartoni C et al., 2010; Gilbertson TA et al., 2014]*. GPR40 is also expressed in the central nervous system, including in neurons of the cerebral cortex, hippocampus, amygdala, hypothalamus, cerebellum and spinal cord *[Ma D. et al., 2007]*.

GPR40 is mainly expressed in pancreatic insulin-producing beta cells where LCFAs promote GSIS and the acute effects of specific FFAs are mitigated by the loss of GPR40 function *[Itoh, Y. et al., 2003]*. The LCFA effects were attenuated on GSIS in the GPR40-deficient mice, while GPR40 overexpression in pancreatic beta cells prevented and enhanced insulin sensitivity in high fat diet (HFD)-induced obese mice *[Nagasumi K.et al., 2009]*.

Docosahexaenoic acid (DHA, 22:6) and eicosapentaenoic acid (EPA, 20:5) are the most potent agonists of GPR40. It is also identified that palmitic acid (PA, 16:0) binds to GPR40 with high affinity *[Miyamoto J. et al., 2016]*. It has been reported that the chronic and acute effect of palmitic acid on insulin sensitivity is exerted in part via GPR40. Indeed, the downregulation of GPR40 mRNA blocking palmitic acid-induced apoptosis in pancreatic beta cells by exendin-4 suppressed c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase (MAPK) cascade *[Kristinsson, H. et al., 2013]*. Pancreatic GPR40 protein level is regulated by glucose and FFAs *[Sykaras, A.G. et al., 2012]*.

A human mutation of the GPR40 gene is associated with reduced lipid-mediated enhancement of GSIS *[Del Guerra S et al., 2010; Fontes G et al., 2010; Walker CG et al. 2011]*. The effects of GPR40 on pancreatic beta cell viability have been controversial. Indeed, a disruption of islet morphology is associated with pancreatic-specific GPR40 overexpression and impaired beta cell function, whereas GPR40 interruption is linked with increased beta cell viability in mice fed on a HFD *[Steneberg P. et al., 2005]*. These investigations promoted the view that GPR40 antagonism could be beneficial in the treatment of diabetes. However, most subsequent preclinical studies contradict these result, indicating that GPR40 agonism has no effects on beta cell viability *[Lan H. et al., 2008; Kebede M. et al., 2012, Nagasumi K. et al., 2009]*, or even protects beta cells *[Zhang Y. et al., 2007; Wu P. et al., 2010; Wagner R. et al., 2013]*.

Expression of GPR40 in the pancreas was linked to the capacity of FFAs to intensely amplify glucose-stimulated insulin secretion *[Itoh et al., 2003; Salehi et al., 2005; Nagasumi K. et al., 2009]*. These results may be useful to evaluate the action of synthetic GPR40 ligands in animal models of glucose dysregulation and for the treatment of diabetes in humans *[Stoddart et al., 2008; Mancini and Poitout, 2013]*. Recently, studies have been suggested that selective activators of peroxisome proliferator-activated receptor (PPAR-γ) may activate GPR40 *[Kotarsky et al., 2003; Smith et al., 2009]* linking directly to the beneficial effect of pioglitazone on lipotoxicity *[Wu et al., 2010]*.

1.2.2 GPR41/FFA3

In 2003, it was reported that GPR41 is activated by SCFAs *[Brown AJ et al., 2003; Le Poul E et al., 2003]*. It is coupled with Gi/o protein and it is expressed in adipose tissue, gut and the peripheral nervous system by regulating intestinal gluconeogenesis and sympathetic activity. In fact, GPR41 is reported to be involved in energy regulation in response to SCFAs produced from the gut microbiota *[Inoue D et al., 2014]*. GPR41 ligands include acetate (C2), propionate (C3) and butyrate (C4) and its ligand affinity is "propionate > butyrate > acetate" *[Brown A.J. et al., 2003; Le Poul E. et al., 2003]* but Zaibi et al. showed that acetate, rather than butyrate, can stimulates leptin secretion by mesenteric adipocytes in wild type mice *[Zaibi et al., 2010].*

GPR41 is expressed in human *[Brown AJ et al., 2003; Le Poul E. et al., 2003]* and mouse adipose tissue *[Xiong Y. et al., 2004]*. In human, SCFAs are generated in the lower intestine primarily by fermentation of dietary fibres contributing significantly to caloric intake *[Brown A. J. et al., 2013]*. SCFAs act as signalling molecules which may regulate leptin production in accordance with dietary intake. Adipocytes can sense SCFA levels and positively regulate leptin production in response to elevated SCFA concentrations.

Other studies showed that the propionate-stimulated activation of GPR41 increased the release of leptin in mice after oral administration. *[Xiong Y. et al., 2004]*.

1.2.3 GPR43/FFA2

GPR43 is identified as an SCFA receptor that can be activated by acetate (C2), propionate (C3) and butyrate (C4) *[Tolhurst G. et al., 2012; De Vadder F., 2014]*. Acetate and propionate are the most efficient for activating GPR43 *[Flint H. J., 2008; Wolever T. M. et al., 1989]*.

GPR43 is a Gi/o- and Gq-dual-coupled receptor that the activation coupled with Gi/o by SCFAs inhibits cAMP production, activates the extracellular signal-regulated kinase (ERK) cascade, whereas the coupling with Gq increases intracellular Ca2+ levels, and promotes activation of the mitogen-activated protein kinase (MAPK) cascade *[kimura I. et al., 2014]*.

In Gpr43-deficient mice, GLP-1 secretion is reduced by SCFAs in vitro and in vivo studies, and they have improved glucose tolerance *[Hong, Y.H.; et al., 2005]*.

GPR43 is expressed in the adipose tissue, intestines, and immune tissues *[Brown AJ et al., 2003; Inoue D. et al., 2012]*. Several studies indicate that GPR43 expression in adipose and gastrointestinal tissues suggest that its activation may be involved in regulating obesity and energy accumulation *[Bellahence M. et al., 2013]*. In particular, GPR43 mRNA is expressed in subcutaneous, perirenal and epididymal adipose tissues as well as in 3T3-L1-derived adipocytes and mature adipocytes *[Brown AJ et al., 2003]*. Hong et al. showed that GPR43 expression was significantly higher in WAT of mice with HFD-induced obesity. The activity of GPR43 promotes energy expenditure and favoured usage of fat through the repression of fat accumulation by inhibition of adipose tissue-specific insulin signalling. The most important source of GPR43 ligands come from gut microbiota suggesting that GPR43 is dependent on the presence of gut microbiota on the regulation of adipose-insulin signalling by sensing gut

microbial SCFAs *[kimura I. et al., 2014].* GPR43 also stimulates GLP-1 secretion in the colon and directly regulation of insulin secretion in the pancreas, thereby promoting insulin sensitivity.

1.2.4 GPR120/FFA4

The GPR120 gene was discovered in 2003 *[Fredriksson R et al. 2003]*. The first studies on GPR120 as a drug target reported that this receptor is activated by FFAs to promote GLP-1 secretion and protect the GLP-1-secreting cells *[Hirasawa A et al., 2005; Katsuma S. et al., 2005]*. Recently reports on anti-inflammatory and insulin secreting effects showed an association between a dysfunctional receptor variant and increased risk of obesity *[Ichimura A. et al., 2012; Oh DY et al., 2010]*. FFAs are primary endogenous agonists of GPR120.

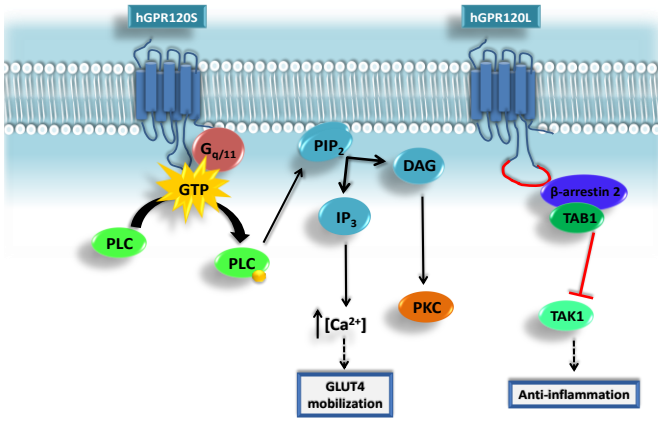
GPR120 is expressed in adipose tissue and expression increases during adipocyte differentiation in murine 3T3-L1 cells and human adipocytes, indicating that the receptor is implicated in adipocyte differentiations *[Gotoh C. et al., 2007; Miyauchi S. et al., 2009]*. GPR120 has been associated with a number of physiological functions related to inflammation and metabolic disorders *[Ulven T. et al., 2015]*. This suggests that GPR120 has varied roles in energy regulation and immunological homeostasis [*Miyamoto J. et al., 2016*].

GPR120 is a receptor for MC- and LCFAs *[Hirasawa, A. et al 2005]* and importantly it is activated by various n-3 or n-6 PUFAs, including DHA, EPA and palmitoleic acid (POA, 16:1) *[Hirasawa, A. et al 2005]*, at micro molar concentrations.

Recently, TUG-891 [3-(4-((4-fluoro-49-methyl-[1,19-biphenyl]-2-yl) methoxy) phenyl) propanoic acid] was described as agonist for GPR120. This synthetic agonist shows similar

signalling properties of the α-linolenic acid on human GPR120 in various end points as stimulation of Ca^{2+} mobilization, β-arrestin-2 recruitment, and extracellular signal-regulated kinase phosphorylation. TUG-891 has less selectivity for GPR120 in mouse system including differentiated 3T3-L1 adipocytes and Raw264.7 macrophages which express this LCFA receptor [*Gotoh et al., 2007; Oh et al., 2010*]. Indeed, it has been reported that TUG-891 weakly stimulated insulin-independent glucose uptake in 3T3-L1 cells and inhibited LPS-induced TNFα secretion from RAW264.7 cells [*Oh et al., 2010*].

The human GPR120 exists in two isoforms: as a 361-residue short chain and a long chain with 16 additional residues inserted in the third intracellular loop: hGPR120S and hGPR120L, respectively. The recruitment of β-arrestin-2 leads to anti-inflammatory effects through scavenging of TAB1 [transforming growth factor-βactivated kinase 1 (TAK1)-binding protein 1] and inhibition of TAK1 at a meeting point in the lipopolysaccharide and tumour necrosis factor pathways, it is mediated by GPR120 long isoform. Hormone secretion and translocation of glucose transporter 4 (GLUT4) seem to be mediated through Gq/11 and calcium mobilization by GPR120 short isoform (**Scheme 1**) *[Oh DY. Et al., 2010; Moore K. et al., 2009; Watson SJ et al., 2012]*.



Scheme 1. GPR120 intracellular pathway based on its isoform.

1.2.5 GPR84

GPR84 was originally discovered by expressed sequence tag data mining *[Wittenberger, T. et al., 2001]* and cloned from a cDNA library prepared from human peripheral blood neutrophils. It was initially thought to be expressed mainly in bone marrow, lung, and peripheral blood leukocytes *[Yousefi S. et al., 2001]*.

GPR84 is an MCFA receptor that is activated by undecanoic acid (C11) and lauric acid (C12) *[Wang J. et al., 2006]*. It is linked with the pertussis toxin-sensitive Gi/o pathway and expressed particularly by various immune cells *[Wang J et al., 2006]*.

GPR84 expression is markedly induced in monocytes/macrophages upon activation by LPS, and MCFAs act through GPR84 to amplify the stimulation of lipopolysaccharide (LPS)-induced IL-12 p40 production *[Overton H. A. et al., 2006; Brown A. J. et al., 2003]*. Indeed, activation of GPR84 in RAW264.7 cells, a murine macrophage-like cell line, amplifies LPS-stimulated IL-12 p40 production *[Wang J. et al., 2006]*. GPR84 KO mice increase the production of T helper 2 (Th2) cytokine *[Venkataraman C. et al., 2005]*. However, the physiological roles of GPR84 are still unknown [*Suzuki M. et al., 2013*]. Based on these findings, it is suggested that GPR84 expression plays a role predominantly in the immune system. Nevertheless, the function of GPR84 has not been addressed in adipose tissue *[Nagasaki H. et al., 2012]*.

Recently, the interest in GPR84 has increased due to studies in which co-culture of model macrophages and adipocytes have been found to result in marked up-regulation of GPR84 expression by the adipocytes, both in murine and human fat cell systems *[Nagasaki H. et al., 2012; Trayhurn and Denyer, 2012]*.

Although recognised as a receptor responsive to medium-chain saturated free fatty acids *[Wang J. et al., 2006]*, GPR84 remains the least studied and least understood of the currently described receptors for FFA *[Milligan G. et al., 2014].*

GPR84 is highly expressed in monocytes and neutrophils upon lipopolysaccharide stimulation. GPR84 was up-regulated in a 3T3-L1 and in the adipose tissue of obese subjects and mice fed with HFD *[Nagasaki H. et al., 2012]*.

GPR84 aggravates the pathogenesis of obesity and T2D via TNFα released by infiltrating macrophages in adipose tissue. Thus, GPR84 plays a central role in metabolism and immune responses and mediates a crosstalk between immune cells and non-immune cells, such as adipocytes *[Miyamoto J. et al., 2016]*

In the pathogenesis of IR, chronic activation of inflammatory pathways plays a central role. The inter-communication macrophages and adipocytes provides a key mechanism underlying the common disease states of decreased insulin sensitivity *[Schenk S. et al., 2008]* which involves the migration of macrophages into the AT followed by subsequent switching-on of pro-inflammatory pathways in the macrophages that result in secretion of cytokines. These events promote inflammation and decreased insulin sensitivity in adjacent cells targeted by insulin *[Schenk S. et al., 2008, Shoelson S.E. et al., 2006]*. In adipocytes, GPR84 is involved in the inflammatory changes happening after reaching the status of ‘‘diabesity’’, because over-nutrition causes a shift in adipocytes to express pro-inflammatory genes, few of which are common to M1 macrophages *[Lumeng C.N. et al., 2007].*

The interesting regulatory role of GPR84 in the contribution of macrophages infiltration to AT function might be a target to limit the effects of increased fat mass and adiposity in metabolic and other co-morbid diseases. *[Milligan G. et al., 2014].*

1.3 Biological activity of fatty acids

Fatty acids from the diet affect metabolic processes leading to the synthesis of other fatty acids with a carbon chain greater or less in length and a different number of double bonds. Some enzymes catalyse these reactions: desaturases, which insert a double bond, and elongases which lengthen the carbon chain. Indeed, fatty acids can be classified according to chain length into SCFAs containing less than 6 carbon atoms, MCFAs with 7–12 carbon atoms which contain a single cis-alkene, and LC with more than 12 carbon atoms and LC/PUFAs containing several methylene-interrupted cis-alkenes.

The PUFAs are subdivided into n-6 and n-3 fatty acids on the basis of the distance of the first alkene from the terminal end. They are essential for a healthy body but cannot be de novo synthesized in humans and must be obtained directly through the diet or via other PUFAs.

Linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), can serve as precursors and are therefore known as essential fatty acids, although their conversion to some of the longer congeners is inefficient *[Walker CG, et al. 2013]*.

Several specific fatty acids serve as precursors of potent signalling molecules. Arachidonic acid (ARA, 20:4n-6) is LA metabolite and is released from cell membranes on demand. ARA is a substrate for cascades initiated by cyclooxygenase (COX) enzymes to form prostanoids, by lipoxygenases to form leukotrienes and lipoxins, or by cytochrome P450 enzymes to form epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids, with individual members of each class often acting on specific receptors *[Back M et al., 2011; Korotkova M. et al., 2014, Woodward DF. et al., 2011]*.

The n-3 fatty acids EPA and DHA are the major ALA metabolites. They act as substrates for similar cascades giving rise to resolvins, protectins, and maresins that also are potent activators of specific receptors *[Serhan CN., 2014; Serhan CN. et al., 2011].*

The two series are incorporated in biological membranes as components of phospholipids and glycolipids for maintaining the integrity of cell membranes. N-3 PUFA are essential also in the development of cognitive ability and physical activity *[Youdim k. et al, 2000; Yeuda S. Et al., 1999]* and are involved in cellular signalling by regulating gene expression and neuronal functions *[Pollitt E. et al, 1998]*.

The biological effects of PUFAs are mediated by the interaction between families; in fact, the metabolic pathways of these families compete for a shared enzymatic system which includes the same enzymes for elongation and desaturation reactions that act on precursors producing the relevant metabolites.

1.3.1 Peroxisome proliferator-activated receptors (PPARs)

PPARs are transcription factors activated by natural and synthetic ligand that form heterodimers with another nuclear receptor, the retinoid x receptor (RXR), and they are involved in target gene regulation, energy homeostasis and in adipocyte differentiation and inflammation. These families of receptors consist of three different isoforms: PPARα, PPARγ and PPARδ *[De luca C. e t al 2008]*.

PPARγ is mainly present in AT, the colon and macrophages. Two isoforms of PPARγ are detected in human and in mice: the isoform mostly found in AT is the PPAR-γ2 *[Fajas et al J.,*

2001].

In an in vitro study on the regulation of adipogenesis through GPR120, when its expression was knocked down using siRNA, also the expression of PPAR-γ2 further declined during adipogenesis *[Gotoh C.et al., 2007]*. Activation of PPARγ in adipocytes, despite increasing body fat mass, improves adipocyte function *[Lehrke and Lazar, 2005]*.

Some dietary fats and their metabolites bind to PPARγ with moderate affinity suggesting that their physiological role is to act as a sensor for the integrated flow with more fatty acids. Indeed, PPARγ is highly expressed in WAT where his activation promotes an increased accumulation of lipids acting in a key role in adipogenesis.

The anti-inflammatory role of PPARγ is strongly linked to systemic insulin sensitivity, as documented by the effects of anti-diabetic drugs, thiazolidinediones (TZD), and acting as specific PPARγ agonists. Indeed, the specific upregulation of PPARγ in WAT induces strong antidiabetic effects *[Sugii S. et al., 2009]*. Chronic peripheral administration of PPARγ agonists has led to an improvement in glucose control at the expense of a higher caloric intake, an increase of weight and body fat *[Larsen P.J. et al., 2003; Joosen et al, 2006; Home P.D. et al., 2009]*; instead the administration of antagonist has led to protection against obesity *[Nakano R. et al., 2006]*.

Recently it has been observed that PPARγ is also expressed in the hypothalamus which is involved in the central regulation of energy balance, suggesting that activation of PPARγ by rosiglitazone and other TZDs contributes to the regulation of energy balance. The activation by endogenous lipid antagonists induces hyperphagia and leptin resistance with HFD. Whereas blocking the activation of by PPARγ antagonists results in a negative energy balance and an amelioration of insulin sensitivity in HFD *[Ryan KK. et al., 2011]*.

1.4 Adipose tissue (AT)

AT is distributed throughout the body mainly in the subcutaneous region (subcutaneous adipose tissue, SAT) or in the thorax and abdominal cavities (visceral adipose tissue, VAT) *[Smorlesi A. et al., 2012].* WAT is composed of white adipocytes as well as other cell types, including preadipocytes, endothelial cells and immune cells such as macrophages. White are filled with a single lipid droplet (unilocullar adipocytes) and equipped with a small cytosolic compartment; areas mainly composed of brown adipocytes, which are multilocular cells with respect to the lipid droplets and which are rich in large mitochondria, are considered brown adipose tissue (BAT) *[Smorlesi A. et al., 2012]*. Indeed, different cell morphologies correspond to different functions *[Cousin B. et al., 1992; Guerra C. et al., 1998; Walden T.B. et al., 2012].*

WAT produces a number of protein and non-protein secreted molecules which include cytokines, hormones, growth factors and matrix proteins that affect the nearby cells and also peripheral tissues as well as the brain. Therefore, WAT-derived signals may influence various processes including food intake, energy expenditure, metabolism, immunity and blood pressure *[Trujillo M.E. et al., 2006, Sell H. et al., 2012]*.

The concept of WAT as an endocrine organ gained universal acceptance because of the discovery of the hormone leptin *[Zhang YY et al., 1994]* which is involved in a wide spectrum of biological functions. WAT is considered the most plastic organ among the metabolic tissues, and it can represent 5–60% of total body weight *[Lee M.J. et al., 2013, Kissebah A.H. et al., 1994]*. The number of adipocytes is largely static in adult humans and independent of bodyweight variations, even in response to massive weight loss. This means that the number of

adipocytes is established during childhood and adolescence *[Kissebah A.H. et al., 1994]* and only almost 10% of fat cells are renewed annually in adult humans *[Spalding K.L. et al., 2008]*.

Men accumulate fat in both subcutaneous and visceral depots *[Despres J.P. et al., 2006]*, unlike women, but do not show high rates of subcutaneous fat accumulation except in the case of the morbidly obese *[Kissebah A.H. et al., 1994]*.

The intrinsic metabolism of WAT is a key element for the whole body in relation to the storage of energy in triacylglycerols (TAG) during the postprandial state, as well as the mobilization of energy stores during fasting or exercise, and in the control of circulating FA levels *[Flachs P. et al., 2013]*. Lipolysis of TAG from intracellular lipid droplets in adipocytes is under complex hormonal control and is mediated by numerous co-operating enzymes *[Girousse A. et al., 2012; Zechner R. et al., 2012]*.

WAT is also a site of de novo FA synthesis (de novo lipogenesis), in rodents and in humans, where up to 40% of whole-body de novo FA synthesis from glucose may take place in WAT *[Chascione C. et al., 1987]*. In practise, this depends upon environmental conditions *[Trayhurn, 1981]*. Principally, FA synthesis and the uptake of glucose mediated by GLUT4 into adipocytes are linked to whole body insulin sensitivity. Indeed, it has been hypothesized that some de novo formed lipids in adipocytes might support insulin signalling in other tissues *[Czech M.P. et al., 2013]*.

AT serves as a main source of novel lipid mediators like various isomers of palmitic-acid-hydroxystearic-acid (PAHSA) that can improve glucose tolerance stimulating directly insulin secretion in pancreas, incretin secretion in intestinal cells, and insulin-stimulated glucose transport in adipocytes.

The mitochondrial content of mature white adipocytes is several-fold higher than in

preadipocytes and depends on the anatomical location of a fat depot *[Flachs P. et al., 2013]*. Indeed, mitochondria play a critical controlling role in the main metabolic pathways, i.e. de novo FA synthesis, glyceroneogenesis, lipolysis and FA re-esterification in adipocytes *[Flachs P. et al., 2013]*. Obesity and IR are associated with a low capacity of both mitochondrial oxidative phosphorylation *[Flachs P. et al., 2013; Kusminski C.M. et al., 2012; Naukkarinen J. et al., 2014]* and de novo FA synthesis *[Diraison F. et al., 2002]* in WAT.

1.4.1 Lipid signalling in AT

A large family of endogenous lipid mediators contributes to the immune and metabolic state of WAT. Eicosanoids/prostanoids are involved in the control of metabolism and inflammation *[Yore M.M. et al., 2014]*. Overall fatty acids may modulate the inflammatory response *[Calder, 2005; Lee et al., 2003]*.

In general, the n-6-derived ARA gives rise to pro-inflammatory mediators, whereas the n-3-derived EPA and DHA produce signalling molecules with anti-inflammatory or resolving action *[Serhan CN., 2014; Scher JU.et al., 2009].*

Eicosanoids are produced via cyclooxygenases, lipoxygenases, and cytochrome P450 pathways. They are powerful local mediators of signal transduction and modulate the inflammatory response and metabolism of WAT. Immune cells and adipocytes are the main producers of eicosanoids. In particular, adipocytes can also synthesize the main prostanoids and leukotrienes and express eicosanoid receptors. Prostanoids are involved in adipocytes differentiation and regulation of lipolysis in an autocrine and paracrine manner. They are

produced by the action of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), which is a dominant pathway in producing eicosanoids in WAT *[Jaworski K. et al., 2009; Fain J.N. et al., 2000; Fredholm B.B. et al., 1973; Richelsen B. et al., 1987].* It has been demonstrated that eicosanoids activate the PPAR family of transcription factors [*Yu K. et al., 1995*]. Indeed, the products of lipoxygenases and hepoxilins and activators of PPARα and PPARγ were able to induced differentiation in 3T3-L1 preadipocytes *[Yu K. et al., 1995; Hallenborg P. et al., 2010]*. Furthermore, products of lipoxygenase activity are important for the regulation of both adipogenesis *[Yu K. et al., 1995; Hallenborg P. et al., 2010; Nugent C. et al., 2001]* and WAT inflammation.

Different mechanisms have been attributed to the action of n-3 PUFAs:

- competition between n-3 and n-6 PUFA for the enzymes necessary for inflammatory mediator biosynthesis: COX-2 and prostaglandin E2 (PGE2) *[Lands, 1992],*
- activation of anti-inflammatory PPARγ *[Im, 2012; Kliewer et al., 1997],*
- inhibition of toll-like receptors (TLR2/3/4) *[Lee et al., 2003],*
- synthesis of pro-resolving metabolites of DHA and EPA (resolving E1 and neuroprotection D1) *[Serhan CN. et al. 2011]*.

Therefore, several anti-inflammatory mechanisms have been suggested for n-3 PUFAs, such as, the inhibition of COX-2-mediated PGE2 production by EPA *[Calder, 2012; Im, 2012]*. A wide variety of lipid mediators derived from EPA and DHA have been identified in human SAT *[Claria J. et al. 2013]*.

1.4.2 Cytokines and Chemokines

Adipokines are protein signals which include the major adipocyte hormones, leptin and adiponectin, both of which have multiple functions. A number of adipokines, including interleukin-1β (IL-1β), TNFα, interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), are linked to inflammation and their synthesis and secretion are generally increased in the obese state *[Hotamisligil, 2006; Rajala and Scherer, 2003; Rosen and Spiegelman, 2006; Trayhurn and Wood, 2004]*.

Chemokines are a family of cytokines that induce leukocyte chemotaxis. They are directly involved in the development of allergic and autoimmune diseases. More than 50 chemokines have been discovered, which exhibit various physiological and pathological properties *[Mantovani A et al., 2004; Proudfoot AE., 2002]*. Recent studies have shown that chemokines are expressed in preadipocytes and adipocytes, and have demonstrated also that the infiltration of bone marrow-derived macrophages into obese AT is involved in the development of IR *[Tsuguhito Ota, 2013]*.

Based on the motif patterns involving two N-terminal cysteine residues, chemokines can be classified into four subfamilies: CXC, CC, C, and CX3C (where X is any amino acid residue). The CXC chemokines are mostly chemotactic for neutrophils and are known for their association with acute inflammation, while most CC chemokines act on monocytes, T cells, eosinophils, and basophils, mediating chronic inflammation and allergy *[Mantovani A et al., 2004; Proudfoot AE., 2002]*. It is increasing evident that the chemokine system is involved in the chronic sub-acute inflammation that is the common underlying condition of obesity, IR, and T2D.

Particularly, the interaction of MCP-1 with its receptor CCR2 is considered central in obesity-induced IR *[Kanda H. et al. 2006; Weisberg SP. et al. 2006]*.

MCP-1 is produced by a variety of cells, including human adipocytes *[Gerhardt CC. et al., 2001; Rollins BJ. et al., 1997]*, frequently in response to inflammatory stimuli [*Rollins BJ. et al., 1997*]. The increase of MCP-1 during obesity, in particular in visceral fat pads, might contribute to the pathogenesis of IR *[Kanda H. et al., 2006]* and inhibits differentiation of murine adipocytes in vitro *[Sartipy P et al., 2003]*.

The expression of MCP-1 increases during adiposity stimulating the recruitment of macrophages which further increases the expression of cytokines to aggravate inflammation-induced IR [*Kahn S. E. et al., 2006*]. MCP-1 is expressed both before and after differentiation, with the mRNA level increasing post differentiation *[Trayhurn P., 2004]*.

MCP-1's role in IR is exerted by regulating the inflammatory response, insulin sensitivity, lipid metabolism, macrophage polarization and infiltration, and the phosphorylation of extracellular signal-regulated kinase-1/2 (ERK-1/2) and p38 MAPK *[Nio Y. et al., 2012]*.

IL-1β is a pro-inflammatory cytokine and its secretion is regulated by inflammasome activity *[Chen L. et al. 2015]*. IL-1β is extensively implicated in the development of T2D, in relation to IR and to impaired insulin secretion in peripheral tissues and macrophages which leads to reduced insulin sensitivity of β-cells *[Su D. et al., 2009; Boni-Schnetzler M. et al., 2013]*. In different cells such as endothelial cells, the concentration of IL-1β and monocytes are increased during hyperglycaemia *[Koenen T. B. et al., 2011]*.

IL-1β antagonism and blockade of the IL-1β receptor are potential targets for the treatment of T2D *[Donath, 2014; Bing, 2015]*. This pro-inflammatory cytokine might increase systemic inflammation and inhibit insulin action in the major insulin-target cells, such as macrophages

[Hardaway A. L. et al., 2013].

Recently, IL-1β has been shown to inhibit insulin signal transduction in adipocytes, reducing expression of signalling proteins [*Gao D. et al., 2011*]. The suppression of adiponectin production induced by IL-1β may also be impaired insulin sensitivity [*Alomar S et al., 2015*].

IL-1β has a wide stimulatory effect on the expression and release of cytokines and chemokines in human adipocytes. Indeed, IL-1β enhanced a varied range of these factors including CXCL10, CXCL11, CCL11, CCL19 and IL15, while their expression was not altered by macrophage-condition media [*Alomar S et al., 2015*].

In the early 1990s, studies demonstrated the release of the pro-inflammatory cytokine TNFα from adipocytes. Indeed, TNFα was one of the first adipokines to be identified. It is itself produced by adipocytes, being linked to the induction of IR *[Hotamisligil GS et al., 1993]*. This pro-inflammatory cytokine plays an extensive role in AT function, exerting a range of actions such as the induction of apoptosis *[Prins JB. et al., 1997]* and the stimulation of lipolysis *[Ryden M. et al., 2004]* increasing the serine/threonine phosphorylation of insulin receptor substrate-1 (IRS-1) *[Shoelson S. E. et al., 2006; Hotamisligil G. S.et al., 2000]*. TNFα can increase glucose uptake in visceral and subcutaneous adipocytes by activating the adenosine monophosphate activated protein kinase (AMPK) pathway, while it initiates IR in visceral adipocytes by activating JNK1/2 *[Chen L. et al. 2015]*. In addition, the plasma levels of TNFα are higher in males than in females, as well as in obese individuals compared with lean ones. This suggests that obese males are more likely to suffer from IR and related diseases *[El-Haggar S. M. et al., 2015]*.

TNFα has also been reported to influence the production of several adipokines as the expression of IL-6 *[Fasshauer M et al., 2003]* and MCP-1 *[Gerhardt CC. et al., 2001; Sartipy P. et al.,*

2003] is stimulated, whereas adiponectin expression is inhibited by the cytokine *[Fasshauer M et al. 2002]*. Importantly, a link between TNFα and IR in obesity has been established *[Hotamisligil GS. 2003; Sethi JK. et al., 1999]*, the cytokine production being increased in fat cells of rodent models of obesity *[Hotamisligil GS. et al., 1993]*.

TNFα has been shown to be synthesized and secreted from human adipocytes *[Kern PA. et al., 1995],* and the local expression of the cytokine is higher in AT of subjects with obesity-related IR *[Kern PA et al., 2001]*.

IL-6 is secreted by several tissues and is recognized as an inflammatory mediator in AT that causes IR by reducing the expression of GLUT4 and IRS-1. IL-6 acts through activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling pathway and increasing the expression of suppressor of cytokine signalling 3 (SOCS3) *[Lukic L. et al., 2014, Serrano-Marco L. et al., 2012]*.

Adiponectin is produced mainly by WAT. It acts as an anti-inflammatory cytokine in obesity, IR, or T2D where its level is reduced, but it was found to be increased in osteoarthritis (OA) and type 1 diabetes (T1D), where it acts as a pro-inflammatory cytokine *[Stofkova A. et al., 2009; Passos M. et al., 2014]*. Adiponectin exerts its action via two receptors leading to the improvement of IR by controlling the metabolism of glucose. Adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) are expressed respectively mostly in skeletal muscle and the liver *[Boni-Schnetzler M. et al., 2013; Bermùdez V. J. et al., 2013]*. AdipoR1 is likely to reduce the expression of the genes that encode hepatic gluconeogenic enzymes and molecules involved in lipogenesis by activating AMPK. AdipoR2 increases the expression of the genes that contribute to glucose consumption by activating PPARα signalling *[Yamauchi T. et al., 2007; Crimmins N. A. et al., 2007]*. Indeed, adiponectin improves hepatic IR by reducing

glycogenesis and lipogenesis, and increases glucose consumption *[Chen Li et al., 2015]*

1.4.3 Macrophages: role in inflammation

Macrophages are plastic monocyte-derived cells that exert different molecular and functional phenotypes after exposure to various bioactive molecules and environments *[Ariel et al., 2012]*. They are polarized into two extremes which promote pro-inflammatory or alternative anti-inflammatory pathways (Scheme 2) *[Martinez et al., 2009]*. These alternatively activated macrophages are named M1 and M2 *[Mantovani et al., 2005]*. The M1 type macrophages function to activate inflammation by expressing high levels of inflammatory cytokines (TNFα, IL-1β, Il-6) and chemokines (CXCL2, 9, 20, 11 and CCL2, 3, 4) and enzymes which generate reactive oxygen species and nitric oxide *[Mantovani et al., 2005]*, while M2 type macrophages act at the end to resolve inflammation by releasing lower levels of inflammatory mediators but a high level of IL-10 *[Ariel et al. 2012; Mantovani et al., 2005; Martinez et al., 2009]*.

As mentioned above it has been reported that activation of GPR120 promotes the switching to M2 type macrophages leading to anti-inflammatory responses in adipose and liver tissues *[Oh et al., 2010]*. Macrophages aggregate around dead adipocytes and form syncytia namely "crown-like structures" (CLS) and act as scavengers of residual adipocyte lipids by forming multi-nucleate giant cells. Adipocyte necrotic death could represent the initial stimulus that leads to ATM accumulation *[Cinti S. et al., 2005]*.

ATMs can comprise up to 40% of the cells in obese WAT, while their numbers is related with the degree of obesity *[Weisberg S.P. et al., 2003; Xu H. et al., 2003]* and represent the strongest

predictor of T2D progress in obese patients.

Macrophages may infiltrate WAT in response to MCP-1, which is mainly released from adipocytes as part of a scavenger function in response to hypoxia and adipocyte necrosis. Indeed, targeted deletion of the CCL2 gene reduced macrophage accumulation and inflammation in WAT, as well as IR related to obesity *[Kanda H. et al., 2006]*.

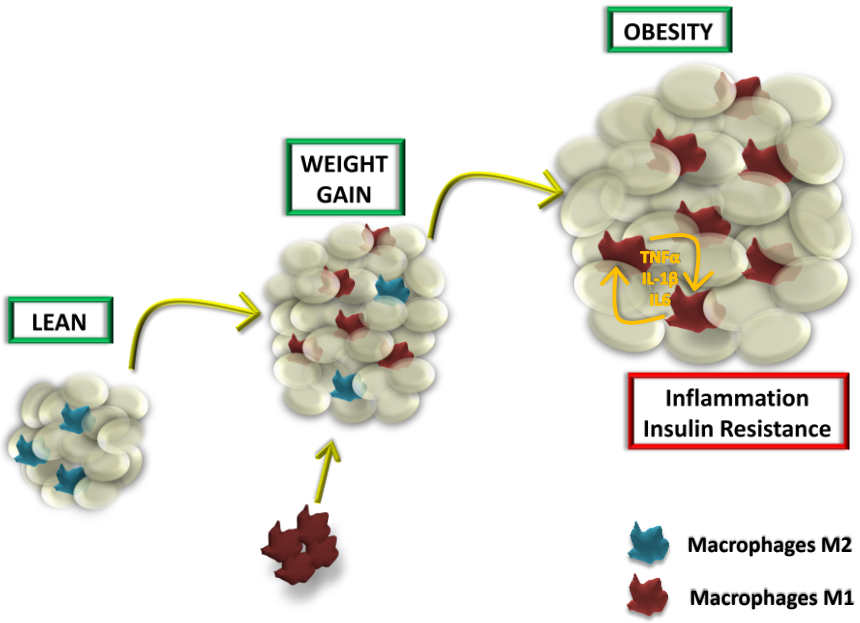
Cell signalling between adipocytes and macrophages in WAT is influenced by dietary FA and FA-derived lipid mediators *[Oh D.Y. et al., 2010]*. N-3 PUFAs exhibit their anti-inflammatory effects via signalling by GPR120 resulting in improved insulin sensitivity in obese mice *[Oh D.Y. et al., 2010; Oh Y. et al., 2014]*.

The recruitment of macrophages in WAT is also influenced by other immune cells such as T cells and B cells, eosinophils and mast cells. T cells influence macrophages infiltration and polarization. They are classified into two groups based on their inflammatory properties: T helper 1 (Th1) which produce pro-inflammatory mediators and T helper 2 (Th2) which produce anti-inflammatory factors. Based on this finding, in the obese state the number of pro-inflammatory Th1 cells increase, while anti-inflammatory Th2 cells are reduced in AT *[Feuerer M. et al., 2009; Nishimura S. et al., 2009; Winer S. et al., 2009].*

VAT compared to SAT accumulates more inflammatory ATMs and is more metabolically active which includes basal and stimulated lipolysis *[Kosteli A. et al., 2010, Medrikova D. et al., 2011]*. In obesity high rates of basal lipolysis could induce WAT inflammation and accumulation of pro-inflammatory M1 ATMs by activating PRRs *[Long E.K. et al., 2013; Claria J. et al., 2012]*. Despite immune cells and adipocytes act like a functional unit, the mechanistic links between them, ATMs and adipocytes, remain mainly unknown *[Kosteli A. et al., 2010; Medrikova D. et al., 2011]*.

A study related to LPS stimulation on human primary preadipocytes and adipocytes showed

that these cells exhibit immune cell-like behaviour. Adipocytes were found to express multiple receptors, involved in T-cell (co)stimulation, particularly upon treatment with LPS. These receptors included adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1), Intercellular adhesion molecule 1-3 (ICAM-1-3) *[Meyer K., 2011]*.



Scheme 2. Linkage between inflammation and obesity/insulin resistance

1.4.4 Implication for obesity and IR

IR is a condition in which skeletal muscle, liver, and white adipose tissue (WAT) which are sensitive to insulin, become less sensitive to the hormone and its downstream metabolic actions under normal serum glucose concentrations *[Chawla A. et al. 2011]*. Indeed, IR encompasses a wide spectrum of disorders: obesity, hypertension, hyperglycaemia, polycystic ovary syndrome and ovary syndrome *[Liang F. X. et al. 2010, Laakso M. et al. 2014]*, related to

defective insulin receptor signal transduction and mitochondrial *function [Abdul-Ghani M. A. et al. 2010, Ahn J.et al. 2014]*, microvascular dysfunction *[Muris D. M. J. et al. 2013, Karaca U. et al 2014]*, and inflammation *[Shoelson S. E. et al., 2006; Konner A. C. et al., 2013; Cai D. S., 2013]*.

It has been demonstrated that IR exists in the prediabetic state, often predating the onset of diabetes mellitus by many years *[Olefsky JM, et al. 2010]*. Obesity with sedentary lifestyles is the dominant cause of the development of IR in subjects with the metabolic syndrome and T2D *[Haffner S et al., 2003 Kahn BB, et al. 2000]*.

Obesity induces an inflammatory state associated with many clinical complications [*Ferrante, 2007*] and is related to a state of chronic low-grade inflammation that in the long term is triggered by nutrients and metabolic surplus *[Hotamisligil, 2006]*. In addition, WAT is an endocrine organ with adipocytes releasing adipokines which are involved in pro and anti-inflammatory states *[Ouchi et al., 2011]*.

Obesity is the result of relative overnutrition. In fact, net overnutrition causes lipid accumulation in adipocytes which activates c-Jun N-terminal kinase (JNK) and nuclear factor kappa B (NF-κB) signalling pathways following an increased production of pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 *[Shoelson S. E. et al., 2006, Sharma M. et al., 2013]*.

In most cases, AT is the first initiator of this inflammatory response in the body *[Berg AH et al., 2005 Tilg H. et al., 2006].* Indeed, obesity is associated with increased circulating levels of inflammatory markers such as C-reactive protein, IL-6, IL-8, TNFα and haptoglobin *[Bullo et al., 2003; Engström et al., 2003; Yudkin et al., 1999]*.

Inflammation induced by obesity is a process characterized by macrophage infiltration into the AT of obese individuals *[Lumeng et al., 2007; Weisberg et al., 2006]*. The finding that AT from obese mice and humans is infiltrated with macrophages provided an advance in

understanding of how obesity propagates inflammation *[Xu H et al 2003, Weisberg SP et al. 2006]*. AT contains bone-marrow-derived macrophages and the content of these ATMs track with the degree of obesity *[Xu H et al 2003, Weisberg SP et al. 2006, Lumeng CN. et al., 2007]*.

Adipose tissue macrophages (ATMs) are considered responsible in part for the induction of IR *[Lesniewski et al. 2007]* and it has been argued for almost all AT-TNFα expression – despite the production of TNFα from adipocytes *[Cancello et al. 2005]*. M2-polarized ATMs were found probably to protect adipocytes from inflammation *[Lumeng et al. 2007]*. Indeed, anti-inflammatory cytokines such as IL-10 protect adipocytes from TNFα induces IR and diet-induced obesity leads to a switch in the activation state of ATMs to an M1 pro-inflammatory pathway *[Lumeng et al., 2007]*. Indeed, macrophage-secreted factors, through increases of the inflammatory transcription actor NF-κB blocking insulin action in adipocytes via down-regulation of GLUT4 and IRS-1 *[Lumeng CN. et al., 2007]*, are suggested to stimulate adipocyte inflammation and induce IR *[Permana PA. et al., 2006]* leading to impaired glucose uptake.

In the presence of macrophages and macrophage-secreted factors, the inflammatory state can impair adipogenesis, and also lead to increased production of IL-6, IL-1β, MCP-1 and other inflammation-related signals *[Constant et al., 2006; Lacasa et al., 2007]*. In fact, the main focus of several studies has been on the effect of macrophages on gene expression in mature adipocytes *[Constant et al., 2006; Permana et al., 2006; Shah et al., 2009; Suganami et al., 2005; Varma et al., 2008; Yamashita et al., 2008; O'Hara et al, 2009].*

Studies have been conducted to identify the endogenous pathophysiologic signals that stimulate pro-inflammatory pathways in ATMs. In this regard, it has found that SFAs largely stimulate macrophage inflammatory pathways through a TLR4-dependent process *[Kennedy A et al., 2009; Nguyen MT. et al., 2007; Suganami T. et al., 2007]* Indeed, it seems that SFAs are one

of the relevant pathophysiologic triggers to activate macrophages in vivo *[Saberi M. et al., 2009; Kennedy A. et al., 2009; Nguyen MT. et al., 2007; Suganami T. et al., 2007; Shi H et al., 2006]*. Although SFAs stimulate macrophage inflammatory pathways, PUFAs do not. The n-3 PUFAs (DHA and EPA) are actually anti-inflammatory, and they can inhibit the stimulatory effects of SFAs or LPS *[Browning LM. et al., 2003; Calder PC. et al., 2005; Oh DY. et al., 2010*; *Simopoulos AP. et al., 2002; Zhao G. et al., 2005]*.

Recently, studies have focused on GPR fatty acid sensors/receptors, *[Hirasawa A. et al., 2005; Itoh Y. et al., 2003]*, in particular on GPR120 *[Hirasawa A. et al., 2005]* because of its unique pattern of tissue expression, as described in a previous section. Indeed, GPR120 is highly expressed in CD11c-positive macrophages, but not in CD11c-negative cells, and is induced by HFD/obesity *[Oh DY. et al., 2010]*. The results showed that n-3 PUFAs inhibit LPS, TLR2 and TNF-α-mediated inflammatory responses in macrophages and that the effects of n-3 PUFAs are suppressed by GPR120 knockdown. These are important results, demonstrating that GPR120 functions as the macrophage n-3 PUFAs receptor/sensor. Although n-3 PUFAs can be processed in some cells to bioactive lipids (lipoxins, resolvins, protectins and maresins), which might participate in the longer term processes, which resolve inflammation- *[Ariel A. et al., 2007; Schwab JM. et al., 2007],* these agents do not stimulate GPR120. Furthermore, when wild type (WT) and knockout (KO) animals were placed on HFD with or without n3-FA supplementation, the data showed that n-3 PUFAs had marked anti-inflammatory and insulin-sensitizing effects in the WT animals but there was not the same action in the GPR120 KOs. Taken together, these studies demonstrated that GPR120 is the relevant receptor, both in vitro and in vivo, for the anti-inflammatory, insulin-sensitizing effects of n-3 PUFAs *[Oh DY. et al., 2010]*.

Adipocytes display immune cell functions and inflammatory responses in a way that is independent of macrophages which participate directly in the inflammatory response in AT, and they also stimulate the production of inflammation-related factors in fat cells *[O'Hara et al 2009; Alomar S. et al., 2015]*.

Based on the fact that ATMs have a crucial role in inflammatory state macrophage-conditioned medium (MCM) was used to investigate the effects of macrophages on total gene expression specifically in human adipocytes *[O'Hara et al, 2009]*. The MC medium was shown to lead to significant up or down-regulation of over 5,000 transcripts in human adipocytes at 4 or 24 h periods studied. Indeed, the microarrays analyses demonstrated an up-regulation of a series of inflammation-related genes in human fat cells by macrophage-conditioned medium; particularly, members of the matrix metalloproteinase (MMP) gene family, especially MMP1, MMP3 and MMP10, were found to be powerfully up-regulated *[O'Hara et al, 2009].* Pathway analysis display that MC medium affects adipocyte genes linked to inflammation, also showing that NF-κB is central to the macrophage-stimulated inflammation pathway, in accordance with previous results *[Permana PA et al. 2006]*.

There are some connections between the pathways affected by MCM in preadipocytes and in adipocytes, including macrophage infiltration, glucose uptake, and lipid accumulation and formation. The pathway most affected in preadipocytes, as with adipocytes, was that linked to inflammation, and this is consistent with a role for preadipocytes in the inflammatory response in AT *[O'Hara et al, 2012].* Involvement of preadipocytes in inflammation has been observed previously, including mediation of lipopolysaccharide-induced inflammation in adipocytes *[Chung et al., 2006]*, and increased expression of several inflammatory-related genes following treatment of preadipocytes with MC medium *[Lacasa et al., 2007; O'Hara et al,*

2012]. In a gene expression study, the levels of IL-6, IL-1β, IL-8 and MCP-1 mRNA each showed an increase in the presence of activated MCM *[Lacasa et al., 2007]*, similar to the increase in expression of these genes found with microarrays. IL-1β and TNFα is known to be the main inflammatory cytokines expressed and secreted by macrophages *[Eder, 2009]*. Studies using IL-1β neutralising antibody showed that IL-1β, a major pro-inflammatory cytokine in preadipocytes, plays a central role in the up-regulation of the production of IL-6 *[O' Hara A. et al., 2011]* The different response between MCM and IL-1β suggests that factors released from macrophages may inhibit, as well as stimulate, the expression of some inflammation-related genes, and in particular neutralize the stimulatory effect of IL-1β.

Analysis of microarray datasets on the role of MCM in inflammation showed an increase in GPR84 expression at both 4h and 24 of human adipocytes incubated with MCM. Indeed, the mRNA level being increased 13.5 fold at 24h compared to the adipocytes incubated with unconditioned medium *[Trayhurn P. et al., 2012]*. This finding is consistent with the fact that GPR84 gene expression is stimulated by TNFα and macrophage secreted factors in 3T3-L1 *[Nagasaki H. et al., 2012]*.

In contrast, GPR120 gene expression in human adipocytes was markedly decreased after exposure to MCM by reducing its expression by as much as 15-fold after 24h. Therefore, macrophage-secreted factors induce a down-regulation expression of the expression of the gene encoding the receptor/sensor for n-3 PUFAs *[Trayhurn P. et al., 2012]*.

Interestingly, macrophage secretions up-regulated GPR84 and while simultaneously down-regulating GPR120 expression in human adipocytes. These effects might lead to an amplification of the fat cell inflammatory response *[Trayhurn P. et al., 2012]*.

1.4.5 n-3 PUFA: anti-inflammatory effect by GPR120 activation

A role for GPR120 in the development of obesity was implicated by identification of a link between absent or dysfunctional GPR120 and increased risk of obesity in both humans and mice *[Ichimura A. et al., 2010]*. GPR120 is highly expressed in macrophages which drive chronic low-grade inflammation in obesity and mediate anti-inflammatory effects of EPA and DHA. Indeed, repression of macrophage-induced inflammation was found to ameliorate insulin sensitization in AT *[Oh D.Y.et al., 2010]*.

In humans, GPR120 dysfunction leads to obesity, resulting in fatty liver and glucose intolerance associated with decreased adipocyte differentiation and lipogenesis and enhanced hepatic lipogenesis *[Ichimura A. et al., 2012]*. GPR120 activation via LCFA was significantly associated with obesity when there was a mutation from R (arginine) to H (histidine) at 270 in the amino acid sequence of protein *[Miyamoto J. et al., 2016]*. These results provide indications on the contribution of n-3 PUFAs which are often proposed as dietary supplements. The n-3 group of PUFAs, present in substantial amounts in oily fish *[Calder PC, 2013]*, could be useful for the activation of GPR120 with potential health benefits *[Oh DY et al., 2010; Saltiel AR, 2010]*.

GPR120 is expressed in the hypothalamus and appears to mediate anti-inflammatory effects to reduce appetite and body weight. Hypothalamic inflammation is associated with improper control of calorie intake and precedes obesity *[Cintra DE. et al., 2012]*. This is supported by GPR120-mediated anti-inflammatory effects observed in immortalized hypothalamic neurons *[Wellhauser L. et al., 2014]*.

GPR120 may affect appetite and food intake by regulating the secretion of hormones, such as GLP-1 *[Hirasawa A. et al., 2005]*, glucagon *[Suckow AT. et al., 2014]*, CCK *[Tanaka T. et al., 2008]*, and ghrelin *[Engelstoft MS. et al., 2013; Gong Z. et al., 2014; Lu X et al., 2012]*, and by mediating anti-inflammatory effects in the hypothalamus *[Cintra DE. et al., 2012]*. Co-regulation of several hormones may well produce synergistic effects *[Sadry SA. et al., 2013]*.

Furthermore, stimulation of FFA induced GLP-1 and CCK secretion in mouse enteroendocrine STC-1 cells, as a murine enteroendocrine cell line, *[Hara T. et al., 2009; Sidhu S.S. et al., 2000]*, while Gpr120 knockdown abolished FFA-induced effects on incretin secretion and [Ca2+]i levels [*Tanaka T. et al., 2008*]. The effect of FFAs on the plasma levels of GLP-1 and insulin were examined by the administration of FFAs into the mouse colon *[Hirasawa A. et al., 2005]*. The lipid accumulation during adipogenesis in 3T3-L1 cells, as a murine preadipocyte cell line, induced GPR120 expression *[Gotoh, C. et al., 2007]*, and Gpr120 knockdown in 3T3-L1 cells and the embryonic fibroblasts in GPR120 deficient mice inhibited adipogenic gene expressions and prevented lipid accumulation. These findings indicate that GPR120 plays a role in the differentiation and maturation of adipocytes *[Miyamoto J. et al., 2016].*

GPR120 may have a specific capacity to mediate the action of LCFAs promoting the secretion of the incretin GLP-1 from enteroendocrine cells of the gut *[Hirasawa A. et al., 2005]* and indications that the activation of GPR120 produces extensive anti-inflammatory and resulting insulin-sensitising effects *[Oh DY et al., 2010]*. Specifically, the activation of GPR120 has been reported to suppress the release of pro-inflammatory cytokines from macrophages, which, given the importance of inflammation in obesity-related IR, has led to the assumption that the activation of GPR120 may therapeutically improve IR *[Hirasawa A. et al., 2005]*. Recent publications have provided further physiological and potential genetic support to favour

agonists of GPR120 as a potential therapy in diabetes. In the studies carried out by Taneera et al. *[Taneera J. et al. 2012]*, the GPR120 receptor gene was identified as a gene strongly associated with diabetes *[Milligan G. et al., 2013]*.

N-3 PUFA treatment inhibited inflammation and improved systemic insulin sensitivity in wild type mice, but was without effect in GPR120 gene deficient mice *[Oh et al. 2010]*. Another study on the anti-inflammatory effects of DHA via the GPR120 signalling pathway, using the same cell types and stimulus, shown that DHA in a concentration-and time-dependent manner reduced COX-2 expression and decreased PGE2 synthesis induced by LPS, and also that GPR120 knockdown annulled the effects of DHA on COX-2 induction, PGE2 production, and IL-6 gene expression *[Lietal. X., 2013].*

GPR120 activation by DHA inhibits both the Toll-like receptors (TLR) and TNFα pro-inflammatory cascades. TLR2/3/4 GPR120 activation by DHA had anti-inflammatory effects in macrophages *[Oh D.Y.et al., 2010]* related with the suppression of TLR signalling via the β-arrestin-2 and the inhibition of TNFα-activated kinase 1, which is involved in pro-inflammatory TNFα signalling.

In particular, in macrophages GPR120 exists in a short isoform. DHA stimulation of GPR120 on macrophages results in the recruitment of β-arrestin2 to the plasma membrane where it co-localizes with GPR120. Then the complex is internalized and is localized in the cytoplasmic compartment where β-arrestin2 associated with TAB1. This interaction blocks the link between TAB1 and TAK1 inhibiting TAK1 activation and the pro-inflammatory downstream signaling: IKKβ/NFκB and JNK/AP1 system (Scheme 3) *[Oh D.Y.et al., 2010; Tanaka T. et al., 2008]*.

GPR120 is present in its long isoform in adipocytes. Stimulation of GPR120 by DHA or EPA was found to sensitize insulin action and enhance glucose uptake in 3T3-L1 cells by stimulating

GLUT4 translocation, an effect that was blocked by Gq/11 knockdown but unaffected by β-arrestin knockdown (Scheme 3) *[Oh D.Y.et al., 2010].*

This important role in the control of inflammation promotes the possibility that GPR120 could have therapeutic potential in inflammatory diseases including obesity and T2D *[Oh DY et al., 2014].*

Furthermore, this study demonstrated that DHA via GPR120 coupling with β-arrestin-2 repressed TLR4 signalling via Akt/JNK phosphorylation and the nuclear translocation of p65, which supports the involvement of GPR120 in the anti-inflammatory action of DHA on LPS-induced COX-2 induction in macrophage, and also established the action of DHA was independent of PPARs *[Li X., et al., 2013].*

Other studies observed that ALA was less efficient than DHA in RAW273.6 cells *[Hudson et al. 2013]*, which suggest extra pharmacological targets for DHA, such as PPARγ via oxo-derivatives of DHA *[Groeger et al., 2010; Li H. et al., 2005]*.

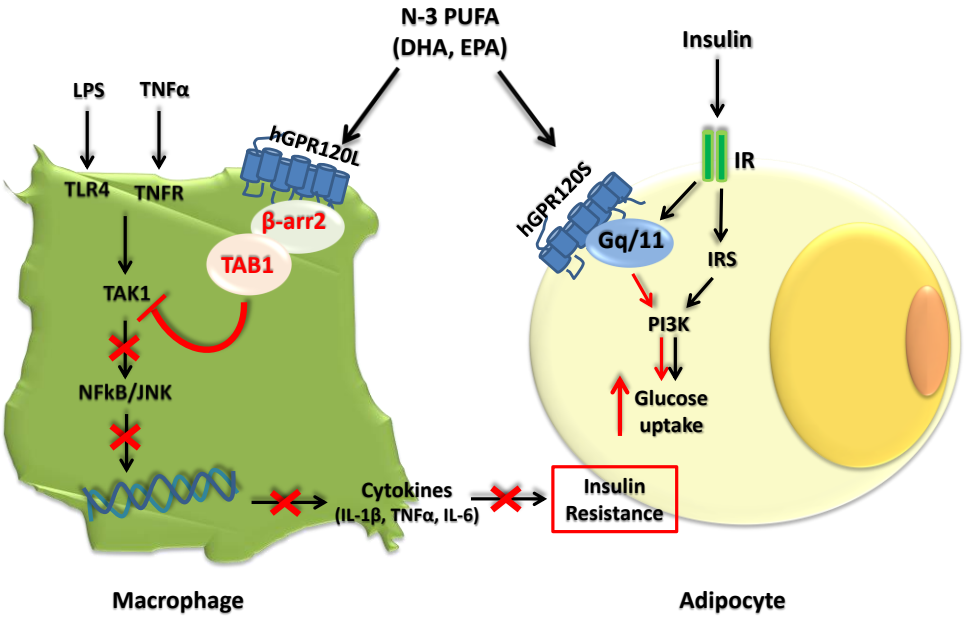
Additionally, a new target of n-3 PUFA (DHA and ALA) was recently identified and the saturated fatty acid, PA, activated ERK phosphorylation in RAW264.7 macrophages, but only DHA and ALA-mediated ERK phosphorylation were abolished by GPR120 knock down *[Oh et al., 2010]*.

Hudson et al. (2013) established that the response is less sensitive with endogenous GPR120, as compared with ectopic over expression systems, using the GPR120 selective agonist, TUG-891 and ALA. The fact that TUG-891 does not produce as large an effect as DHA in the regulation of inflammatory mediator release perhaps indicates that the high concentration of DHA required for some of its anti- actions might be produced through mechanisms other than GPR120 activation, involving COX-2 or PPAR-γ *[Li et al., 2005; Groeger et al., 2010].*

As mentioned above, activation of GPR120 by n-3 PUFA induced M2 polarization and inhibited macrophage migration into AT which resulted in an anti-inflammatory state *[Oh et al., 2010]*.

In man was found that GPR120 exon sequencing in obese subjects shows a deleterious non-synonymous mutation that inhibits GPR120 signaling activity and this mutation is associated with the risk of obesity in European populations *[Ichimura et al. 2012]*. Studies show that in human adipocytes the GPR120 stimulation induces adipocyte differentiation *[Gotoh et al., 2007]*, increases glucose uptake and GLUT4 translocation to the plasma membrane reducing plasma glucose level [*Oh et al., 2010*].

Extensive experimental evidence in rodent studies show that dietary n-3 PUFA can prevent HFD-induced obesity, IR, dyslipidemia and WAT inflammation *[Flachs P. et al., 2011; Storlien L.H. et al., 1987; Ruzickova J. et al., 2004; Rossmeisl M. et al. 2009]*. They can also reverse some pathologies associated with obesity *[Kuda O. et al., 2009; Jelenik T. et al., 2010; Todoric J. et al., 2006]*. The combinations of n-3 PUFA with various anti-diabetic drugs or mild calorie restriction might potentially provide a treatment strategy for diabetic patients *[Flachs P. et al., 2011; Kuda O. et al., 2009].* The effects of n-3 PUFAs on obesity reside in their potent anti-inflammatory action as well as in the modulation of eicosanoid production in various tissues including WAT *[Flachs P. et al., 2009; Bannenberge et al., 2011; Calder P.C. et al., 2011]*. In general, their beneficial effects, especially in the obese and inflammatory state, are associated with a modulation of WAT metabolism and function *[Flachs P. et al., 2009]*. Therefore, dietary intake of n-3 PUFA stimulates the expression and secretion of adiponectin in WAT of HFD-fed mice *[Flachs P. et al., 2006; Neschen S. et al., 2006]* as well as in humans *[Wu J.H. et al., 2013]* inducing mitochondrial biogenesis and FA oxidation in adipocytes *[Flachs P. et al., 2005]*.



Scheme 3. Anti-inflammatory pathways in macrophages and adipocytes via GPR120 activation by n-3 PUFAs

2. Aims of the study

The overall aim of these studies was to evaluate the effect of free fatty acids on inflammatory markers and the involvement of GPR84 and 120 in modulating the inflammatory response in obesity as assessed in human adipocytes.

2.1. In vitro study

The specific aims were to establish:

- The key factors which influence the expression of the GPR fatty acid sensors in human white adipocytes in culture, and particularly GPR120 and GPR84. The effect of TNFα and IL-1β, rosiglitazone, dexamethasone, insulin, and FFA, such as DHA, ARA and LA, has been examined.
- The role of GPR120 signalling in modulating downstream inflammatory response in adipocytes. This was explored by examining the response to the GPR120 natural agonist, DHA, on the expression and secretion of inflammatory cytokines and chemokines, and in particular whether there is the expected anti-inflammatory response.

2.2 Clinical study

The aim of the clinical study was to investigate whether an intake of 2 g/d of either Krill Oil (KO) or Fish Oil (FO) for four weeks, is able to modify the plasma concentration of different FFAs, such as EPA, DHA, ARA, POA and TNFα plasma level in normal weight (NW), overweight (OW) and obese (OB) subjects.

3. Material and Methods

3.1 Cell culture

Human white preadipocytes were obtained from Promocell (Germany) together with the appropriate cell culture media. They were isolated from the SAT of a female Caucasian donor, aged 29 years, with a BMI of 27. On receipt, the cells were plated (5000 cells/cm^2) in 12-well plates and incubated in a proprietary growth medium containing 5% foetal calf serum, endothelial cell growth (0.004 ml/ml), hydrocortisone (1 µg/ml), heparin (90 µg/ml) and epidermal growth factor (10 ng/ml).

When the cells had reached confluence (6–7 days), growth media was removed and the cells were incubated in pre-adipocyte differentiation medium for 72 h to induce differentiation. The preadipocyte differentiation medium contained recombinant human insulin (0.5 µg /ml), dexamethasone (400 ng/ml), IBMX (44 mg/ml) thyroxine (9 ng/ml) and ciglitazone (3 µg /ml). After 72 h the cells were switched to Adipocyte Nutrition Medium, which contains 3% foetal calf serum, insulin (0.5 mg/ml) and dexamethasone (400 ng/ml). The medium was changed every 2 or 3 days and the cells were used at 14 days after the induction of differentiation, by which time they contained multiple lipid droplets (Image 1).

All reagents were from Sigma-Aldrich (Sigma, Poole, UK) unless otherwise specified. The adipocytes were incubated with the following agents at 'low' and 'high' dose levels: human recombinant TNFα (5 ng/ml and 100 ng/ml), rosiglitazone (100 nM and 1 µM-; Sequoia Research.Product.UK), dexamethasone (2 nM and 20 nM), Insulin (1 nM and 20 nM), DHA (25

µM and 100 µM), ARA (25 µM and 100 µM), LA (25 µM and 100 µM) for either 4 or 24 h (Alomar et al., 2015) as shown in scheme 4. In the studies investigating the effects of insulin and dexamethasone, these hormones were removed from the culture medium 24 h before the start of the experiment (and were absent from the control cells).

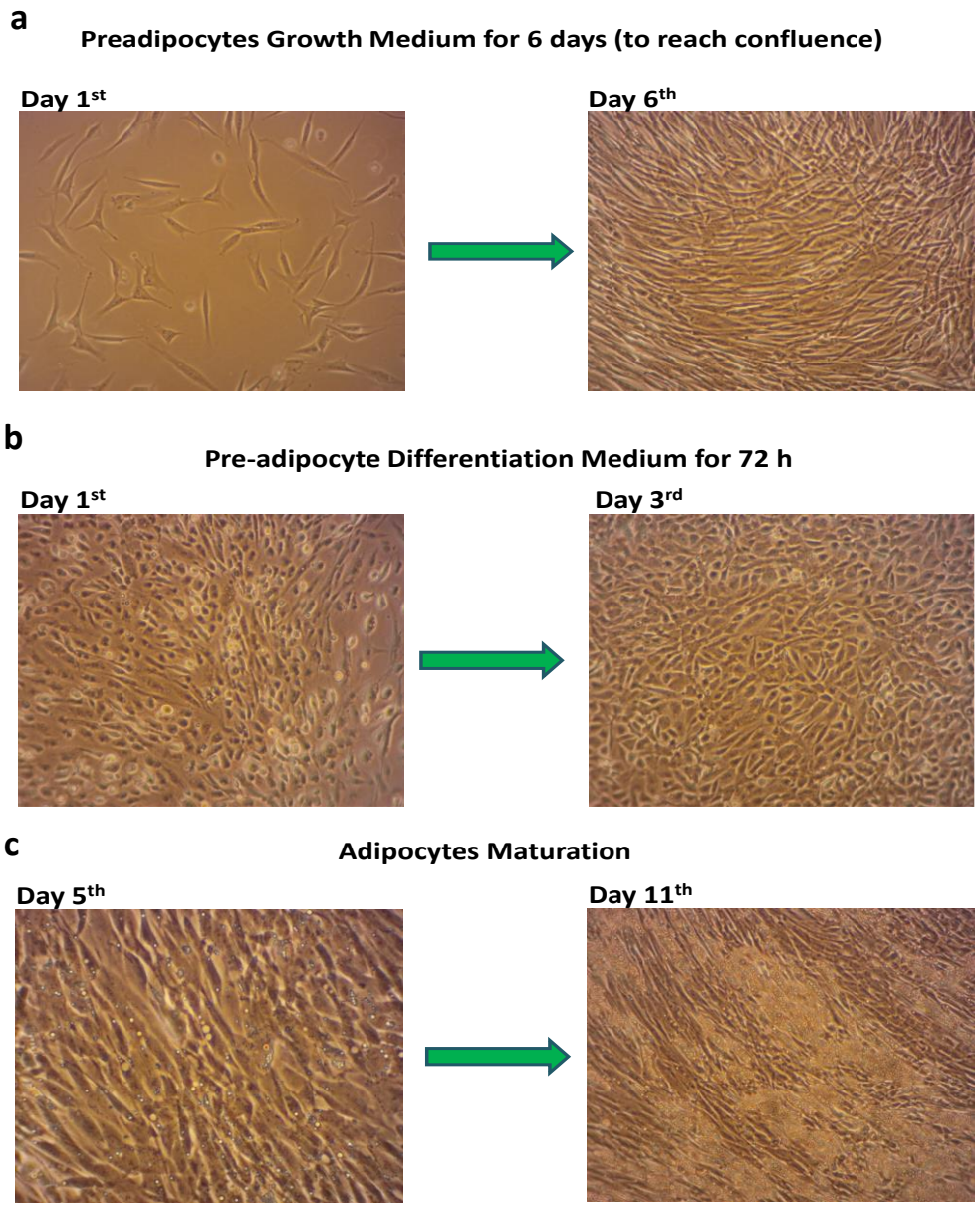
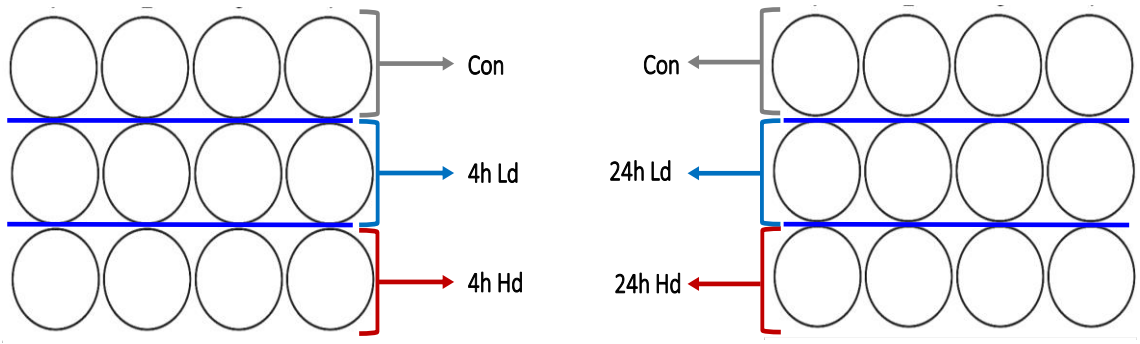
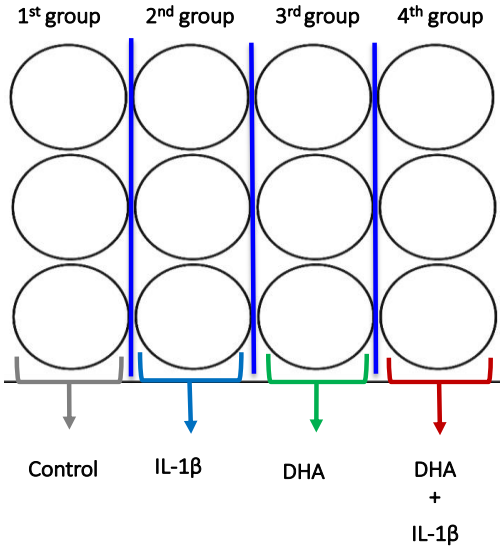


Image 1. Photos of Human preadipocytes taken during different steps: growth (a), differentiation (b) and maturation (c). On day 12th human mature adipocytes were treated with several compounds listed above.



Scheme 4. Strategy of human adipocytes treatments listed above. All agents were added at two doses: low dose (LD) and high dose (HD). Four sets of cells were used for each of the experimental groups. Control adipocytes received vehicle.

Two set of human adipocytes were treated as the scheme 5.



Scheme 5. First and second groups were incubated with supplemented-adipocyte nutrition media for 30 minutes; third and fourth groups were preincubated with supplemented-adipocyte nutrition media plus DHA (100 µM) for 30 minutes. Only second and fourth groups were added with IL-1β (0.5 ng/ml) after the incubation time for 4 h and also after the incubation time for 24 h in DHA+IL-1β treated cells Control adipocytes received vehicle. Sixth sets of cells were used for each of the experimental groups.

At the end of the incubation period the medium was removed, frozen and stored at -20°-C until analysis. The adipocytes were immediately washed with ice-cold PBS, frozen in TRI-Reagent (Sigma, Poole, UK) and stored at -80°-C. The medium was stored at -20°-C until

required for analysis at which point it was thawed and kept on ice during the plating of samples; the medium was not centrifuged prior to analysis so the presence of vesicular structures containing soluble and membrane-associated cytokines cannot be excluded.

3.1.1 RNA extraction

Adipocytes were thawed and homogenised in the TRI-Reagent and total RNA was extracted using RNeasy Micro Kits (QIAGEN, UK). The quantity and purity of the RNA were measured with a UV-Vis spectrophotometer (NanoDrop 1000, Wilmington, USA). The average absorbance ratios (260/280 nm and 260/230 nm) were close to 2.0 for all samples. The integrity of the isolated RNA was analysed with an Agilent 2100 Bioanalyser (Agilent Technologies, Germany) and the Integrity Number for RNA samples was approximately 10.

3.1.2 Real-time PCR analysis

Total RNA was DNAse-treated with a TURBO-DNA-free kit (Ambion, Life Technologies, USA) and 0.8 µg/40ul was reverse transcribed using Taqman reverse transcription reagents (Invitrogen, Applied Biosystems, UK).

Real-time PCR was performed between 60 and 80 ng of cDNA, in duplicates or triplicates, using Gene Expression Master Mix and predesigned TaqMan Gene Assays consisting of specific Taqman human probes (Applied Biosystems, Life Technologies, USA):

GPR120 and GPR84 probes were set up for all treatments; *IL6*, *ADIPOQ*, *MCP1* and *IL1B*

probes for the treatments described in scheme 5; and ACTB (β-actin) is used as the reference gene. PCR reactions were set up in duplex format where the FAM-labelled Taqman probe for the gene of interest was mixed with the VIC-labelled Taqman probe for the control gene (ACTB). PCR amplification was performed using a real-time PCR detection system (ABI StepOneplus, Applied Biosystems, Life Technologies, USA) with a two-step thermal cycling: 95°-C for 10 min, followed by 40 cycles of 95°-C for 15 s and 60°-C for 1 min. All procedures were carried out in accordance with the manufacturer's recommendation. Basic relative quantification of expression was determined using the comparative $2^{-\Delta\Delta Ct}$ method and expressed as fold-changes in the target gene (normalized to ACTB as the reference gene) in treated adipocytes and related to the expression of the control adipocytes (normalised to the mRNA level in the control cells = 1.0).

3.1.3 Protein arrays

MSD assay kits (MesoScale Discovery, Rockville, USA) were used to measure the release of cytokines and chemokines into the cell culture medium (Alomar et al., 2015) and in human plasma. These sandwich ELISAs enable protein targets to be measured rapidly in small sample volumes. The following 4 and one-multi-spot plates were employed: human MMP 3-Plex Ultra-Sensitive Kit, V-PLEX Cytokine Panel 1 Human Kit and V-PLEX Human TNF-α Kit. The plates were pre-coated with capture antibodies on independent, well-defined spots and the assay was performed according to the manufacturer's instructions. Proprietary Meso Scale software was used to analyse the data. The lowest limit of detection in the arrays was 0.01 pg/ml.

3.2 Human study

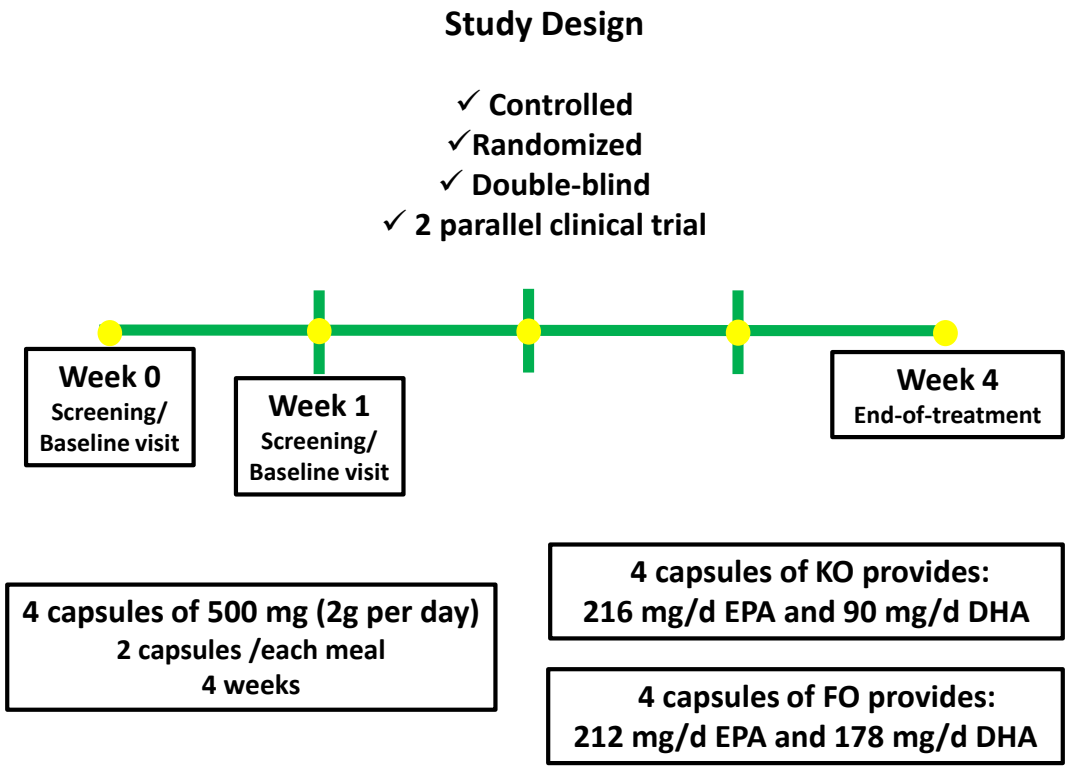
Generally healthy male and female subjects (n=63), 35 to 64 years of age, with waist circumference of 102 cm or greater (men) or 88 cm or greater (women) were included in the study. Stratification of the subjects has been carried out by BMI values: normoweight BMI <25; overweight 25 < BMI <30; obese 30 < BMI <35 (**Table 1**).

	OO (n = 19)	FO (n = 23)	KO (n = 21)
Male, n	3	4	3
Female, n	16	19	18
Normoweight, n, male/female	1/3	1/3	1/6
Overweight, n, male/female	1/6	2/5	1/4
Obese, n, male/female	1/7	1/11	1/8
Age, year, mean ± SEM	47.4 ± 8.5	49.6 ± 8.7	49.4 ± 8.5
Body mass index, kg/m2, mean ± SEM	30.6 ± 1.3	31.6 ± 0.9	30.1 ± 1.0

Table 1. Baseline demographic and anthropometric characteristics of subjects by treatment group. Stratification of the subjects has been carried out by BMI values: normoweight BMI <25; overweight 25 < BMI <30; obese 30 < BMI <35. There were no significant differences between groups for any variable.

3.2.1 Study procedures

Qualified subjects were assigned to 1 of 3 groups: 2 g/d of either KO (Superba krill oil, Aker BioMarine ASA, Oslo, Norway), FO (Omega-Pure, Houston, Texas, USA), or Olive Oil (control). Subjects were instructed to consume four 500 mg capsules per day, preferably 2 capsules with each of 2 meals, for 4 weeks. This study was a randomized, double-blind, controlled trial. The trial included 3 visits: 2 screening/baseline visits (weeks -1 and 0) and 1 end-of-treatment visit (week 4). Four capsules of the KO supplement provided 216 mg/d EPA and 90 mg/d DHA, and the FO supplement provided 212 mg/d EPA and 178 mg/d DHA (Scheme 6).



Scheme 6. Study design of clinic study.

3.2.2 Extraction of total lipids.

All solvents used were HPLC grade (Carlo Erba, Milano, Italy) and were purchased from Sigma Chemicals Co., St. Louis, USA: Total lipids were extracted from human plasma using the Folch method. The method consists of a lipid extraction from a tissue homogenate in a chloroform-methanol 2:1 solution with 2 µg of Vitamin-E. Samples are kept in the dark for 1 hour after which water was added (equal to the volume of methanol present). Samples are left another hour in the dark and then were centrifuged for 1 h at 900 x *g*, to permit the separation of the chloroform phase from the aqueous-methanol layer.

The lower chloroform phase containing lipids was collected and evaporated under vacuum in a rotary evaporator at room temperature.

3.2.3 Fatty acid analysis of plasma.

Aliquots of lipid extracts from plasma were treated with and addition of 10 ml of n-hexane and 7 ml of H_2O, samples were acidified with 0.35 ml of 37% HCI- to a pH 3-4, and were then centrifuged for 1 hour at 900 x *g*. The hexane phase containing free fatty acids was collected, the solvent evaporated, and the residue was dissolved in 0.5 ml of CH_3CN/0.14% of CH_3COOH (v/v). Separation of fatty acids was carried out with an Agilent 1100 HPLC system (Agilent, Palo Alto, Calif., USA) equipped with a diode array detector.

3.3 Statistical analysis

Data from all experiments were analysed with GraphPad Prism software version 5.00 for Windows (GraphPad, San Diego, CA, USA). Differences between more than two groups were determined by one-way ANOVA with a Bonferroni post-test for selected columns of Bonferroni selected pairs. Differences between two groups were compared with an unpaired two-tailed Student 't'test. Significance was set at $p<0.05$. The results are depicted as the means ± SEM.

4. Results

A previous study of microarray datasets on the effect of macrophage secretions on GPR120 and GPR84 gene expression in human adipocytes showed that exposure of the adipocytes to macrophage-conditioned medium (MCM) for 4 h and 24 h increases GPR84 expression at both 4 and 24 h of incubation, the mRNA level being increased 13·5-fold at 24 h relative to the adipocytes incubated with unconditioned medium [*Trayhurn P. et Denyer G., 2012*]. In contrast, there was a marked reduction in GPR120 expression in human adipocytes exposed to MCM, the mRNA level being reduced by as much as 15-fold after 24 h. In a following study human adipocytes were treated and incubated with IL-1β (2 ng/ml) at 4h and 24h. The data showed a very major increase in GPR84 mRNA level while the mRNA level of GPR120 decreased at both 4h and 24h of incubation relative to the untreated adipocytes (**Figure 1**).

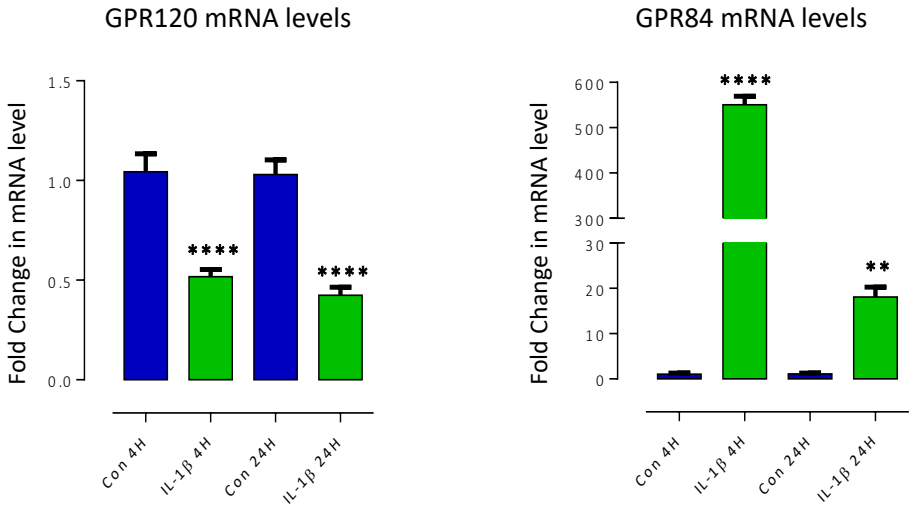


Figure 1. Effect of IL-1β (2 ng/ml) on GPR120 and GPR84 gene expression in Human Adipocytes at 4h and 24h of incubation. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$).

From these findings, human adipocytes were next incubated with TNFα low dose (LD) (5 ng/ml) and high dose (HD) (100 ng/ml) and at two time points (4 h and 24 h). The data observed showed a marked decrease of GPR120 gene expression and this more evident at 24 h and at the high concentration of TNFα (**Figure 2a**).

In contrast, GPR84 mRNA level was substantially increased with both doses at 4 h, there being a >200-fold increase with the higher dose. There was also an increase in GPR84 mRNA level after 24 h of incubation, but a ttest analysis revealed that this was statistically significant only when adipocytes were treated with the LD of TNFα (**Figure 2b**); the absence of a significant increase with the HD reflects the considerable variation in this group.

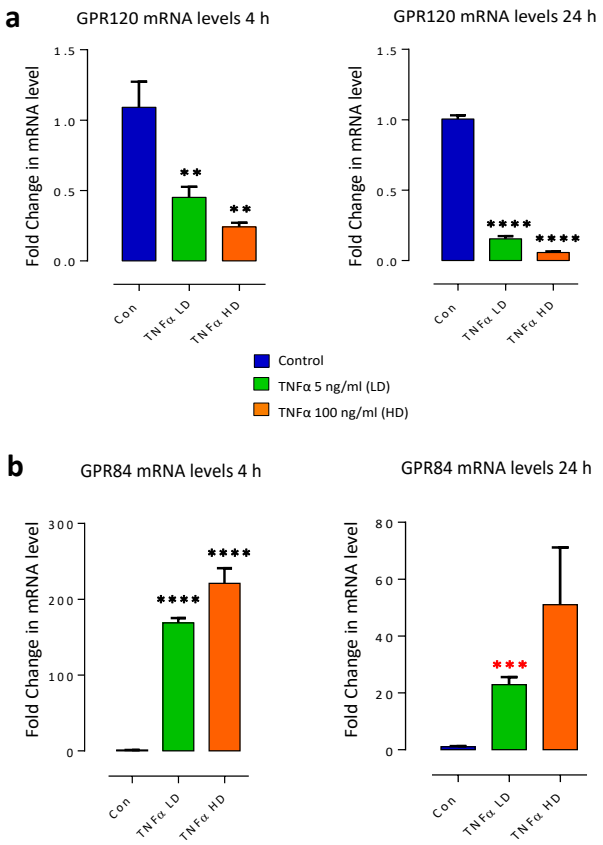


Figure 2. Effect of TNFα, LD (5 ng/ml) or HD (100 ng/ml), on GPR120 **(a)** and GPR84 **(b)** gene expression in Human Adipocytes at 4 h and 24 h of incubation. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$; *** unpaired two-tailed t-Student test).

Data analysis of the protein concentration released into the culture medium of human adipocytes treated with IL-β show a markedly increase of ICAM-1, VCAM-1 and metalloproteases, MMP-1 and MMP-3, at both time points with a concentration of 2ng/ml (**Figure 3**).

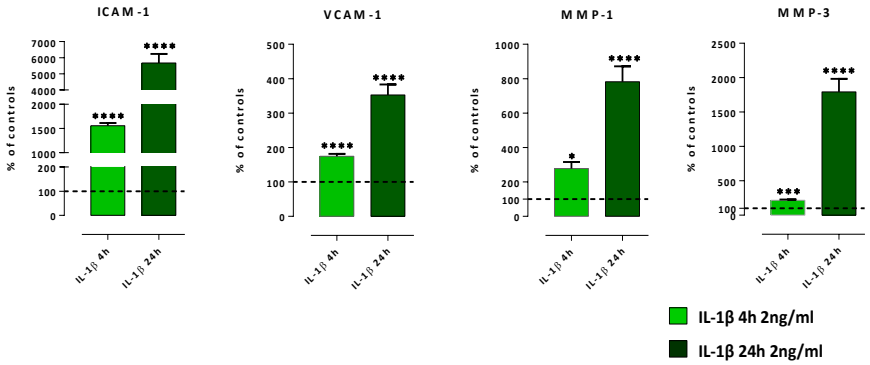


Figure 3. Protein concentrations of ICAM.1, VCAM-1, MMP-1 and MMP-3 released into the human adipocytes culture medium. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, p <0.05)

The same proteins measured on culture media of human adipocytes after 24 h treatment with 5 ng/ml or 100 ng/ml of TNF-α, showed a significant increase dose dependent of ICAM-1, VCAM-1, MMP-1 and MMP-3 concentrations with respect to untreated adipocytes (**Figure 4**).

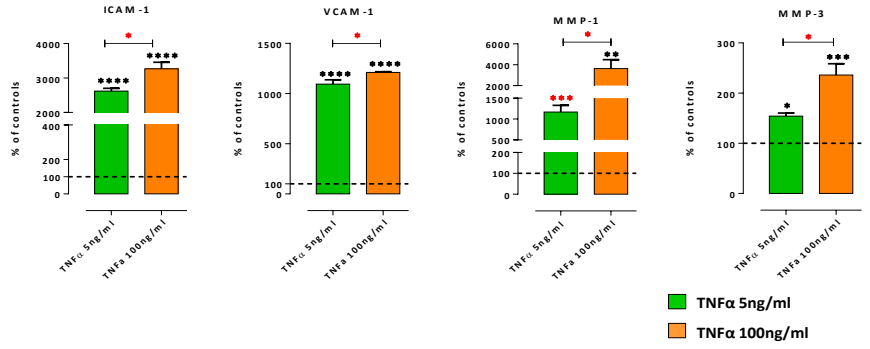


Figure 4. Protein concentrations of ICAM.1, VCAM-1, MMP-1 and MMP-3 released into the human adipocytes culture medium. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, p <0.05; *** and * unpaired two-tailed t-Student test).

In the next experiment, the effect of the PPARγ agonist, rosiglitazone, was examined. Rosiglitazone significantly increased the GPR120 mRNA level at both time points and concentrations (100 nM and 1 µM) relative to untreated adipocytes (**Figure 5a**). There was, however no statistically significant effect of Rosiglitazone on GPR84 gene expression in human adipocytes (**Figure 5b**).

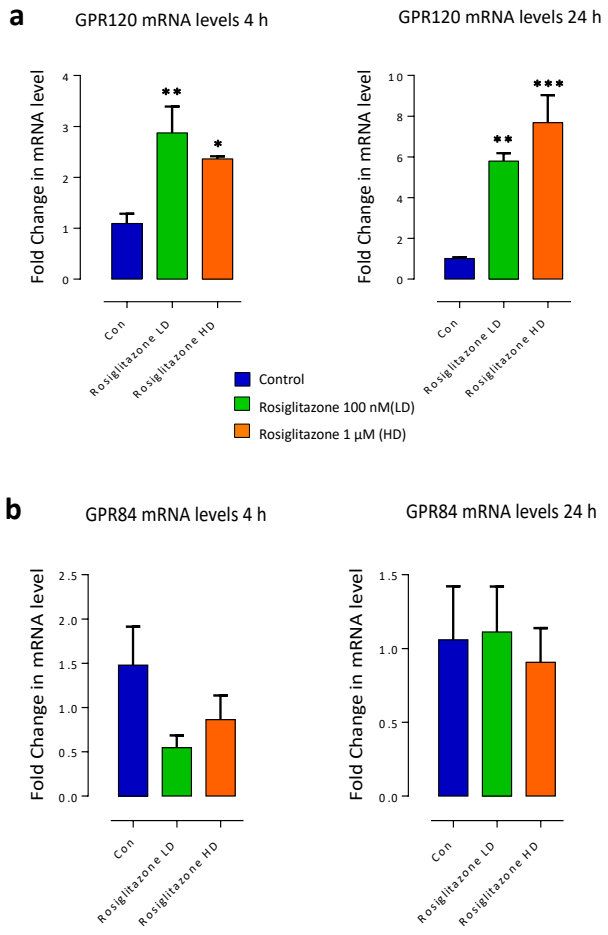


Figure 5. Effect of rosiglitazone, LD (100 nM) or HD (1 µM), on GPR120 **(a)** and GPR84 **(b)** gene expression in Human Adipocytes at 4 h and 24 h of incubation. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$).

Adipocytes were also incubated with dexamethasone with LD (2 nM) and HD (20 nM) at 4h and 24 h. The steroid drug did not affect GPR120 gene expression after either 4 h and 24 h of incubation with both doses with respect to untreated adipocytes (**Figure 6a**). GPR84 mRNA level decreased significantly with 20 nM dexamethasone at 24 h of incubation but not at 4 h (**Figure 6b**).

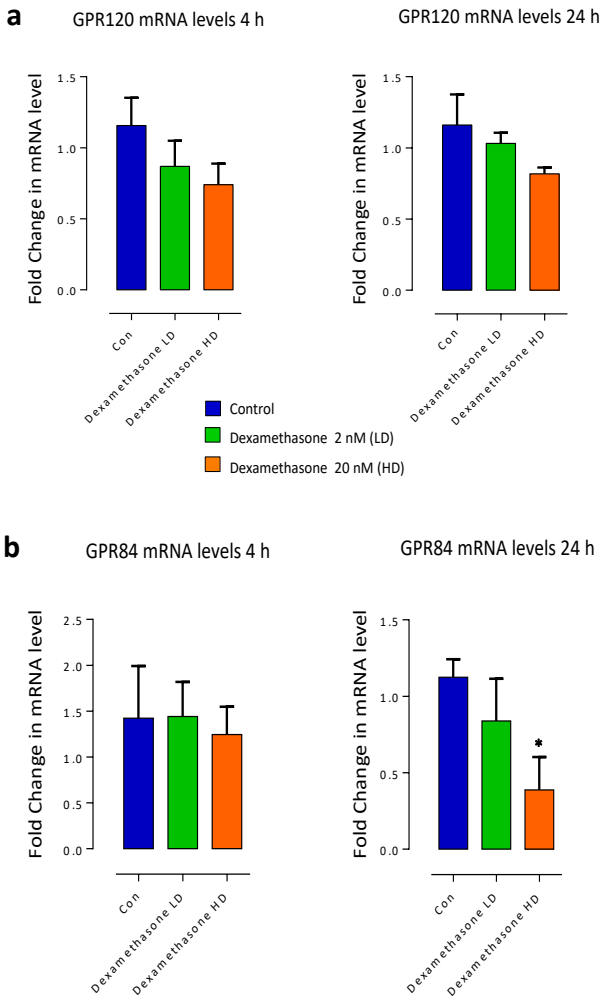


Figure 6. Effect of dexamethasone, LD (2 nM) and HD (20 nM), on GPR120 **(a)** and GPR84 **(b)** gene expression in Human Adipocytes at 4 h and 24 h of incubation. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$).

Insulin did not significantly affect the gene expression of GPR120 at 24 h (**Figure 7a**) and GPR84 with LD (1 nM) or HD (20 nM) at 4 h (**Figure 7b**) or 24 h of incubation (data not shown). The only significant effect was an increase in GPR120 mRNA level when human adipocytes were treated with the hormone with 20 nM at 4 h of incubation (**Figure 7a**).

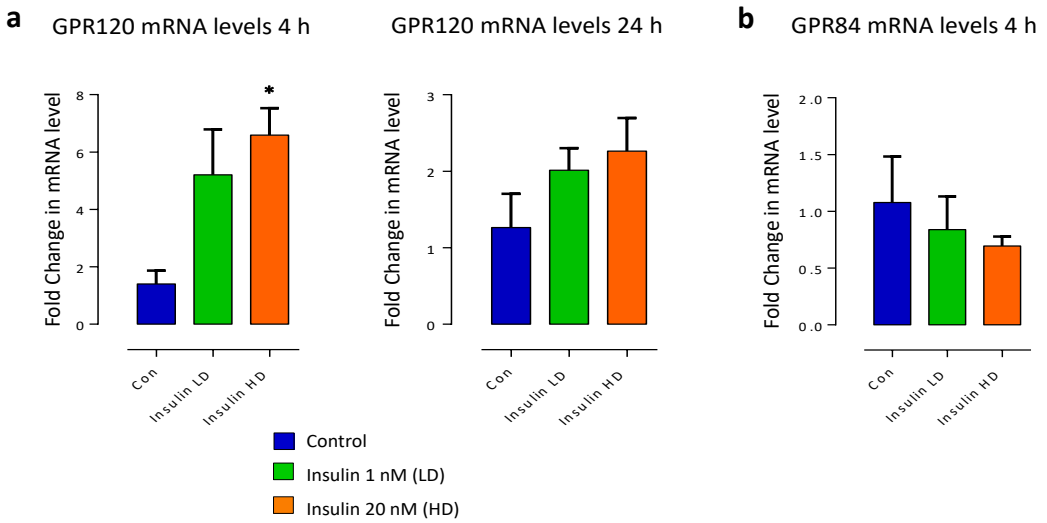


Figure 7. Effect of insulin, LD (1 nM) or HD (20 nM), on GPR120 **(a)** and GPR84 **(b)** gene expression in Human Adipocytes at 4 h and 24 h of incubation. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$).

In the next set of experiments, human adipocytes were incubated with the free fatty acids DHA or ARA or LA.

GPR120 gene expression was significantly increased at 24 h with the HD concentration, while no significant differences were detected when DHA was incubated for 4 h with both concentrations (Figure 8a).

There was no significant effect of DHA on GPR84 gene expression at both time points and concentrations (Figure 8b).

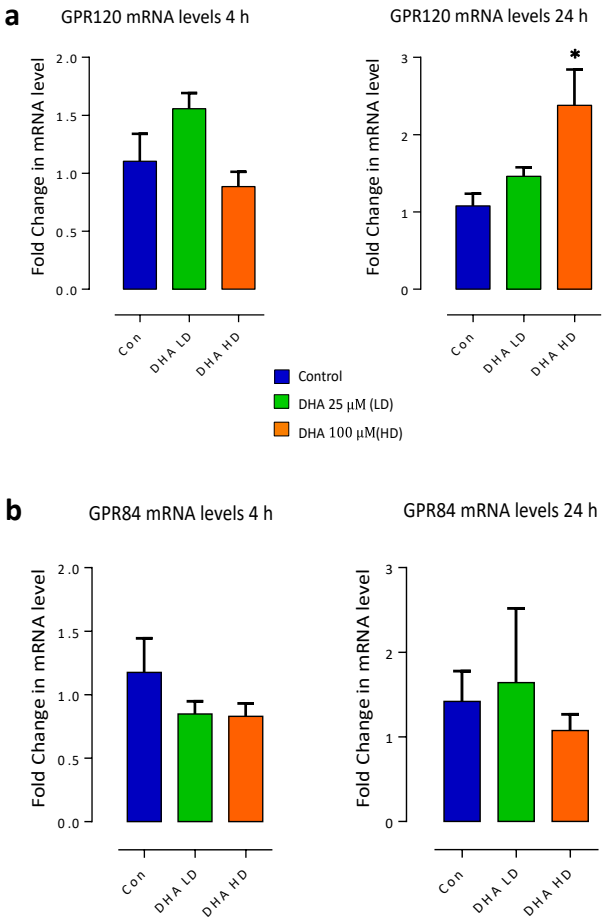


Figure 8. Effect of DHA, LD (25 µM) or HD (100 µM), on GPR120 **(a)** and GPR84 **(b)** gene expression in Human Adipocytes at 4 h and 24 h of incubation. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$).

In contrast, ARA markedly decreased GPR120 gene expression at 24 h in a dose-dependent manner, but not at 4 h of incubation with either 25 µM or 100 µM of the FA (**Figure 9a**). No effects of ARA on GPR84 mRNA levels were found (data not shown). Treatment with LA, a precursor of ARA, did not show any effect on GPR120 and GPR84 gene expression (data not shown).

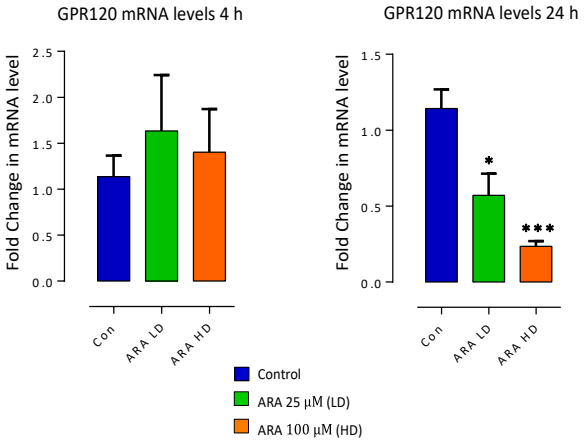


Figure 9. Effect of ARA, LD (25 µM) or HD (100 µM), on GPR120 gene expression in Human Adipocytes at 4 h and 24 h of incubation. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$).

MCP-1 protein concentration was increased on cultured media of human adipocytes treated with 100 µM of ARA but not with 100 µM of DHA at 24 h of incubation relative to untreated cells (**Figure 10**).

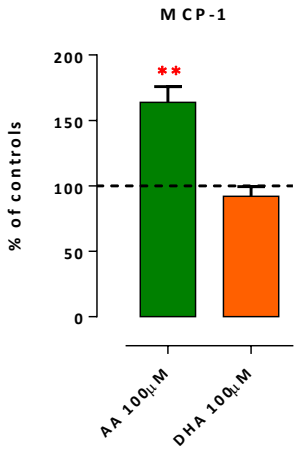


Figure 10. Protein concentration of MCP-1 released into the human adipocytes culture medium treated with 100µM of ARA and DHA at 24 h of incubation. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$).

Based on these findings in the subsequent experiments human adipocytes were incubated with DHA (100 µM) or IL-1β (0.5 ng/ml), or a combination of both at 4 h and 24 h. The aim was to evaluate whether incubation with DHA was able to prevent the changes in GPR120 and GPR84 gene expression, and in the expression of key inflammation-related genes, induced by IL-β challenge.

IL-1β alone decreased significantly GPR120 gene expression only at 24 h relative to untreated cells (**Figure 11a**), while markedly increased GPR84 gene expression 250-fold and 25-fold at 4 h and 24 h respectively; (**Figure 11b**) as in the previous studies. DHA alone did not influence

GPR120 or GPR84 gene expression (**Figure11 a-b**). DHA pre-incubation didn't prevent the decrease of GPR120 mRNA level triggered by IL-1β treatment; in contrast, DHA was able to prevent the increase of GPR84 gene expression by IL-b (**Figure 11 a-b**).

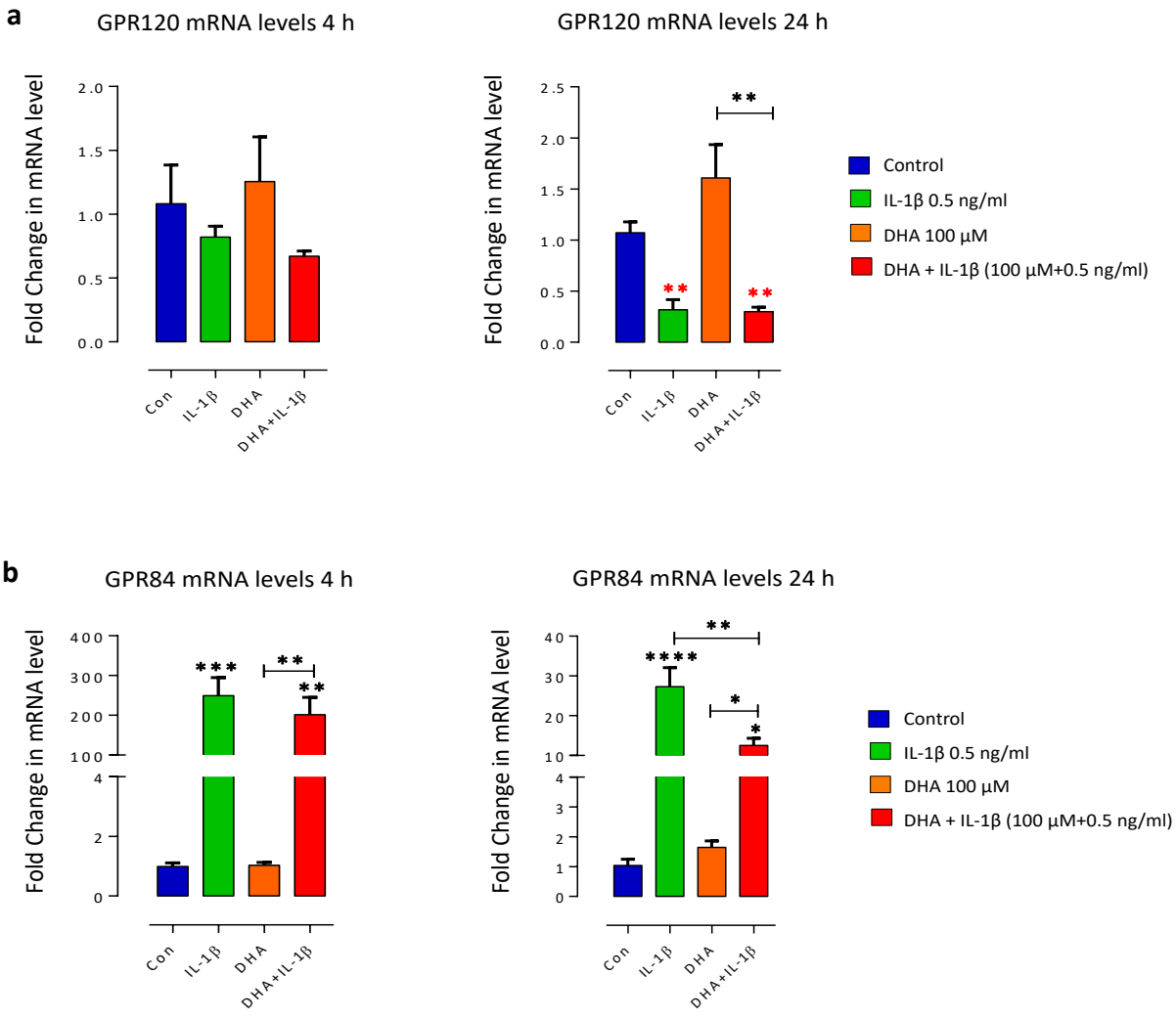


Figure 11. Effect of IL-1β (0.5 ng/ml), DHA (100 µM) and co-treatment of IL-1β+DHA (0.5 ng/ml+100 µM) on GPR120 **(a)** and GPR84 **(b)** gene expression in Human Adipocytes at 4 h and 24 h of incubation. (One-way ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$; ****** unpaired two-tailed t-Student test).

Furthermore, it is evident that there are marked increases mediated by IL-1β on *MCP1* mRNA level at both time points of incubation; DHA does not affect the gene expression this chemokine at 4 h while there was a small reduction at 24 h (**Figure 12**).

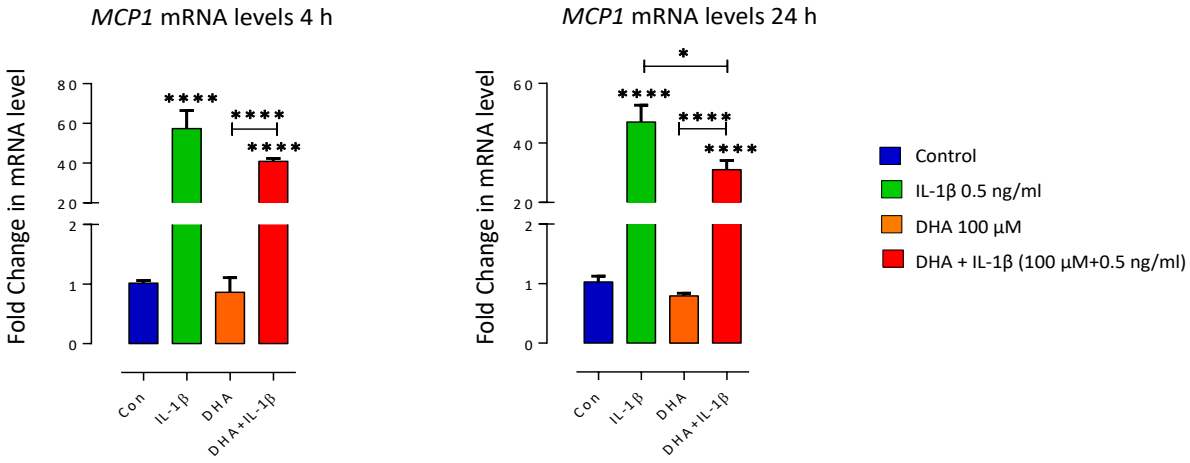


Figure 12. Effect of IL-1β (0.5 ng/ml), DHA (100 µM) and co-treatment of IL-1β+DHA (0.5 ng/ml+100 µM) on *MCP1* gene expression in Human Adipocytes at 4 h and 24 h of incubation. (One-way ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$).

Treatment with IL-1β increased markedly *IL6* gene expression at 4 h and 24 h of incubation, while DHA (100 µM) alone decreased significantly *IL6* gene expression only at 24h. On the other hand, pre-incubation with DHA did not significantly alter *IL6* expression induced by IL-β treatment (Figure 13).

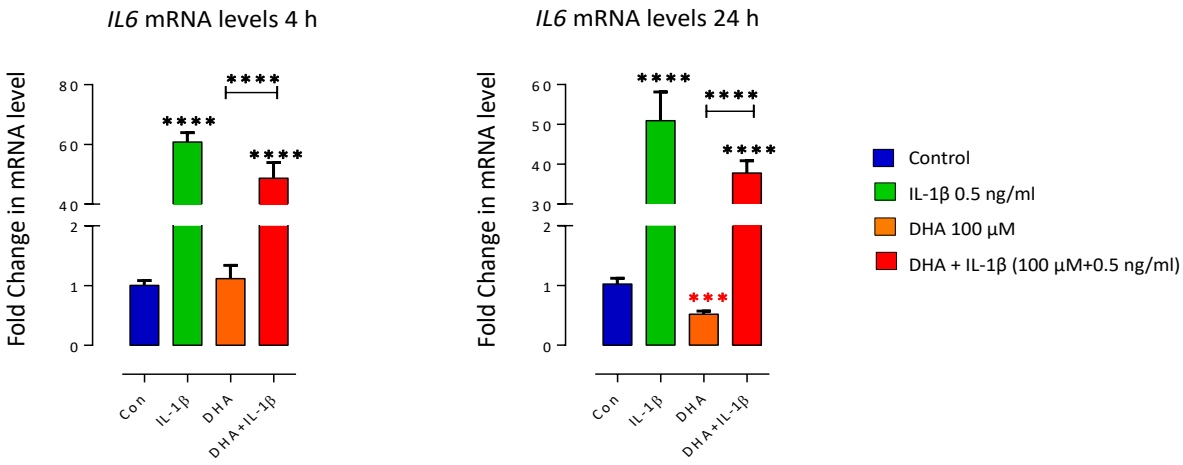


Figure 13. Effect of IL-1β (0.5 ng/ml), DHA (100 µM) and co-treatment of IL-1β+DHA (0.5 ng/ml+100 µM) on *IL6* gene expression in Human Adipocytes at 4 h and 24 h of incubation. (One-way ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$; *** unpaired two-tailed t-Student test).

IL-1β increased markedly *IL1B* gene expression at 4 h (>5000-fold mRNA level) but less so at 24 h (~ 30-fold) of incubation revealing a possible positive feed-back mechanism. Surprisingly, Incubation with DHA increased *IL1B* significantly at 24 h, and incubation of DHA in combination with IL-1β treatment did not affect *IL1B* gene expression at both time points. (Figure 14).

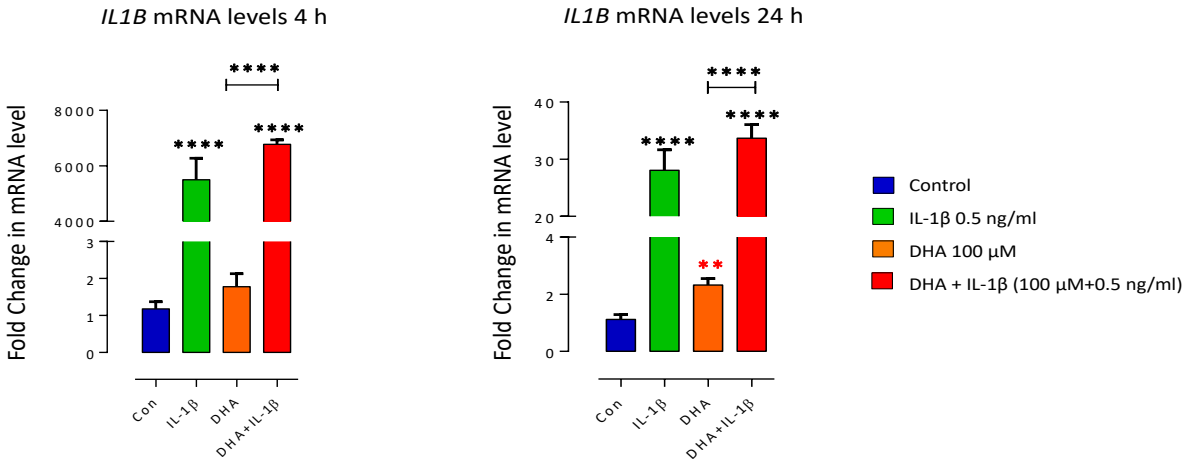


Figure 14. Effect of IL-1β (0.5 ng/ml), DHA (100 µM) and co-treatment of IL-1β+DHA (0.5 ng/ml+100 µM) on *IL1B* gene expression in Human Adipocytes at 4 h and 24 h of incubation. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$; ** unpaired two-tailed t-Student test).

IL-1β treatment decreased significantly *ADIPOQ* gene expression at 24 h of incubation with respect to untreated adipocytes, while Incubation of adipocytes with DHA significantly increased *ADIPOQ* gene expression at both 4 h and 24 h. In addition, incubation with DHA in IL-1β-treated cells completely prevented the negative effect of IL-1β on *ADIPOQ* gene expression (Figure 15).

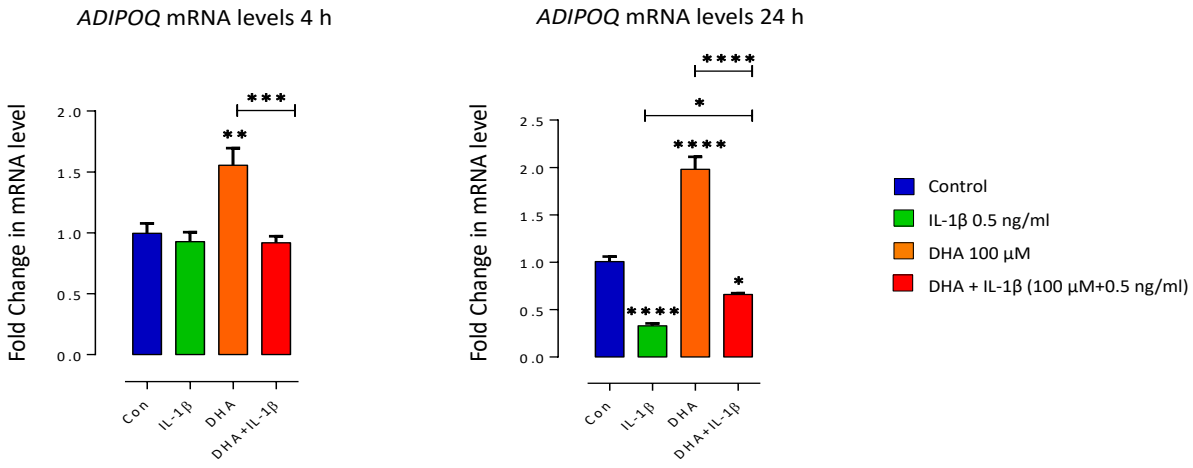
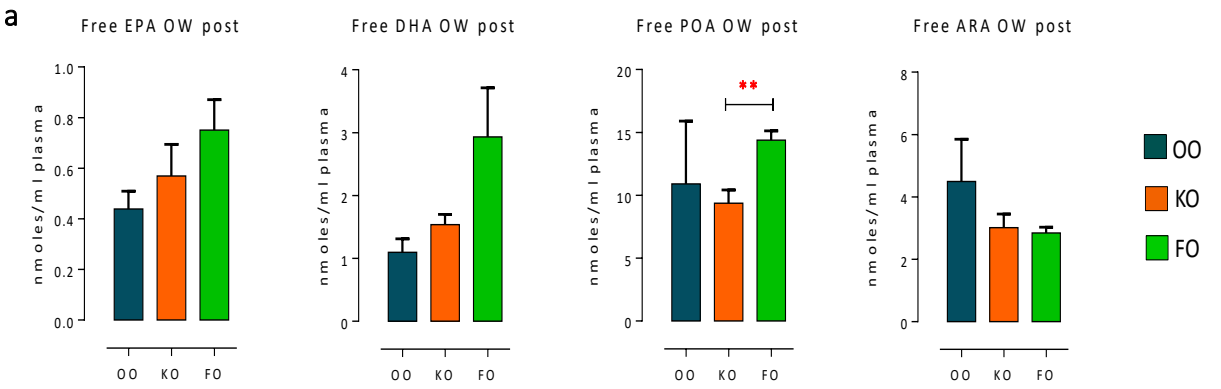


Figure 15. Effect of IL-1β (0.5 ng/ml), DHA (100 µM) and co-treatment of IL-1β+DHA (0.5 ng/ml+100 µM) on *ADIPOQ* gene expression in Human Adipocytes at 4 h and 24 h of incubation. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$).

In order to evaluate whether concentration of DHA in the free form present in human plasma changes according to its dietary intake, we carried out a pilot study where the subjects, stratified according to their BMI in normal weight, overweight and obese groups, were treated with 2 different types of EPA/DHA supplements, one as a fish oil (FO), i.e. in the triglyceride form, and the other as Krill oil (KO), where EPA and DHA are mostly esterified into phospholipids. As controls, one group was treated with olive oil (OO). Plasma free fatty acids, particularly those relevant for the effects on GPRs, namely EPA, DHA, POA and ARA were taken into consideration. The data showed that in NW subjects plasma FFAs did not change significantly with any treatment (data not shown), while in OW and OB, POA increased significantly only in FO supplemented subjects respect to KO in OW, while in OB in both KO and OO groups. Free ARA decrease significantly in Ob treated with FO with respect to OO, while vs KO it decreased but not significantly. DHA even though not significantly increased in the FO group in NW, OW and OB. EPA, probably because of the circulating low concentration, did not change significantly in all groups and subjects (**Figure 16a-b**).



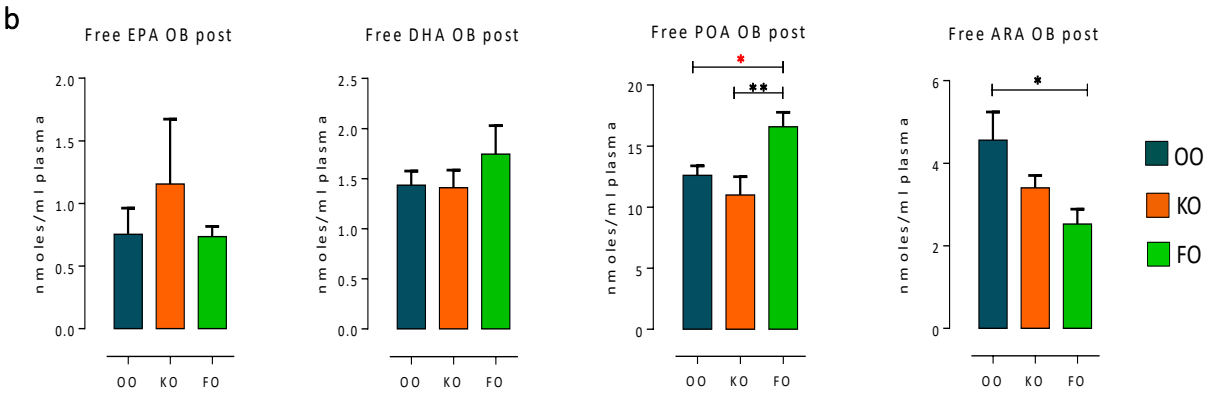


Figure 16. FFAs plasma levels (nM) after (post) treatment with different oils: Krill Oil (KO), Fish Oil (FO) and Olive Oil (OO) in OW (**a**) and OB (**b**) subjects as depicted in Table 1. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, p <0.05; ** and * unpaired two-tailed t-Student test)

Due to the divergent effects observed in adipocytes of DHA and ARA in modulating gene expression of GPR120, we also analysed the ratio DHA/ARA. The data showed that FO supplementation increased the DHA/ARA ratio in Ob significantly with respect to OO and KO groups, while in NW only KO increased significantly this ratio vs OO, and in OW, both KO and FO increased significantly vs OO group (**Figure 17**).

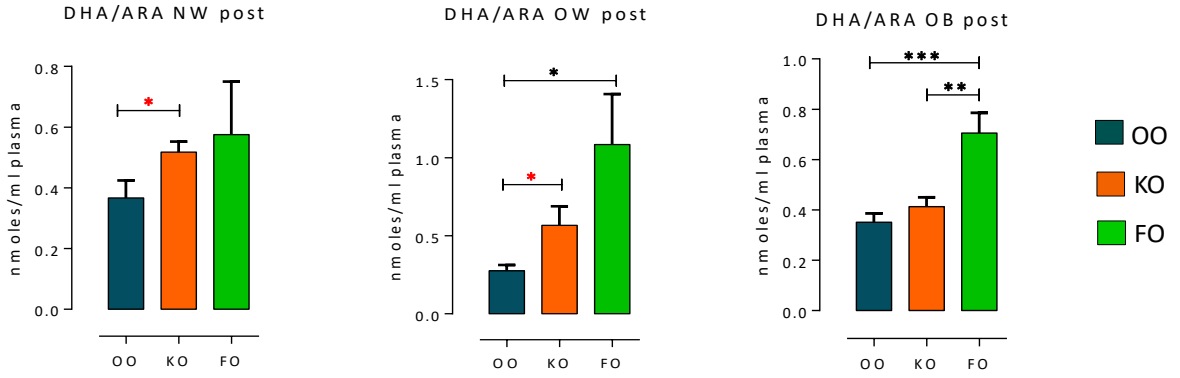


Figure 17. DHA/ARA ratio (nM) after (post) treatment with different oils: Krill Oil (KO), Fish Oil (FO) and Olive Oil (OO) in NW, OW and OB subjects as depicted in Table 1. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, p <0.05; * unpaired two-tailed t-Student test).

We also tested whether EPA/DHA supplementation modulated circulating TNFα. We found that TNFα concentration in plasma of OB was significantly higher than in OW and NW, and in OW higher than in NW (**Figure 18a**). All treatments did not change significantly the circulating TNFα in NW subjects. While in OB both FO and KO reduced TNFα significantly vs OO with the effect more pronounced in the FO group (**Figure 18b**).

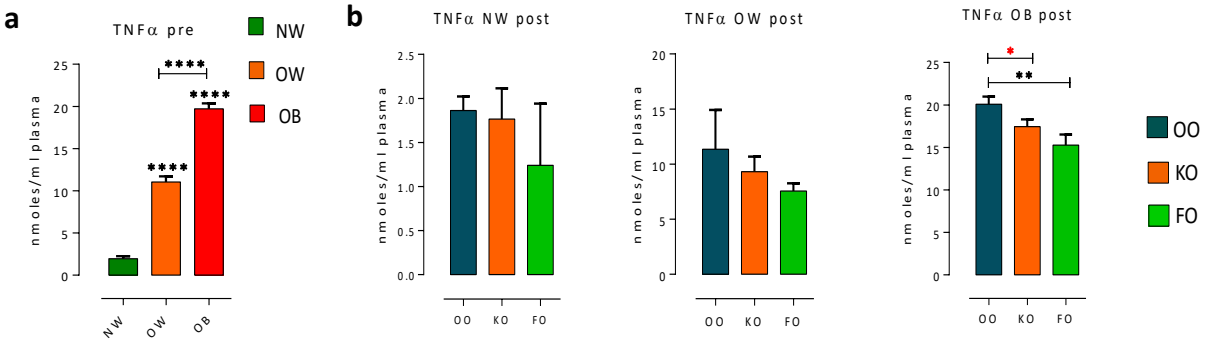


Figure 18. TNFα plasma concentration before (pre) treatment in NW, OW and Ob subject(**a**) and after (post) treatment with different oils: Krill Oil (KO), Fish Oil (FO) and Olive Oil (OO) in NW, OW and OB subjects (**b**) as depicted in Table 1. (ANOVA with a Bonferroni post-test for selected columns with ± SEM, $p < 0.05$; * unpaired two-tailed t-Student test).

5. Discussion

The fate of dietary fatty acids is finely regulated by specific cellular receptors capable of sensing the quantity and the quality of fatty acids reaching different organs via lipoproteins or as FFAs.

Therefore, there are different lines of control on how fatty acids are distributed and metabolised in different tissues according to the physiological needs. GPRs seem to play a fundamental role in signalling at governing the fate of dietary fatty acids in adipocytes. Previously *[Trayhurn P. et Denyer G., 2012]*, it has been shown that the two GPRs, GPR120 and GPR84 are regulated by macrophage-conditioned medium with a dramatic down regulation and upregulation of GPR120 and GPR84 respectively. These data suggested that these GPRs are deeply influenced by macrophage activities, proposing a physiological role of these receptors in preventing or sustaining inflammation. As a support of this hypothesis, incubation of adipocytes with pro-inflammatory cytokines showed similar effects. In addition, these data may also suggest that the anti-inflammatory activity of GPR120 is blunted by the marked decrease of its gene expression and therefore even an enhanced exposure to its ligands may not be effective in reverting the inflammatory process, with the increased production of proteins strongly related to inflammation such as ICAM and VCAM and MMPs. Also, other factors seem to influence GPR gene expression as well fat deposition in adipocytes. We have shown that rosiglitazone a potent PPARγ agonist, and thereby an inducer of lipid accumulation, is able to significantly increase GPR120 expression. This is consistent with the concept that PPARγ may indirectly induce an anti-inflammatory action as well

increase insulin sensitivity by increasing GPR120 gene expression, which may synergistically help the adipocyte to accumulate fat. As a matter of fact, the incubation with a strong anti-inflammatory agent such as dexamethasone did not influence GPR120 gene expression but rather reduced GPR84 gene expression. Therefore, our data suggest that GPR120 and GPR84 are not always complementary, as is the case where an increase of GPR120 resulted in a decrease of GPR84 as demonstrated in pro-inflammatory environment. However, in other physiological conditions the system may respond independently resulting in 2 different ways to modulate their activities. In fact, with incubation with insulin, GPR120 mRNA level was found to be increased already after 4 h of incubation, while GPR84 mRNA did not decrease significantly.

Several lines of evidences have shown that DHA is a strong ligand of GPR120 *[Oh DY., 2010]* and through its activation exerts its anti-inflammatory and insulin sensitization activities. Our results showed that DHA was able to increase GPR120 gene expression only at high concentration and after 24h of incubation, while in the study where the effect of DHA - *[Oh DY., 2010]* on GPR120 expression was described 4 h was sufficient with the same concentration of the FA. We may speculate that gene expression might be a delayed event with respect to the DHA effect. Interestingly, we did not detect any effect on GPR84 expression, which may suggest that there is not cross talk between these 2 GPRs.

In contrast, arachidonic acid strongly decreased GPR120 gene expression also at low concentrations and again, no effect was evident on GPR84 expression. This event is strongly associated to the increasing release of MCP-1 protein, normally in response to inflammation and potentially by stimulating the recruitment of macrophages.

These data highlight that rather than the absolute concentration of DHA, the DHA/ARA ratio might more important than an overall ratio of omega-3/omega-6 fatty acids. As a matter of fact, several reports in the literature pointed out that DHA and ARA are the most important players for eicosanoid and endocannabinoid *[Matias I., 2008]* biosynthesis modulation, and our present data seem also to suggest it is also relevant for GPR activities *[Murru E, Banni S, Carta G., 2013]*.

The lack of preventing GPR120 gene expression downregulated triggered by pro-inflammatory stimuli by DHA may suggest that in a state of chronic inflammation, its anti-inflammatory activities may be mediated by preventing chemokines and GPR84. Indeed, there is a significant decrease of GPR84 and *MCP1* mRNA level after 24 h of incubation with DHA which might suggest an indirect action of DHA mediated by resolvins and protectins *[Serhan CN., 2014]* rather than a direct effect on GPR120. Our data showed that DHA in physiological condition supressed *IL6* after 24h of incubation highlighting the possible indirect effect of DHA to keep an anti-inflammatory homeostatic control, inhibiting the gene expression of this pro-inflammatory cytokine.

In contrast, the data is showing an increase of *IL1B* gene expression after 24 h of incubation in adipocytes treated with DHA. This data might be explained by a specific effect of DHA as a free fatty acid, since it has been shown that exposure of adipocytes to a relatively high concentration of FFAs may trigger a pro-inflammatory process *[Zhang WY et al., 2006]*. Future studies should investigate whether this effect is dose dependent and varies according to the type of fatty acid.

Interestingly, IL-1β markedly upregulated *IL1B* mRNA level at 4 h and much less at 24 h of incubation suggesting that the increased release of IL-1β into the medium promotes an

additional increment of *IL1B* gene expression in adipocytes with a positive feed-back mechanism.

Our data demonstrated that DHA is able to increase *ADIPOQ* gene expression in physiological conditions and this contrasts with its downregulation triggered by incubation with IL-1β. Thus suggesting that DHA plays a direct and indirect role in regulating glucose uptake and fatty acid oxidation in adipocytes via modulation of *ADIPOQ* gene expression.

The GPR FFA receptors are sensitive to circulating levels of FFAs. We therefore evaluated in a pilot study whether dietary EPA and DHA were able to modulate circulating FFAs such as EPA and DHA but also other possible relevant fatty acids that influence GPR120 gene expression, namely POA and ARA. In addition, we use 2 different EPA and DHA formulations, one containing EPA and DHA in the TAG form (fish oil) and the other one as KO, where EPA and DHA are mostly esterified to PC. We chose these two formulations because in previous studies it has been shown that EPA and DHA in the form of KO are more efficient than FO in facilitating the incorporation of the FAs into tissue PLs, thereby affecting endocannabinoid biosynthesis in obese subjects, thus modulating energy and lipid metabolism *[Murru E., Banni S., Carta G., 2013]*. Actually, our data showed that FO was more efficient in increasing free DHA and POA, while in the case of KO it was EPA (even though not significantly). Interestingly, along with the increase of DHA and POA was associated a decrease of circulating TNFα. Probably, the changes were evident only in the obese subjects because they had higher levels of circulating FFAs, and higher concentration of TNFα. In normal weight as well over-weight subjects, as it has been shown for endocannabinoid biosynthesis, dietary EPA and DHA did not exert any effect, implying that their activities are exerted only in physiopathological conditions.

Therefore, our data suggest that KO being mostly incorporated into PLs decrease circulating FFAs and thereby DHA and POA, with a possible less pronounced effect on GPR120 and consequent less efficient ability to reduce inflammation, while the opposite is so for fish oil. These data point out the importance not only on the type of dietary fatty acids but also on the formulation which may strongly influence their activities in lipid and energy metabolism in the obese by affecting the endocannabinoid system and the inflammatory response by targeting GPR120.

One more mechanism by which EPA and DHA is able to influence inflammation is through the formation of resolvins and protectins *[Serhan CN., 2014]*, which may explain, along with the limited number of individuals recruited, the non-significant differences. Therefore, future studies should be carried out in larger cohorts and possible modulation of resolvins and protectins biosynthesis should be taken into consideration. However, since several reports suggest that even more important than the absolute values of single fatty acids, the ratio between DHA and ARA might be more relevant. As a matter of fact, we also found in adipocytes a divergent activity on GPR120 gene expression from the two PUFAs. In addition, we found that free ARA decreased with FO treatment and the analysis of the ratio DHA/ARA clearly showed an effect of FO supplementation. Interestingly, this increase mirrored the decrease of TNFα. We may speculate that free DHA and ARA by modulating GPR120 activity may affect in an opposite way inflammation, which implies the importance of circulating FFAs in qualitative and quantitative terms in regulating the inflammatory response. From our data it seems also that POA may play a relevant role, probably more marked in controlling insulin sensitivity as previously demonstrated *[Murru E., Banni S., Carta G., 2013].* It has been shown that KO is more efficient than FO in being able to decrease endocannabinoid concentrations

because of the increased bioavailability of DHA in PLs *[Murru E., Banni S., Carta G., 2013]*, mediating the positive effects on fatty liver and adipose tissue distribution *[Murru E., Banni S., Carta G., 2013]*. Here, our data suggest that probably not only the incorporation in PLs of DHA and consequent decrease of ARA may mediate the effects of dietary EPA and DHA, but also modulation of plasma levels of DHA, ARA and POA in the non-esterified form may, by influencing GPRs gene expression, exert anti-inflammatory and insulin sensitising activities.

We are currently evaluating whether the detected changes of gene expressions in human adipocytes paralleled the amount of GPRs and cytokines and chemokines proteins.

Future studies will aim at evaluating whether in experimental animals and humans the modification of fatty acid profile in different lipid fractions may mediate different omega-3 effects and thereby whether different omega-3 formulations may be employed for specific targeted metabolic dysfunction and/or inflammation.

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