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Fatty acids unsaturation and oxidative stress in membrane function, pregnancy, and metabolic syndrome.

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Abstract

Fatty acids have biological activities that go far beyond their function as energy sources. A correct dietary ratio between the nutritionally essential ω-6 and ω-3 polyunsaturated fatty acids (PUFA) is fundamental for a physiological production of second messengers, such as eicosanoids and other autacoids, and to ensure a controlled biological response to numerous physiological and pathological processes. Besides, PUFA, because of the unsaturation of their carbon chains, play an important role in maintaining the structure and a correct fluidity state of the membranes, the latter is a factor that directly regulates the activity of membrane-bound proteins, including ATPase pumps. However, the higher the degree of PUFA unsaturation the higher is their peroxidability. Lipid peroxidation (LPO) is a degrading process that, proceeding through a chain reaction mechanism, produces various cytotoxic and pro-inflammatory substances involved in the etiopathogenesis of numerous pathologies, including cerebro- and cardio-vascular diseases and type 2 diabetes mellitus. Several studies suggest protective effects of PUFA on cardio-metabolic risk, but in recent years some studies have also reported potential negative effects.
Based on these premises I collaborated on four studies aimed at evaluating: i) the effects of dietary supplementation with a multivitamin and ω-3 PUFA (MVP) on maternal biomarkers during the second and third trimester of pregnancy; (ii) the effects of ω-3 PUFA enrichment in endothelial cell membranes on LPO and the activity of the Na, K pump, which plays an important role in regulating the vascular tone of endothelial myocytes; (iii) the effects of diet-induced weight loss on the lipid composition of lipoproteins, peripheral cytokine levels and metabolic endotoxemia in obese subjects with metabolic syndrome (MetS); and vi) the chemical characterization of plasma lipoproteins by Raman microspectroscopy (RS). The first was a multicenter, randomized, and parallel study demonstrating that MVP supplementation led to a significant increase in concentrations of erythrocytic docosahexaenoic acid (DHA), omega-3 index, and vitamin D levels, while it did not affect the state of oxidative stress during pregnancy. These findings are important considering the essential roles of DHA in the developing brain, in visual development, and immunomodulation of the fetus. The results of the second study showed that as concentrations of EPA and DHA increase, the degree of fluidity and susceptibility to peroxidation increases in endothelial cell membranes. Besides, it has been observed that Na, K pump activity peaked at a concentration of 3.75 μM of the two PUFA, and then gradually decreased. This study highlights that low concentration EPA and DHA minimizes peroxidation potential and optimizes pump activity. The third study revealed a caloric-restriction-induced substantial transfer of triacylglycerols from VLDL and LDL to HDL that could impair the functionality of the latter, along with positive effects on inflammatory markers in a small group of subjects with MetS. In addition, an interesting positive correlation was also observed among peripheral cytokines levels after diet and peripheral levels of cholesteryl ester transfer protein, an enzyme with a key role in lipoprotein remodelling. Finally, the latest study allowed the determination of the chemical composition of the main classes of lipoproteins through RS identification and characterization of the spectra of cholesterol, triacylglycerols, apolipoproteins and carotenoids in a very small amount of samples. This study paves the way for the application of this technique in clinical nutritional studies and on a better understanding of several metabolic and pathological conditions.

Riassunto

Gli acidi grassi hanno attività biologiche che vanno ben oltre la loro funzione di fonti di energia. Un corretto rapporto dietetico tra gli acidi grassi polinsaturi nutrizionalmente essenziali (PUFA) delle famiglie ω-6 e ω-3 è fondamentale per una produzione fisiologica di secondi messaggeri, come gli eicosanoidi e altri autacoidi, e per garantire una risposta biologica controllata a numerosi processi fisiologici e patologici. Inoltre, i PUFA, a causa dell'insaturazione delle loro catene carboniose, svolgono un ruolo importante nel mantenere la struttura e un corretto stato di fluidità delle membrane, quest'ultimo è un fattore che regola direttamente l'attività delle proteine legate alla membrana, comprese le pompe ATP-asiche. Tuttavia, maggiore è il grado di insaturazione dei PUFA, maggiore è la loro perossidabilità. La perossidazione lipidica (LPO) è un processo degradativo che, procedendo attraverso un meccanismo di reazioni a catena, produce varie sostanze citotossiche e pro-infiammatorie coinvolte nell'eziopatogenesi di numerose patologie, tra cui malattie cerebro- e cardio-vascolari e diabete mellito di tipo 2. Diversi studi suggeriscono effetti protettivi dei PUFA sul rischio cardio-metabolico, ma negli ultimi anni alcuni studi hanno anche riportato potenziali effetti negativi.

Sulla base di queste premesse ho collaborato a quattro studi volti a valutare: i) gli effetti dell'integrazione della dieta con multivitaminico e ω-3 PUFA (MVP) su biomarcatori materni durante il secondo e il terzo trimestre di gravidanza; ii) gli effetti dell'arricchimento in PUFA ω-3 delle membrane cellulari endoteliali sull'LPO e l'attività della pompa Na, K, che svolge un ruolo importante nella regolazione del tono vascolare dei miociti endoteliali; iii) gli effetti della perdita di peso indotta dalla dieta sulla composizione lipidica delle lipoproteine, sui livelli periferici di citochine e sull'endotossiemia metabolica nei soggetti obesi con sindrome metabolica (MetS); e vi) la caratterizzazione chimica delle lipoproteine plasmatiche mediante microspettroscopia Raman (RS). Il primo è stato uno studio multicentrico, randomizzato e parallelo che ha dimostrato che l'integrazione con MVP ha portato a un aumento significativo delle concentrazioni di acido docosaesaenoico eritrocitario (DHA), dell'omega-3 index e dei livelli di vitamina D, mentre non ha influenzato lo stato di stress ossidativo durante la gravidanza. Questi risultati sono importanti considerando i ruoli essenziali del DHA nel cervello in via di sviluppo del feto, nello sviluppo visivo e nell'immunomodulazione. I risultati del secondo studio hanno mostrato che con l'aumentare delle concentrazioni di EPA e DHA, il grado di fluidità e la suscettibilità alla perossidazione aumenta nelle membrane cellulari endoteliali. Inoltre, è stato osservato che l'attività della pompa Na, K raggiugeva un picco ad una concentrazione di 3,75 μM dei due PUFA, per poi diminuire gradualmente. Questo studio evidenzia che l'EPA e il DHA a bassa concentrazione riducono al minimo il potenziale di perossidazione e ottimizzano l'attività della pompa. Il terzo studio ha rivelato un trasferimento sostanziale indotto da restrizione calorica di triacilgliceroli da VLDL e LDL alle HDL che potrebbe compromettere la funzionalità di quest'ultime, insieme a effetti positivi sui marcatori infiammatori in un piccolo gruppo di soggetti con MetS. Inoltre, dopo il dimagramento si è osservata un'interessante correlazione positiva tra i livelli di citochine periferiche e i livelli della proteina di trasferimento degli esteri del colesterolo, un enzima con un ruolo chiave nel rimodellamento lipoproteico. Infine, l'ultimo studio ha permesso la determinazione della composizione chimica delle principali classi di lipoproteine attraverso l'identificazione e caratterizzazione degli spettri di colesterolo, triacilgliceroli, apolipoproteine e carotenoidi in una piccolissima quantità di campioni mediante RS. Questo studio apre la strada all'applicazione di questa tecnica negli studi nutrizionali clinici e a una migliore comprensione di diverse condizioni metaboliche e patologiche.

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Abbreviation List

AA = Arachidonic Acid

ALA = alpha-Linolenic Acid

COX = Cyclooxygenases

CHD= Coronary Heart Disease

CRP = C-Reactive Protein

CVDs= Cardiovascular Diseases

DAMP= Danger-Associated Molecular Patterns

DHA = Docosahexaenoic Acid

DPA = Docosapentaenoic Acid

EDH= Endothelium-Dependent Hyperpolarization

EFA= Essential Fatty Acids

EPA = Eicosapentaenoic Acid

FA = Fatty Acids

FFAR= Free Fatty Acid Receptor

GC-FID= Gas-Chromatography – Flame Ionization Detection

GC-MS= Gas-Chromatography - Mass Spectrometry

HDL = High-Density Lipoproteins

HFD = High-Fat Diet

HIF-1α = Hypoxia Inducible Factor 1α

HIF-1β = Hypoxia Inducible Factor 1β

HNF4 = Hepatocyte Nuclear Factor 4

4-HNE = 4-Hydroxynonenal

IBD = Intestinal Bowel Diseases

IHD = Ischemic Heart Disease

IKK = Inhibitor of Nuclear Factor-κB (IκB) kinase

IL = Interleukin

IR = Insulin Resistance

LA = Linoleic Acid

LC-MS = Liquid-Chromatography - Mass Spectrometry

LC-PUFA = Long-Chain PUFA (*See Below*)

LDL = Low Density Lipoprotein

LGI = Low-Grade Inflammation

LOX = Lipoxygenases

LPO = Lipid Peroxidation

LXR= Liver X Receptor

MD = Mediterranean Diet

MDA = Malondialdehyde

MetS = Metabolic Syndrome

MUFA = Monounsaturated Fatty Acids

NAFLD = Non-Alcoholic Fatty Liver Disease

NEFAs = Non-Esterified Fatty Acids

NF-kB = Nuclear Factor kappa B

OxLDL = Oxidized LDL (*See Above*)

PA = Palmitic Acid

PAMP= Pathogen-Associated Molecular Patterns

PPARs = Peroxisome Proliferator Receptors

PRR= Pattern Recognition Receptors

POA = Palmitoleic Acid

PUFA = Polyunsaturated Fatty Acids

PURE = Prospective Urban Rural Epidemiological Study

ROOH = Hydroperoxides

ROS = Reactive Oxygen Species

RXR = Retinoic Acid X Receptor

SdLDL = Small Dense LDL (*See Above*)

SFA = Saturated Fatty Acids

SPMS = Specialized Pro-Resolving Mediators

SREBPs = Sterol Response Element Binding Proteins

TAG = Triacylglycerols

T2DM = Type 2 Diabetes Mellitus

TFA = *Trans* Fatty Acids

TLR = Toll-Like Receptor

TNFα = Tumor Necrosis Factor Alpha

VLDL = Very Low-Density Lipoprotein

VSMC = Vascular Smooth Muscle Cell

WAT = White Adipose Tissue

CHAPTER 1

1.1. Introduction

Fatty acids have biological activities that go far beyond their function as energy sources. These substances, in fact, affect cellular and tissue metabolism, function, and reactivity to hormonal signals and other signals through the regulation of different biological activities, such as the function of the membrane proteins, intracellular signaling pathways, the activity of transcription factors, gene expression and the production of bioactive lipid mediators. A correct dietary ratio between the nutritionally essential polyunsaturated fatty acids (PUFA) of the ω-6 and ω-3 families is essential to determine a physiological production of second messengers and autacoids, as well as to ensure a controlled biological response to inflammatory and immune processes, pain regulation, bone turnover, platelet aggregation, blood

clotting, smooth muscle contraction, kidney function and proliferation of cancer cells. In addition, during pregnancy PUFA are necessary to support optimal visual, neural, and behavioral development of the newborn/baby. PUFA, because of the unsaturation of their carbon chains, play an essential role in maintaining the structure and function of cell membranes also through their contribution to the fluidity state of membranes, a factor that directly regulates the activity of membrane-bound proteins, including ATP-ase pumps. However, the higher is the degree of PUFA unsaturation the higher is their peroxidability. Lipid peroxidation (LPO) is a process promoted by the attack of an oxidant to lipids, especially PUFA, which proceeds through a chain reaction mechanism and results in the production of many cytotoxic and pro-inflammatory substances. In addition, LPO induces pro-atherogenic and pro-inflammatory changes of lipoproteins that are involved in the etiopathogenesis of atherosclerosis and vascular diseases. Numerous scientific data indicate that a chronic low-grade inflammatory state is involved in peripheral insulin resistance (IR) and etiopathogenesis of various non-communicable diseases, including cerebro- and cardio-vascular diseases and type 2 diabetes mellitus (T2DM). IR results in hypertriglyceridemia and remodeling of high-density lipoproteins (HDL) that alters their metabolism and functions. Several studies suggest protective effects of PUFA on cardio-metabolic risk, but in recent years some reports

have also reported potential negative effects.

The object of this thesis are the results of the research I have carried out in the wide field concerning fatty acids and their role in some physiological and pathological processes. In particular, I collaborated on research that specifically covered:

1. The study of the effects of supplementation with micronutrients plus ω-3 PUFA during pregnancy on nutritional biomarkers of mothers and infants' anthropometric parameters at delivery, as well as on oxidative status of mothers.
2. The concentration-dependent effects of ω-3 LC-PUFA on the function of human endothelial cells membrane's, in particular with regard to Na,K-ATPase activity.
3. The effects of diet-induced weight loss on the relationship between lipoprotein lipid composition, the peripheral levels of cytokines and metabolic endotoxemia in overweight and class I obese subjects with metabolic syndrome.
4. The chemical characterization of the plasma lipoproteins with Raman spectroscopy.

1.2. Background

1.2.1 Fatty acids

In the diet, blood flow, cells, and tissues of humans there is a variety of fatty acids. Fatty acids have biological activities that go far beyond their function as energy sources. These compounds, in fact, affect cellular and tissue metabolism, function and reactivity to hormonal signals and other signals through the regulation of different biological activities, such as the structure and function of the membrane, intracellular signaling pathways, the activity of transcription factors, gene expression and the production of bioactive lipid mediators.

Digestion and absorption of dietary fatty acids is very efficient; however, they can also be synthesized in the human body from non-lipid precursors such as glucose or other fatty acids; except for essential ones that need to be introduced with the diet. Fatty acids are transported into the bloodstream as components of more complex lipids (e.g., triacylglycerols, phospholipids, cholesteryl esters) within lipoproteins, and to a lesser extent as non-esterified fatty acids (NEFAs) bound to albumin.

Fatty acids are classified according to the presence and number of double bonds in their carbon chain as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). Unsaturated fatty acids can be further categorized into four main families according

to the position of the first double bond starting from the methyl-end of the fatty acid chain:

- ω-3 (or n-3) fatty acids include mainly α- linolenic acid (ALA) and its derivatives eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA);
- ω-6 (or n-6) fatty acids include mainly linoleic acid (LA) and its derivative arachidonic acid (AA);
- ω-7 (or n-7) fatty acids include mainly palmitoleic acid (POA);
- ω-9 (or n-9) fatty acids include mainly oleic acid.

ALA and LA are essential nutrients.

SFA are present in significant proportions in many cell membrane phospholipids, especially in ceramides and sphingolipids. Membrane lipid rafts are generally rich in SFA–containing phospholipids and sphingolipids. In addition, the acylation of proteins with myristic and palmitic acids allows the anchoring of proteins involved in cellular signaling to the inner leaflet of the plasma membrane and protein traffic between different organelles. The liver plays a major role in regulating the metabolism of lipids and lipoproteins. SFA modulate gene expression through the regulation of transcription factors involved in lipid metabolism, such as sterol response element binding proteins (SREBPs), liver X receptor (LXR) /retinoic acid X receptor (RXR), and hepatocyte nuclear factor 4, (HNF4) [1]. High intakes of dietary SFA increase plasma cholesterol, particularly low-density lipoprotein (LDL) cholesterol,

through the modulation of these transcription factors in the liver with mechanism not yet fully clarified [2,3]. In addition, SFA are ligands for Toll-like receptor 4 (TLR4), thereby they induce inflammation response through Nuclear factor kappa B (NF-κB) activation [4]. These activities contribute to the determination of the risk of cardiometabolic diseases by diet rich in SFA.

Oleic acid (18:1ω-9) is the most abundant MUFA in the diet and in complex lipid of Human. Palmitoleic acid (16:1ω-7) is found in low amounts in many plants oils and animal fats and is quite abundant in macadamia oil and in fatty fish and fish oils. Many cell membrane phospholipids contain significant proportions of oleic acid and some palmitoleic acid. Oleic acid, the main fatty acid of olive oil, seems to have anti-inflammatory, hypolipemic and hypoglycemic properties [5]; however, its effects on inflammatory processes are modest and probably due to polyphenolic substances of olive oil rather than oleic acid. The effects of oleic acid on cholesterolemia and glycemia are often observed when it is used to replace SFA in the diet, and are likely to be due to partial removal of the negative effects of SFA rather than a direct action of oleic acid [1].

In mice, palmitoleic acid acts as a lipid messenger released by adipose tissue (lipokine) with effects on insulin sensitivity of skeletal muscle and accumulation of liver fat [6]; in addition, it interacts with the SREBPs and inflammatory system [7].

PUFA play an important role in maintaining the structure

and function of cell membranes. The dependence of membrane fluidity on the extent of unsaturation of fatty acids in membrane lipids is a well-characterized phenomenon. The physical state of membrane lipids directly regulates the activity of membrane-bound proteins, such as the translocators of small molecules, ion channels, receptor-associated protein kinases, sensor proteins and Na/K dependent adenosine triphosphatases (ATP-ases). Long-chain (C ≥ 20, LC) ω-3 PUFA, are more unsaturated than LC-ω-6 PUFA, therefor an increase in the ratio between LC-ω-3 PUFA and LC-ω-6 PUFA resulted in an increase of membrane fluidity.

Individual PUFAs have specific biological roles. LA (18:2 ω-6) is the most prevalent ω-6 PUFA in the human diet, followed by AA. LA is the metabolic precursor of γ-linolenic acid, di-homo-γ-linolenic acid (20:3 ω-6), and AA; however, the conversion of LA to AA is low in humans (approximately 0.2%) [8]. LA is essential in skin barrier function and AA in brain development and function. LA has also cholesterol and blood pressure lowering effects when it replaces dietary SFA [9]. AA of membrane phospholipids is involved in cell signaling and is the precursor of endocannabinoids (compounds with various biological properties [10]), second messengers, and eicosanoids. Eicosanoids, which include prostaglandins, thromboxanes, and leukotrienes, are produced specifically from C 20 PUFA by cyclooxygenases (COX) lipoxygenases (LOX) or cytochrome P450

monoxygenases-mediated pathways and have many biological roles [11]. AA play a major role in eicosanoids biosynthesis. They often have opposite effects to each other, ensuring a controlled biological response on inflammatory and immune processes, pain regulation, bone turnover, platelet aggregation, blood clotting, smooth muscle contraction, kidney function, and proliferation of cancer cells. However, too high levels of AA in membrane phospholipids can create a pro-coagulant, proinflammatory, pro-allergic, and pro-tumor environment [1].

ALA is the metabolic precursor of eicosapentaenoic acid (EPA; 20:5 ω-3), docosapentaenoic acid (DPA; 22:5ω-3) and docosahexaenoic acid (DHA; 22:6ω-3). Conversion of ALA to EPA and DHA is also approximately 0.2% [8]. In general, membrane phospholipids contain very low amounts of ALA, modest amounts of EPA and greater amounts of DPA and DHA; while the membranes of the cerebral cortex, retina, testis, and sperm are particularly rich in DHA. The structure of ω-3 PUFA, especially DHA, have a strong influence on the physical properties of membranes into which they are incorporated, on membrane protein function, and on lipid raft formation. Furthermore, DHA can influences the biosynthesis of neurotransmitters and N-docosahexaenoyl-ethanolamine (synaptamide, an endocannabinoid-like derivative of DHA that promotes neurite growth, synaptogenesis and glutamatergic synaptic activity), as well as the transmission of signals [12,13]. Furthermore, an

adequate ω-3 PUFA, especially DHA, intake is fundamental during the pregnancy to sustain optimal visual, neural, and behavioral development of the infant/child [14]. However, both EPA and DHA seem to play key roles in the brain function not only in infancy but also throughout the life course [1].

EPA and AA are parent compounds for eicosanoid production. In addition, DHA is the precursor of other autacoids termed D-series resolvins, docosatrienes and neuroprotectins, that are also produced by COX and LOX and act as anti-inflammatory and inflammation resolving compounds, especially in the brain [15]. In general, most of the autocoids formed from ω-3 PUFA are considered anti-inflammatory and/or pro-resolving, whereas those formed from ω-6 PUFA are classified as proinflammatory [15]; however, recent evidences indicate that ω-6 PUFA can be also metabolized into anti-inflammatory and pro-resolving compounds [16].

PUFA, like other fatty acids, also regulate the transcription factors LXRs and SREBPs inducing the suppression of hepatic lipogenic gene expression [1,17]. Besides, LC-PUFA acts as endogenous ligand of peroxisome proliferator activated receptors (PPARs), LXR, RXR, HNF4α, and free fatty acids receptors (FFAR), but often ω-6 PUFA have opposite effects to those of ω-3 PUFA [18–20]. In particular, the effects of ω-3 PUFA modulate inflammation, metabolism fatty acid and triacylglycerol, and adipocyte differentiation [21]. EPA and

DHA, lower serum triacylglycerol levels [22], through the reduction of hepatic secretion of very low-density lipoprotein (VLDL) [23], and lower blood pressure through the production of vasorelaxant eicosanoids and by influencing the secretion of aldosterone and the endothelial production of nitric oxide [24].

Ω-6 and ω-3 fatty acids are not interconvertible, and their metabolic pathways shares the same enzymes; therefore, there is direct competition for metabolism of ω-6 and ω-3 PUFA. Considering that ω-6 and ω-3 PUFA are essential nutrients, are metabolically and functionally distinct and often have important opposite physiological effects, it becomes evident the importance of their correct intake with the diet to achieve a physiologically balanced ratio of these fatty acids in membrane phospholipids. However, intake of LA has risen dramatically over the past century in western countries, due to a substantial increase in consumption of vegetable oils; while overall consumption of n-3 PUFAs has simultaneously decreased [25]. As a consequence, the membrane phospholipids of inflammatory cells of humans consuming Western-type diets typically contain approximately 20% of fatty acids as AA and lower proportions of di-homo-γ-linolenic acid and EPA (about 2% and <1% of fatty acids, respectively) [15]. Increased concentration of EPA and/or DHA in the diet results in higher amounts of those fatty acids in phospholipids of inflammatory cells [26] and other types of cells [27].

The restoration of the balance between ω-3 and ω-6 PUFA in the diet, obtained by taking foods or supplements rich in ω-3 PUFA, and the consequent effects of EPA and DHA on blood lipids, vascular function, blood pressure, and inflammation, are the basis of the protective effects of these fatty acids toward the development of cardiovascular diseases (CVD) [28].

Animal model have revealed the important role of PUFA, especially PUFA ω-3, in the control of several genes in the central nervous tissue in all phases of life, including perinatal period, and these effects appear to be mainly independent of their effects on membrane composition [29].

Unfortunately, higher is the degree of unsaturation of a PUFA higher is its peroxidability. Lipid peroxidation (LPO) is a process promoted by the attack of an oxidant to lipids, particularly PUFA, which through a mechanism of chain reactions produces lipid peroxyl radicals and lipid hydroperoxides. These primary products of LPO give rise to several different secondary cytotoxic products, such as malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE). Physiological low LPO rates stimulate the endogenous antioxidant defense systems resulting in an adaptive response to cell stress. On the contrary, when the extent of LPO overwhelms this repair capacity the accumulation of cytotoxic products of LPO damages proteins and nucleic acids and can promote cellular apoptosis or necrosis. LPO have overabundant health

consequences [30]. 4-HNE has been implicated in the activation of protein kinase cascades leading to the activation of redox-sensitive transcription factors, such as NF-κB and AP-1, and consequently activate several genes of inflammatory marker, such as cytokines and chemokines, and various cellular proteins involved in the survival, differentiation, and death of cells [31]. In addition, at membrane level LPO, causes profound alterations in the structural organization and functions by decreasing fluidity and altering the activities of proteins. Moreover, LPO induces proatherogenic and proinflammatory modifications in serum lipoproteins that are involved in the etiopathogenesis of atherosclerosis and associated vascular diseases. Finally, increased levels of LPO end products are implicated in neurological disorders and cancer development because of their mutagenic and carcinogenic effects.

In conclusion, fatty acids have different physiological functions and can prevent or exacerbate the risk of developing numerous pathologies through mechanisms that have not yet been fully clarified. These functions are performed not only in the regulation of energy and lipoprotein metabolism and membrane function; but also, in the control of both the production of bioactive lipid mediators and the activity of transcription factors and, consequently, gene expression.

1.2.1.1. ω-3 PUFA in pregnancy.

An adequate ω-3 PUFA intake is fundamental during the pregnancy to sustain optimal visual, neural, and behavioral development of the infant/child [32]. Nevertheless, the benefit of supplementation of LC-PUFA with infant formula with respect of standard formula was questioned in a Cochrane systematic review [33]; however, the majority of the studies included in this systematic review analyzed infant formulas contain both DHA and AA, and only few studies with DHA alone, in this way is not possible to detect and isolate the beneficial effects of DHA in infants. The beneficial effect of LC-PUFA was systematically reviewed even in the supplementation trials on breastfeeding women. The outcome taking in consideration were child neurodevelopment, visual acuity, physical growth, infant allergy, and mothers postpartum depression. Compared to placebo, supplementation with DHA did not produce significant effects on childhood neurodevelopment, although children's attention improved at 5 years, while other outcomes had significantly improved [34]. Another systematic review demonstrated a reduce risk for low birthweight for offspring of women supplemented with LC-PUFA [35]. A very recent study of Japanese pregnant women cohort showed that dietary intake of fish and PUFAs (both ω–3 and ω–6) in pregnancy was partly associated with a reduced risk of suboptimal neurodevelopment outcomes at age 6

months and 1 year of infant age, while the dietary ω–6/ ω–3 ratio was positively associated with increased risk for problem solving neurodevelopment domain and a reduced risk for delay gross motor domain [36]. Therefore, the effects of ω-3 LC-PUFA on correct infant's development, may derive from mother diet or supplementation. In addition, DHA and EPA can exert positive effects through the regulation of inflammatory processes and therefore help to keep under control the physiological insulin resistance (IR) typical of pregnancy [37–42]. There are many factors that contribute to the development of peripheral IR, such as gluco-lipotoxicity, oxidative stress, epigenetic factors, activation of various transcriptional mediated pathways and increased levels of various pro-inflammatory cytokines. Among the various pro-inflammatory cytokines, the tumor necrosis factor-alpha (TNF-α) is one of the most important pro-inflammatory mediators that is critically involved in the development of insulin resistance and type 2 diabetes mellitus [43]. The molecular mechanism involved in pregnant physiological IR are not well understood, but appear to be related to the activities of hormone and pro-inflammatory cytokines [37]. A study performed in a small group of subjects showed that the plasma concentration of TNFα was inversely related to insulin sensibility measured with euglycemic hyper-insulinemic clamp in normoglycemic pregnant women [44]. Besides, the maintenance of pregnancy depends on the inhibition of NF-κB, which it is activated by TNF α. An

incorrect level of NF-κB for the appropriate stage of pregnancy may predispose women to obstetric complications (eg. intrauterine growth restriction, pre-eclampsia, and premature labour) [45].

Pregnancy is physiologically associated with a condition of oxidative stress [46], however, this condition is further increased in obese pregnant women [47], and even more in women with pregnancy complications, such as preeclampsia [48]. At molecular level, oxidative stress increase inflammatory processes mainly via inflammasome and NF-κB pathways activation [49,50]. Furthermore, high oxidative stress levels have a detrimental effects during pregnancy and increase the risk of complications [51]. In this perspective, ω –3 LC-PUFA, mainly DHA, are important not only for the proper development of children [30], but also for the modulation of inflammation, mainly NF-κB pathways [60], and oxidative stress of mothers [59]. In fact, given the high susceptibility to LPO due to their high degree of unsaturation [8], an excessive intake of ω–3 LC-PUFA, could increase oxidative stress. Therefore, the safety of an integration of DHA in pregnancy should be accompanied by monitoring oxidative stress and antioxidant defense capacity; for example, by determining lipoperoxidation extent through the measurement of plasma concentrations of reactive oxygen species (ROS), and by measuring the ratio of reduced to oxidized glutathione in erythrocytes as an index of red/ox balance at cellular level [61].

1.2.1.2. ω-3 PUFA in endothelial membranes.

Healthy endothelium is essential for cardiovascular control [52]; on the contrary, dysfunctional endothelium, characterized by reduced vasodilation, and a pro-inflammatory and pro-thrombotic state, plays an important role in the development of hypertension and pathogenesis of CVD [53].

The supplementation with , ω–3 PUFA has shown positive effects on endothelial function in animal and cellular models [54]; while in human studies has led to conflictual results [53].

The positive potential effects of ω–3 PUFA on endothelium include increased anti-inflammatory properties, improved endothelial-dependent vasodilation, and decreased blood pressure and arterial plaque development [52].

In murine models of essential hypertension, endothelial dysfunction can be attributed to marked attenuation of endothelium-dependent hyperpolarization (EDH) and concomitant production of endothelium-derived contracting factors with no or little alteration in the production of nitric oxide. This decrease in EDH has been associated with various changes in the expression profile/function of the actors involved in those responses, including different cellular channels such as potassium channels, connexins, and Na+/K+-ATPase. In essential hypertension, oxidative stress is related to endothelial dysfunction and compromises vasodilation in microcirculation and mesenteric artery, while the prevention of ROS production restores EDH by

enhancing an IK_{Ca}-Na+/K+-ATPase pathway involved in the loss of endothelial tone [55].

Proper Na,K-ATPase activity is critical to maintaining vascular tone [55] and it is well known that lipids that increase membrane fluidity, such as ω-3 LC-PUFA, promote Na,K-ATPase activation [56], as well as that highly unsaturated PUFA peroxidation in membrane lipids has opposite effects [57]. Therefore, it is fundamental to reach a balance between the risk and benefit of ω–3 LC-PUFA membrane enrichment.

1.2.2. Lipoprotein metabolism, chronic low-grade inflammation, and oxidative stress.

Growing evidence indicates that a chronic low-grade inflammatory state is involved in the etiopathogenesis of non-communicable diseases [58,59], including CVDs [59] and type 2 diabetes mellitus (T2DM) [60].

The main causes of low-grade inflammation (LGI), also called 'silent inflammation', are not entirely understood, but several evidences link life style to this condition [58,61,62]. In particular, one of the major factors that promote LGI is the so-called "Western lifestyle" [61], that is characterized by sedentary life and unbalanced nutrition consisting of excessive energy intake, especially in the form of simple fats and sugars, high ratio ω -6 / ω -3, excess alcohol and salt, but very low intake of fiber, micronutrients and dietary bioactive compounds. Such a lifestyle promotes the development of obesity that can further contribute to LGI [61].

Obesity is begun to be defined as an epidemy from World Health Organization since 2017 [63]. According to the latest Obesity Update report of the Organization for Economic Co-operation and Development, in 2017 more than one in two adults and nearly one in six children are overweight or obese. The obesity epidemic continues its widespread rise and new projections until 2030 are keeping the same trend [64].

In obesity, the release of proinflammatory cytokines such as

TNFα and interleukin 6 (IL-6), from the WAT results, up-regulated, these molecules enhance the hepatic production of C- reactive protein (CRP) [65].

Indeed in obese people, WAT can become dysfunctional and not expand properly to store energy [66]. The causes of unhealthy expansion of WAT are not fully understood, but the two main factors are the involvement of inflammasome activity [67] and the excess of threshold of lipid storage in adipocytes, independently of adipocytes inflammation [68,69]. Besides, the unhealthy expansion of WAT produces different metabolic alteration that leads to 'lipotoxicity'. These alterations consist in a decrease of adipogenesis, insulin sensitivity; and increase in adipocytes hypertrophy, LGI and macrophages infiltration [70]. Hypertrophic adipocytes have elevated basal lipolysis rate and consequently released into bloodstream increased levels of non-esterified fatty acids (NEFA) [70,71]. In obesity complicated by metabolic syndrome (MetS) fat mobilization from adipocytes is impaired, and insulin is unable to suppress lipolysis, thus NEFA spill-over from hypertrophic adipocytes to ectopic sites that are not designed to lipid storage function, such as muscle, liver, pancreas and HDL. This lipotoxic mechanism contribute to IR [71–73], and increases the risk of non-alcoholic fatty liver disease (NAFLD) and T2DM and CVDs [70,74]. Furthermore, when plasmatic NEFA are in excess, they stimulate NF-κB leading to a propagation of inflammatory cascade [75]. The NF-κB

activation by NEFA pass through various pathway, one of the most important is activation of pattern recognition receptors (PRR) [74,75].

PRR are a family of receptors recognizing pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS, also called endotoxin), microbial peptides and proteins [76]. PRR, recognize not only microbial molecules, but also endogenous "danger "signals, danger-associated molecular patterns (DAMPs) that include free SFA [75].

Toll Like Receptor 4 (TLR4) and 2 (TLR2) are some of the main members of PRRs that recognize free SFA, principally palmitic acid (PA) and lauric acid [77,78]. PRR, once activated, can induce the innate inflammatory response [79] and consequently, the release of interleukin 1 beta (Il-1β), Il-6, IL-8, IL-12 and TNFα, via stimulation of proinflammatory NF-κB pathway [79–82]. The interaction of TNFα to its receptor in insulin-responsive cell (muscle/liver), activates NF-κB pathways via inhibition of κB kinase (IKK), and impairs the autophosphorylation of insulin receptor substrate (IRS) that results in insulin insensitivity [83,84]. This is considered the immune-metabolic link between obesity and inflammation, that leading to impairment of insulin signaling [84].

In addition, the nutrient overload, leading to excess level of glucose and NEFA, increase the production of acetyl-CoA in Krebs cycle, with overloading of electron transport chain,

and subsequent increases of ROS in the cytoplasm, activating redox-sensitive transcription factors (e.g. NF-κB), which are the principal mediators of the inflammatory cascade [50].

Noteworthy, dietary factor (eg. SFA, LC-PUFA, amino acids, carbohydrates) are involved in LGI and consequent IR [85]. LC-PUFA, namely EPA and DHA, seems to have an anti-inflammatory effect by the inhibition of NF-κB pathway, both upstream and downstream, through the attenuation of the activation of TLR4, which is due mainly by LPS, and to a lesser extent by SFA [80,86]. Human studies show that diet and meal are effective factors able to mediate postprandial endotoxemia [87]. Subjects consuming high SFA diet with respect high MUFA or LC-PUFA or low-fat diet had an increased postprandial plasma LPS concentration [88] that contributes to activation of TLR4, and, consequent of inflammatory NF-κB pathways [80]. The activation of NF-κB pathways, via TLR4 stimulation, is sustained by NEFA, mainly released from WAT adipocytes, and high plasma level of post-prandial endotoxin, stimulated by dietary SFA [89].

As mentioned above, hypertrophic adipocytes induced by high-fat diet (HFD) generated also ROS [90,91]. Both in mice and humans, HFD causes a significant elevation in ROS from mitochondria isolated from skeletal muscle [92]. The ROS accumulation has also been shown in adipose, liver and kidney tissue's mitochondria in animals feed with HFD, as reviewed by Tan and colleagues [93]. ROS can stimulate

signal transduction pathways (mainly via NF-κB), which activates the production of TNF-α and IL-6 [93]. This contribute to impaired insulin signaling, and perpetrating LGI and IR. Obese subjects in comparison with normo-weight controls have high levels of several LPO markers, such as conjugate-dienes, MDA and lipid peroxides, as well as an increased grade of lipoprotein oxidation, in hematic circulation [94]. Furthermore, in patients with MetS and/or T2DM often there is a dyslipidemic state, characterized by increased level of triacylglycerols (TAG), small dense LDL (sdLDL) and reduced level of high-density lipoprotein cholesterol (HDL-C) [95].

Overproduction of VLDL is particularly evident in T2DM patients, with low insulin sensitivity. In addition, the intra-abdominal and ectopic liver fat are significant predictor of liver production on TAG-rich VLDL [95,96]. Therefore, in diabetic dyslipidemia the VLDL production is increased and the catabolic rate of apoB-containing lipoproteins, such as LDL and IDL is reduced, while the catabolic rate of HDL is increased [95]. IR and hypertriglyceridemia could remodel HDL lipoprotein particles because of the altered lipoprotein lipase (LPL), hepatic lipase (HL), cholesteryl ester transfer protein (CETP), and phospholipid transfer protein (PLTP) activities [97] and the strong link between HDL and VLDL metabolism. As a result, HDL have altered protein and lipid composition, in particular a higher TAG to cholesteryl ester (CE) ratio in their hydrophobic core, and consequently an

alteration of their normal biological function and catabolic rate. In addition, these changes in HDL lipid composition increase their susceptibility to oxidation [98]. Furthermore, while high levels of LDL cholesterol increase the risk of developing CVD, on the other hand the preventive role of high HDL cholesterol levels is still controversial. In the past, observational studies had suggested an inverse relationship between HDL cholesterol and both cardiovascular disease and total mortality [99]. However, recent large-scale cohort studies have failed to confirm this association and some studies have found increased cardiovascular events and increased mortality associated with very high levels of HDL [100]. Therefore, more detailed knowledge of the composition, chemical-physical properties and oxidizability of HDL could be a useful, additional means for better understanding of the ethiopathological events that link the alteration of HDL metabolism to the increased risk of CDV in MetS and diabetic patients. Lipoprotein chemical characterization can be determined by using several different methodological approaches, including Raman spectroscopy (RS) [101]. Most modern Raman instruments are coupled with microscopy; therefore, RS is a well-suited tool for the characterization of lipid in very small amount of samples.

1.2.3. Methods to study fatty acids and oxidative stress.

Many analytical methods are used for the qualitative and quantitative evaluation of fatty acids (FAs) in all kinds of biological sample, including gas chromatography, liquid chromatography, near-infrared spectroscopy, and NMR spectroscopy [102]. Among these methods, gas chromatography of fatty acid methyl esters (FAMEs) is one of the most employed. Since FAs exhibit low volatility and strong polarity, they must be converted into FAMEs to increase their volatility and decrease their polarity prior to the gas-chromatographic analysis. Moreover, FAs, especially PUFA, easily undergo polymerization, deacidification, cracking, and other adverse reactions at high temperatures, therefore they must be protected during the all the analytical process, for example by adding to samples powerful antioxidants, such as butylated-hydroxy-toluene (BHT) [103]. In clinical studies, FAs are measured mainly in blood serum or plasma and erythrocyte; however, the profile of the fatty acids, especially of the PUFA, of each of these blood components has a different meaning both from a nutritional and diagnostic point of view. MUFA and SFA can be introduced with diet or synthesized de novo by humans, whereas PUFA derived directly or indirectly only from dietary fats. Hence, the PUFA concentrations in serum reflect the dietary intake of the week that preceded the blood

sampling [104]; whereas, in erythrocytes indicate the dietary intakes of the past three months [105].

Free radicals generated in excessive and uncontrollable amounts under oxidative stress conditions cause damage to DNA, proteins, and lipids, which can severely compromise cell health and contribute to disease development. Oxidative stress give rise to several complex reaction of peroxidation (autooxidation) of these biomolecules that generate a very wide array of end products. Therefore, a correct interpretation of oxidative stress requires more than one laboratory test, each of which must provide complementary information. Methods and approaches to measure oxidative stress in clinical samples include the determination of the levels of pro-oxidant, such as reactive oxygen substances (ROS) and reactive nitrogen substance (RNS) and antioxidants, such as vitamin E, Vitamin C and glutathione, or the assessment of oxidant-induced damages to proteins, DNA and lipids [106]. However, the determination the levels of a pro-oxidant cannot be regarded as a general estimate of such a complex condition as oxidative stress [107]. The same applies to the determination of levels of one or more antioxidants and antioxidant enzyme activities. [107].

Lipid peroxidation (LPO) has been commonly used as an indicator of ROS-mediated damage to lipids. Malondialdehyde (MDA) is one of the best-studied end-products of AA peroxidation in clinical samples that has frequently been used to determine oxidative stress

conditions, especially in scientific research. In fact, the levels of MDA can be measured using the rapid and easy, but poor specific thiobarbituric acid reactive substances (TBARS) method, or by using more specific, but time-consuming and labor-intensive methods that involve the use of high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS). Since peroxidation is one of the most common mechanisms of ROS-induced damage, the dosage of hydroperoxides (ROOH) is frequently used to determine the pro-oxidant state of an individual by spectrophotometric assays easily executed both manually and automatically [108,109]. ROOH are thought to be useful targets to assess pro-oxidant contribution to oxidative stress, because they are the most reactive form of ROS, can initiate lipid peroxidation by attacking PUFA, and are produced by reactions of peroxynitrite and singlet oxygen with lipids, amino acids, peptides, and proteins [110]. ROOH present in blood circulation are considered markers and amplifiers of tissue damage induced by oxidative stress [111–113].

As regards antioxidant capacity assessment, the determination of the redox state of glutathione is one of the useful approaches to integrate lipid peroxidation parameters. In fact, glutathione system is one of the major antioxidants *in vivo* and its activity is closely related to the concentration and the redox state of other low-molecular-weight thiols such as cysteine, cysteine-glycine and

homocysteine [114]. The reducing power of hydro-soluble thiols is necessary to maintain some lipo-soluble antioxidant (such as vitamin E and coenzyme Q) in their reduced form, the only form active in the prevention of ROS-induced lipid peroxidation of lipoproteins and cell membranes [115].

Alternative approaches are based on the susceptibility of lipid specimen to a flux of free radicals, as monitored upon exposure to free radical 'generators' ex vivo. The response of lipid specimens to radical attack may be monitored either at one predefined time-point or at multiple consecutive time-points (kinetically). The kinetic profile of peroxidation can be based on continuous measurement of the production of oxidation products and/or of the consumption of oxidizable lipids and/or oxygen consumption and/or depletion of antioxidants. Many analytical methods have been applied to monitor different biomarkers, including spectrophotometry, chromatography, fluorescent probes, chemiluminescence and electrochemistry [116]. In the laboratory of chemical biology and biochemical nutrition where I did my doctoral thesis work, have been set-up several analytical methods for lipid peroxidation kinetic determination using fluorescence techniques [117–119]. The kinetic curve individuates three phases: a latency phase (lag-time), a propagation phase, and a termination phase. The lag time is commonly used as a criterion for the resistance of lipids to peroxidation, while the maximal rate of propagation phase is a complex function of lipid oxidability and antioxidative potency. Therefore, kinetic

methods offer the possibility of assessing several meaningful parameters from each assay.

CHAPTER 2

2.1 Studies

As above mentioned, my research activity has contributed to the publication of four research articles on the following topics:

1. The study of the effects of supplementation with micronutrients plus ω-3 PUFA during pregnancy on nutritional biomarkers of mothers and infants' anthropometric parameters at delivery, as well as on oxidative status of mothers.
2. The concentration-dependent effects of ω-3 LC-PUFA on the function of human endothelial cells membrane's, in particular with regard to Na,K-ATPase activity.
3. The effects of diet-induced weight loss on the relationship between lipoprotein lipid composition, the peripheral levels of cytokines and metabolic endotoxemia in overweight and class I obese subjects with metabolic syndrome
4. The chemical characterization of the plasma

lipoproteins with Raman spectroscopy.

Below are the publications of the studies in which I collaborated during my doctoral program in Nutritional Science.

nutrients

MDPI

Article

Multiple Micronutrients and Docosahexaenoic Acid Supplementation during Pregnancy: A Randomized Controlled Study

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Abstract: Maternal dietary intake during pregnancy needs to meet increased nutritional demands to maintain metabolism and to support fetal development. Docosahexaenoic acid (DHA) is essential for fetal neuro-/visual development and in immunomodulation, accumulating rapidly within the developing brain and central nervous system. Levels available to the fetus are governed by the maternal diet. In this multicenter, parallel, randomized controlled trial, we evaluated once-daily supplementation with multiple micronutrients and DHA (i.e., multiple micronutrient supplementation, MMS) on maternal biomarkers and infant anthropometric parameters during the second and third trimesters of pregnancy compared with no supplementation. Primary efficacy endpoint: change in maternal red blood cell (RBC) DHA (*wt*% total fatty acids) during the study. Secondary variables: other biomarkers of fatty acid and oxidative status, vitamin D, and infant anthropometric parameters at delivery. Supplementation significantly increased RBC DHA levels, the omega-3 index, and vitamin D levels. Subscapular skinfold thickness was significantly greater with MMS in infants. Safety outcomes were comparable between groups. This first randomized controlled trial of supplementation with multiple micronutrients and DHA in pregnant women indicated that MMS significantly improved maternal DHA and vitamin D status in an industrialized setting—an important finding considering the essential roles of DHA and vitamin D.

Keywords: docosahexaenoic acid; long-chain polyunsaturated fatty acids; maternal biomarkers; micronutrients; neurodevelopment; pregnant women; supplementation; vitamin D

1. Introduction

During pregnancy, an adequate maternal dietary intake is essential to meet the increased nutritional demands required to maintain metabolism and support fetal development [1]. Micronutrients such as folic acid and other B vitamins, vitamin D, vitamin C, calcium, copper, magnesium, iodine, selenium, zinc, and iron all have vital roles throughout all stages of pregnancy [2–4]. Poor dietary intake or deficiencies in both micro- and macronutrients can have adverse effects on pregnancy

outcomes and neonatal health [5], including an increased risk of neural tube defects, preeclampsia, miscarriage, and low birth weight [6,7]. Many women are at risk of insufficient nutrient intake in industrialized as well as developing countries [8–10]. Therefore, micronutrient supplementation is frequently recommended during pregnancy to help improve pregnancy outcomes in the mother and child [11,12]. International guidelines (i.e., from the World Health Organization) currently recommend supplementation of iron and folic acid (0.4 mg/day) during the whole pregnancy for the purpose of improving pregnancy outcomes and for reducing maternal anemia in pregnancy [13]. Recently, there have been extensive scientific and medical discussions around the need to include vitamin D as a standard nutrient to be supplemented during pregnancy, due to low intake. Vitamin D regulates calcium and phosphate body stores and is therefore critical for bone health [14]. Furthermore, low concentrations of blood vitamin D in pregnant women have been associated with pregnancy complications [15,16].

In addition to micronutrients, a balanced macronutrient intake is recommended. In particular, the long-chain polyunsaturated fatty acids (LCPUFAs) found at high concentrations within the brain and central nervous system are essential for the development of the fetal brain [17]. Docosahexaenoic acid (DHA)—representing the largest proportion of LCPUFAs in the brain and retina—plays a key role during the pre- and early postnatal period [17–20]. After the first trimester, when the neural tube has closed and grey matter begins to form [21], DHA begins to rapidly accumulate in the brain [18,22]; accumulation continues for up to two years [23,24].

However, the human body is not efficient at producing essential LCPUFAs [22], and maternal concentrations decrease over the course of gestation [25]. Of note, the levels of DHA available to the fetus during pregnancy are governed by the diet of the mother [17,26–28]. Studies suggest that consumption of a diet rich in omega-3 LCPUFAs including DHA may have a reduced risk of common pregnancy complications such as intrauterine growth restriction, preeclampsia, and preterm deliveries [29–31]. Supplementation with DHA can also increase the expression of fatty acid transport proteins, thus increasing transport through the placenta and improving the fatty acid status of both the mother and child [32,33].

Meta-analyses have demonstrated that there are clinical benefits associated with prenatal multiple micronutrient [34] and LCPUFA supplementation [35] during pregnancy. However, there is limited data on the effects of prenatal supplementation in industrialized countries, particularly when used in combination. Clinical guidelines for pregnant women tend to focus on single nutrients for supplementation [36,37]. Given the interest in the potential beneficial effects of supplementation with micronutrients and DHA during pregnancy, we carried out a randomized trial to evaluate the effects of multiple micronutrients plus DHA supplementation during the second and third trimesters of pregnancy on maternal biomarkers compared with no supplementation in the control group in an industrialized country. The primary variable, i.e., the concentration of DHA (weight percent of total fatty acids (*wt*% TFA)) in maternal red blood cells (RBC), was considered indicative of LCPUFA status. Secondary explorative variables were other biomarkers of fatty acid and oxidative status, vitamin D, and anthropometric parameters of infants at delivery. We included vitamin D status as a secondary endpoint to investigate whether vitamin D supplementation is needed to maintain adequate status, and whether the levels of vitamin D in the supplement would be sufficient to maintain an adequate status. We hypothesized that supplementation might help to improve maternal DHA and vitamin D status in a healthy population of pregnant women, whereas dietary intake would be insufficient to meet the increased needs during pregnancy.

2. Materials and Methods

2.1. Trial Design

This was a multicenter, parallel, randomized controlled trial conducted at two centers in Italy to compare the effects of once daily supplementation with multiple micronutrients plus DHA (hereafter

referred to as multiple micronutrient supplementation, or MMS) versus no supplementation during pregnancy on maternal biomarkers and infant anthropometric parameters. Supplementation began at gestational week 13–15 until delivery. Six visits were conducted during the trial, from screening to final follow-up, as outlined in Figure 1 and Supplementary Table S1. At baseline (Visit 2; gestational week 13–15), women who fulfilled the eligibility criteria were randomized to the supplementation or control group in a 1:1 ratio. The sequential randomization list (generated through a validated SAS program by an independent statistician) was generated according to permutated block codes. A randomization number was assigned to each woman at each site by means of randomization cards. The study was not blinded. All blood parameters were measured at Visits 1, 3, and 4 in all women, while dietary intake was recorded at Visits 2, 3, and 4.

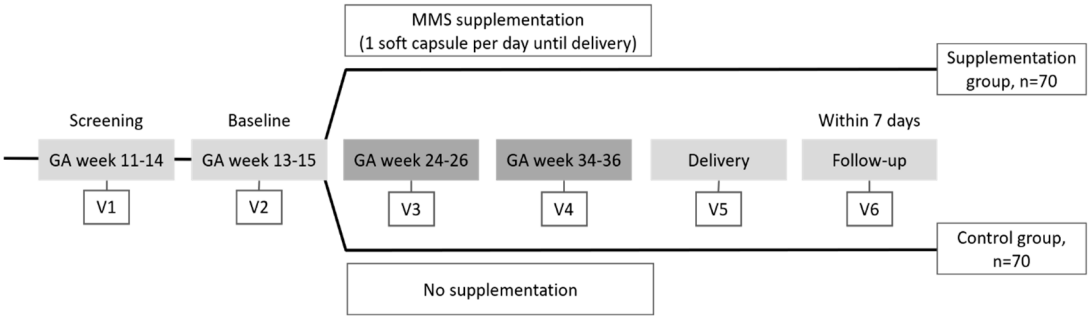

Figure 1. Study design. Visit 1 (V1, screening): pregnant women were screened for study eligibility and blood collection was performed. Visit 2 (V2, baseline): eligible women meeting the inclusion and exclusion criteria were randomized equally to one of the two study groups; nutritional status was assessed using a semi-quantitative FFQ. Visits 3 and 4 (V3 &V4, MMS supplementation or no supplementation): FFQ was administered and blood sampling took place—the red blood cell DHA level measured at Visit 4 was compared with the value measured at Visit 1 to assess the primary endpoint. Visit 5 (V5, delivery): obstetric evaluations were performed in all women and infant anthropometric parameters were measured. Concomitant medications and adverse events were assessed at all Visits. GA, gestational age; DHA, docosahexaenoic acid; FFQ, food frequency questionnaire; MMS, multiple micronutrients and DHA supplementation.

The study was approved by an independent ethics committee (Comitato Etico Milano, Milan, Italy). The Institutional Review board Project no. of the study: 2016/ST/024. The study was approved on 30 March 2016. The study was conducted in accordance with the Declaration of Helsinki and in compliance with all current Good Clinical Practice guidelines, local laws, regulations, and organizations. The trial was registered at ClinicalTrials.gov (ClinicalTrials.gov Identifier: NCT04438928). The trial protocol can be obtained from the corresponding author, upon reasonable request.

2.2. Study Population

Healthy, pregnant Caucasian women aged 18–42 years were screened during their first trimester prenatal visit (gestational age (GA), week 11–14) at Hospital Sacco and Hospital Buzzi in Milan, Italy. The study was proposed to all pregnant women with a singleton pregnancy within the gestational age indicated. Women were included in the study if they were having a singleton pregnancy, hemoglobin level >105 g/L, normal ultrasound examination, and inconspicuous fetal anomaly screening, taking at least 400 µg folate per day, and provided written, signed informed consent for participation in the study. Women were excluded if they had experienced previous adverse pregnancy outcomes, followed a specific diet, or were already taking DHA/multivitamin supplements (except folate or iron). Full inclusion and exclusion criteria are listed in Supplementary Table S2.

2.3. Study Product

The study product was an oral MMS soft gel capsule (Elevit, Bayer) that contained 12 vitamins, six minerals, and DHA (200 mg) to meet the requirements of women during pregnancy, especially during the second and third trimester [38,39] (Supplementary Table S3). One capsule was taken per day with a sufficient amount of liquid, from GA week 13–15 (Visit 2, baseline) until delivery (Visit 5; approximately 27 weeks of supplementation). The control group did not receive a placebo during this time.

2.4. Parameters Assessed

Analyses were performed at the "Luigi Sacco" Department of Biomedical and Clinical Sciences (Università degli Studi di Milano) and ASST Fatebenefratelli Sacco, Milan, Italy. In total, approximately 56 mL of blood was taken in the fasted state from each subject for the efficacy and safety assessments during the whole study. Blood samples were centrifuged for 10 min at 1000 g at 4 °C; plasma for 8-isoprostane and dROMs analysis was separated from the erythrocyte pellet, and the buffy coat was discarded. Erythrocytes for fatty acid and glutathione analyses were washed once with a 0.2 M EDTA + 150 nM NaCl solution through gentle inversion, and then 15 min centrifugation at 2000 g at 4 °C.

The efficacy parameters assessed are outlined in Supplementary Table S4. The change in RBC DHA (*wt*% TFA) from Visit 1 to Visit 4 was the primary maternal variable to assess the beneficial effects of supplementation with micronutrients and DHA during the second and third trimesters of pregnancy. Secondary maternal variables included other RBC fatty acid parameters (TFA, eicosapentaenoic acid (EPA), *wt*% TFA, DHA/TFA ratio, and omega-3 index), calcidiol (25-hydroxyvitamin D), and oxidative stress markers in blood including reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio, plasma reactive oxygen metabolites (ROMs, which are hydroperoxides), and plasma 8-isoprostane. The erythrocyte membrane fatty acid composition was determined by gas chromatography of fatty acid methyl esters [40–42]. The amount of each considered fatty acid was calculated as μg/mL of RBCs and expressed as a percentage of the total fatty acid concentration. The omega-3 index was calculated by summing the percentage of EPA and DHA [43]. Calcidiol levels were measured using radioimmunoassay [44], the GSH/GSSG ratio using fluorimetric assay [1], ROMs using photometric assay [45,46], and 8-isoprostane using competitive enzyme immunoassay with an ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions [47]. Briefly, 500 uL of heparinated plasma were stored at –80 °C with a preservative ethanol solution containing butylated hydroxytoluene (BHT) until analysis. Alkaline hydrolysis was performed to allow total 8-isoprostane (both free and esterified fractions) quantification; after neutralization, proteins were removed by ethanol precipitation and samples were purified by solid phase extraction (SPE) using octadecyl (C-18) silica affinity cartridges. Total 8-isoprostane levels in purified plasma samples were then analyzed by ELISA. Dietary intake was evaluated using a semi-quantitative Food Frequency Questionnaire of five food categories to assess the usual daily intake of foods and nutrients (adapted from Vioque et al. [48], which was validated in pregnant women) at Visits 2, 3, and 4. Dietary intake data and results of a small subgroup analysis in women who underwent a cesarean section (cord blood and placenta samples) will be presented elsewhere.

Safety and tolerability were assessed by evaluating the incidence and severity of adverse events (AEs) and their relationship to trial treatment. Laboratory parameters, physical examination, and vital signs were also recorded.

2.5. Statistical Analysis

Assuming a treatment difference of 1.6 (standard deviation (SD) 3.4), as observed by Bergmann et al. 2008 [49], 70 subjects per arm were required to achieve 80% power with 0.05 of alpha to detect the treatment difference between the supplementation and control groups. To account

for a drop-out rate of 15%, approximately 164 subjects (82 per treatment group) were to be randomized to get 140 evaluable subjects.

The primary efficacy analysis was performed on the per protocol (PP) population (all subjects with efficacy data for the primary efficacy endpoint at Visit 4 who did not have protocol violations). Results were corroborated using data from the intent-to-treat (ITT) population (i.e., all subjects in the safety population who had at least one post-baseline measurement of efficacy data). The safety population comprised all subjects who were randomized into the study, and took at least one dose of the supplement for those randomized to the treatment group.

The primary efficacy endpoint was defined as the change in maternal RBC DHA (*wt*% total fatty acids) from Visit 1 to Visit 4, analyzed using the analysis of covariance (ANCOVA) with treatment as a fixed effect and the Visit 1 value as covariate. Secondary maternal efficacy endpoints were changes from Visit 1 to Visit 4 in blood fatty acid parameters (RBC EPA (*wt*% total fatty acids), DHA/EPA ratio, RBC omega-3 index), 25-hydroxyvitamin D, and antioxidant status (GSH/GSSG ratio, plasma ROMs, 8-isoprostane). All secondary endpoints were analyzed similarly to the primary endpoint. Secondary infant efficacy endpoints (gestational age, head circumference, weight and length measurements, ponderal index, infant skinfold thickness, Apgar score, bone density) were collected at delivery (Visit 5) or within 10 days after delivery for bone density and analyzed using ANCOVA with treatment as fixed effect.

Safety and tolerability variables were assessed by evaluating incidence and severity of AEs, their relationship to trial treatment, and the incidence of abnormal findings in measurement of objective tolerability through vital signs, physical examination, and clinical laboratory findings. Only treatment-emergent AEs (TEAEs) were analyzed, i.e., AEs that began or worsened after randomization.

Two-sided p-values < 0.05 were considered statistically significant. Results are presented as mean ± standard deviation (range), n (%), or LSMEANS (least squares means) of change from Visit 1 (95% confidence interval, CI), as appropriate. All statistical tables, listings, and analyses were produced using SAS® release 9.4 or later (SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Subject Characteristics

The study took place between September 2016 to December 2019. After screening, 176 subjects were randomized to the MMS ($n = 87$) or control ($n = 89$) groups (Figure 2). All subjects were included in the safety population. Forty-six subjects discontinued the study, mainly because of adverse events (32 (69.6%) subjects). The PP population comprised 141 subjects (MMS, $n = 65$; control, $n = 76$). The mean study duration was 24.5 ± 6.49 (1.0–30.9) weeks, and was comparable in both groups. Overall compliance was ≥80% in 63 (72.4%) of MMS subjects, ≤80% in four (4.6%), and unknown in 20 (23%).

Subject baseline demographics, clinical characteristics, and delivery information are shown in Table 1. The mean age was 31.9 ± 4.64 (18–41) years and all subjects were Caucasian. All demographics were similar between groups, with no significant differences. No abnormalities in physical or gynecological examinations were reported at Visit 1 or Visit 2. Although not statistically significant, a higher proportion of subjects in the control group compared with the MMS group experienced delivery complications (16 (23.2%) vs. eight (12.9%) subjects, respectively) or had an induced labor (13 (18.8%) vs. nine (14.5%) subjects). The groups were well balanced regarding infant sex (male 58.1% in the MMS group, 56.5% in the control group).

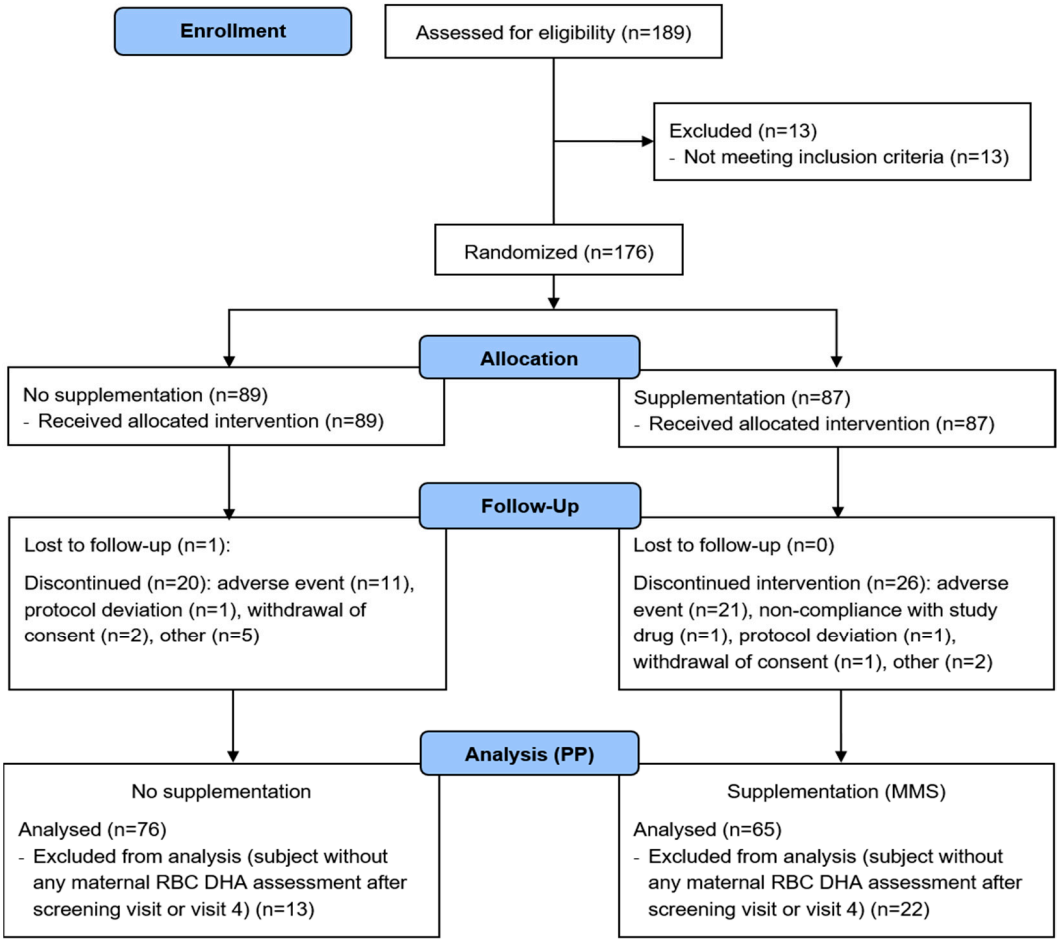

Figure 2. Flow diagram for study participants. DHA, docosahexaenoic acid; MMS, multiple micronutrients and DHA supplementation; RBC, red blood cells; PP, per protocol.

Table 1. Subject characteristics at baseline (values expressed as *n*, mean ± standard deviation, and median (range), unless otherwise stated) and delivery information (values expressed as *n* (%), unless otherwise stated) (per protocol population).

Characteristics	No Supplementation (*n* = 76)	MMS (*n* = 65)
Age (years)	76	65
	32.3 ± 4.72	31.4 ± 4.52
	33.0 (18–41)	32.0 (20–40)
Weight (kg)	76	65
	61.5 ± 9.96	63.2 ± 9.48
	59.0 (45–87)	47.0 (47–95)
Height (cm)	76	65
	164.1 ± 7.08	165.9 ± 5.60
	165.0 (147–184)	165.0 (150–178)
Body mass index (kg/m^2)	76	65
	22.8 ± 3.24	22.9 ± 3.10
	21.7 (18.0–29.7)	22.0 (18.1–29.9)
Previous pregnancy, *n* (%)		
No	30 (39.5)	30 (46.2)
Yes	46 (60.5)	35 (53.9)
Smoking status, *n* (%)		
Never	49 (64.5)	49 (75.4)
Former [a]	27 (35.5)	16 (24.6)

Table 1. *Cont.*

Characteristics	No Supplementation (n = 76)	MMS (n = 65)
Delivery information		
Subjects performing delivery visit	69	62
Type of delivery, *n* (%)		
Vaginal	55 (79.7)	49 (79.0)
Caesarean	14 (20.3)	13 (21.0)
Delivery complications, *n* (%)		
No	53 (76.8)	54 (87.1)
Yes	16 (23.2)	8 (12.9)
Induced labor, *n* (%)		
No	56 (81.2)	53 (85.5)
Yes	13 (18.8)	9 (14.5)
Infant sex, *n* (%)		
Male	39 (56.5)	36 (58.1)
Female	30 (43.5)	26 (41.9)

[a] Stopped smoking prior to pregnancy/when becoming aware of pregnancy consent signature plus one day. MMS, multiple micronutrients and docosahexaenoic acid supplementation.

3.2. Efficacy Endpoints

Primary. Maternal RBC DHA (*wt*% TFA) increased every visit in both groups (Figure 3 and Table 2), but the mean change from Visit 1 to Visit 4 was significantly greater in the MMS group compared with the control group, with an estimated treatment difference of 0.96 (95% CI 0.61, 1.31) ($p < 0.0001$) (Table 2). Furthermore, RBC DHA levels in women at the lower ranges increased by a greater extent in the MMS group (1.1% at Visit 3 and 1.6% at Visit 4 vs. Visit 1) compared to those in the control group (increase of 0.2% at Visit 3 and 0.5% at Visit 4 vs. Visit 1), and reached threshold levels (5% [50]) by Visit 4 (Table 2).

Secondary maternal endpoints. Significant differences were observed in favor of MMS for maternal RBC DHA/TFA ratio (estimated difference 0.01 (95% CI 0.006, 0.013); $p < 0.0001$), omega-3 index (estimated difference 1.00 (95% CI 0.64, 1.37); $p < 0.0001$), and calcidiol (estimated difference 3.96 (95% CI 0.88, 7.04) µg/L; $p = 0.0122$) (Figure 3 and Table 2).

The remaining secondary efficacy endpoints (maternal RBC TFA, RBC EPA (*wt*% TFA, GSH/GSSG ratio, ROMs, 8-isoprostane)) were comparable between groups, albeit slightly higher in the MMS group, with no significant differences (Supplementary Table S5).

Secondary infant endpoints. As outlined in Supplementary Table S6, infant variables were comparable between groups, with no statistically significant differences apart from subscapular skinfold thickness (thicker in the MMS group, $p = 0.0292$) and bone density in m^2 (borderline significantly greater in the control group, $p = 0.0486$).

Dietary intake. Assessment of dietary intake showed that consumption of the macro- and micronutrients measured was comparable between groups at each visit (Supplementary Table S7).

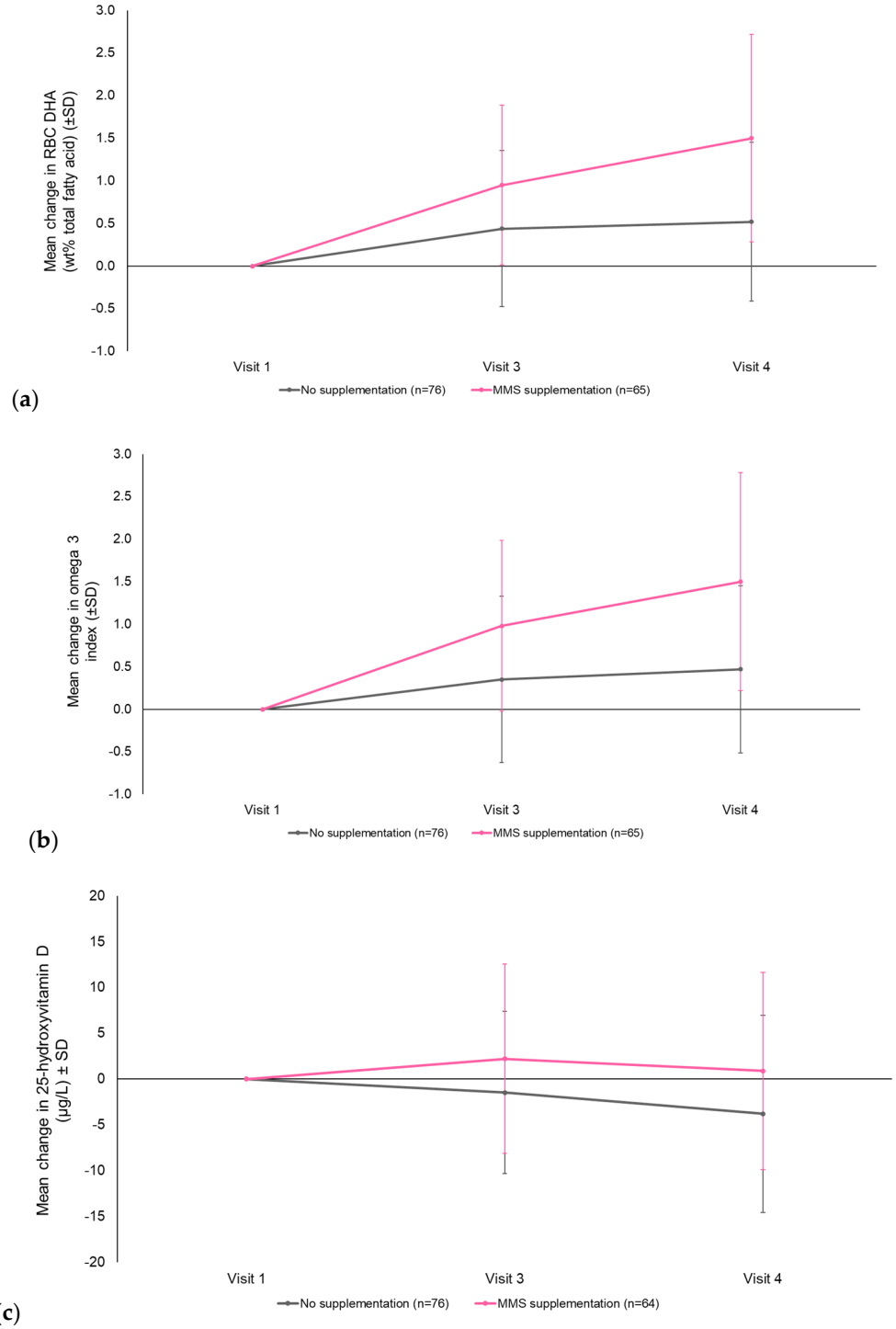

Figure 3. Mean change (± standard deviation) from Visit 1 to Visit 4 in maternal (**a**) RBC DHA (*wt*% TFA) ($p < 0.0001$ in favor of MMS), (**b**) omega 3 index ($p < 0.0001$ in favor of MMS), and (**c**) calcidiol (25-hydroxyvitamin D) ($p = 0.0122$ in favor of MMS) (per protocol population; LOCF approach). Visit 1: Screening (GA Week 11/14); Visit 3: GA Week 24/26; Visit 4: GA Week 34/36. DHA, docosahexaenoic acid; GA, gestational age; LOCF, last observation carried forward; MMS, multiple micronutrients and DHA supplementation; RBC, red blood cells; SD, standard deviation; TFA, total fatty acids; *wt*, weight.

Table 2. Primary and secondary outcomes at each visit (LOCF approach; values presented as mean ± standard deviation (range)) and differences between groups from Visit 1 to Visit 4 (presented as LSMEANS (95% confidence interval), ANCOVA model) (per protocol population).

	No Supplementation (*n* = 76)			**MMS (*n* = 65)**		
	Visit 1	**Visit 3**	**Visit 4**	**Visit 1**	**Visit 3**	**Visit 4**
RBC DHA (*wt*% TFA)	6.1 ± 1.23 (3.8–9.3)	6.6 ± 1.30 (4.0–10.4)	6.7 ± 1.34 (4.3–9.6)	6.1 ± 1.26 (3.4–10.2)	7.0 ± 1.30 (4.5–10.5)	7.5 ± 1.48 (5.0–13.0)
LSMEANS difference/*p* value	—	—	—	—	—	0.96 (0.61, 1.31)/ < 0.0001 *
RBC DHA/TFA ratio	0.06 ± 0.01 (0.04–0.09)	0.07 ± 0.01 (0.04–0.10)	0.07 ± 0.01 (0.04–0.10)	0.06 ± 0.01 (0.03–0.10)	0.07 ± 0.01 (0.04–0.11)	0.08 ± 0.01 (0.05–0.13)
LSMEANS difference/*p* value	—	—	—	—	—	0.010 (0.006, 0.013)/ <0.0001 *
Omega 3 index (%)	6.7 ± 1.38 (4.2–10.1)	7.0 ± 1.43 (4.2–10.7)	7.1 ± 1.45 (4.5–10.0)	6.5 ± 1.40 (3.7–10.9)	7.5 ± 1.43 (4.7–11.1)	8.0 ± 1.59 (5.3–13.6)
LSMEANS difference/*p* value	—	—	—	—	—	1.00 (0.64, 1.37)/ <0.0001 *
Calcidiol (ug/L)	21.6 ± 8.94 (5.5–48.8)	19.9 ± 9.87 (4.6–64.1)	17.8 ± 9.72 (4.0–45.0)	20.5 ± 7.54 (4.4–36.5)	22.8 ± 8.94 (4.0–48.6)	21.4 ± 9.07 (5.5–42.7)
LSMEANS difference/*p* value	—	—	—	—	—	3.96 (0.88, 7.04)/ 0.0122 *

* Two-sided *p* value <0.05 considered statistically significant. Visit 1: Screening (GA Week 11/14); Visit 3: GA Week 24/26; Visit 4: GA Week 34/36. DHA, docosahexaenoic acid; GA, gestational age; LOCF, last observation carried forward; LSMEANS, least squares means (difference = supplementation—no supplementation); MMS, multiple micronutrients and DHA supplementation; RBC, red blood cells; TFA, total fatty acids; wt, weight.

3.3. Safety Analysis

As outlined in Table 3, 125 (71.0%) subjects reported at least one TEAE pertinent to the mother (232 TEAEs overall) and 23 (13.1%) subjects reported them as serious, with a comparable number in each group. In the MMS group, 19 (21.8%) had one TEAE that led to permanent treatment discontinuation. Only three (3.5%) subjects in the MMS group had at least one suspected related TEAE (vomiting, with mild severity). At least one TEAE pertinent to the fetus/child were reported in ten (5.7%) subjects (13 TEAEs overall), and five (2.8%) reported them as serious. A higher proportion of subjects reported a TEAE in the MMS group, but none were considered to be treatment related. One (1.6%) subject had one TEAE pertinent to the fetus/child that led to permanent discontinuation. There was one fatality in the MMS group unrelated to study treatment. No relevant changes in clinical laboratory parameters (i.e., hematology, kidney function, liver function, blood coagulation, CRP) were observed, although there was a decrease in mean ferritin levels in both groups over the course of the study. Physical and gynecological examinations were normal throughout.

Table 3. Summary of participants with treatment-emergent adverse event (safety population; values expressed as *n* (%) subjects).

Parameters	No Supplementation (*n* = 89)	MMS (*n* = 87)	Total (*n* = 176)
Number of TEAEs pertinent to the mother	114	118	232
Any TEAEs pertinent to the mother	64 (71.9)	61 (70.1)	125 (71.0)
At least one suspected related [a]	NA	3 (3.5)	3 (1.7)
At least one serious TEAE	11 (12.4)	12 (13.8)	23 (13.1)
At least one leading to temporary treatment interruption [b]	NA	1 (1.2)	1 (0.6)
At least one leading to permanent treatment discontinuation [c]	NA	19 (21.8)	19 (10.8)
Fatal outcome	0	0	0
Number of TEAEs pertinent to fetus/child	4	9	13
Any TEAEs pertinent to fetus/child	3 (3.4)	7 (8.1)	10 (5.7)
At least one suspected related [a]	NA	0	0
At least one serious TEAE [b]	2 (2.3)	3 (3.5)	5 (2.8)
At least one leading to temporary treatment interruption [c]	NA	1 (1.2)	1 (0.6)
At least one leading to permanent treatment discontinuation [d]	NA	1 (1.2)	1 (0.6)
Fatal outcome	0	1 (1.2)	1 (0.6)

[a] Suspected related adverse events were those events with causal relationship equal to related; [b] No Supplementation group, the TEAEs pertinent to the fetus/child classified as severe were: fetal distress syndrome 1 (1.12%), fetal growth restriction 1 (1.12%); MMS group, the TEAEs pertinent to the fetus/child classified as severe were "Duodenal atresia" (1, 1.15%), "Fetal compartment fluid collection "(1, 1.15%), "Fetal growth restriction" (1, 1.15%) and "Polyhydramnios" (1, 1.15%). No TEAE pertinent to the fetus/child was suspected of being related to the study product; [c] adverse events leading to temporary treatment interruption were those events with action taken equal to drug interrupted; [d] adverse events leading to permanent treatment discontinuation were those events with action taken equal to drugs withdrawn. MMS, multiple micronutrients and docosahexaenoic acid supplementation; NA, not applicable; TEAEs, treatment-emergent adverse events.

4. Discussion

Supplementation with MMS plus DHA throughout the second and third trimester of pregnancy led to a significant increase in RBC levels of DHA, as well as the proportion of DHA compared with EPA and TFA. There was also a significant increase in the omega-3 index, while vitamin D levels increased during the course of the study compared to a decrease in women who did not receive supplementation. In the infant, a significantly greater subscapular skinfold thickness was observed in the MMS group. Safety outcomes were comparable between groups and MMS was well tolerated.

Our findings demonstrate that RBC DHA levels were significantly higher in the MMS group than in the control group. In pregnant women, the target RBC DHA level is 5% [50] (with <4.3% considered very low [51]). In our study, although average RBC DHA levels were above 6% at each visit (with higher levels in the MMS group), the lower ranges indicated that some women in both groups fell below this value. Nevertheless, RBC DHA levels in women at the lower ranges increased by a

greater extent in the MMS group compared to those in the control group over the course of the study, and reached the threshold by the third trimester (Table 2).

The omega-3 index was also significantly higher after supplementation. As RBC EPA values were comparable between groups, the increase in omega-3 index must be the result of an increase in DHA. In cardiovascular disease, the target range for the omega-3 index is 8–11%; it has been suggested that this range might also be suitable during pregnancy and lactation [52]. Reference values of 7.5–10.0% have also been recommended in pregnant women [53]. In our study, while the omega-3 index increased from 6.7% to 7.1% in the control group, the increase was greater (6.5% to 8.0%) in the MMS group. Therefore, supplementation with DHA helped women to reach target levels during pregnancy.

Current nutritional recommendations indicate that pregnant and lactating women should aim to achieve an average dietary intake of at least 200 mg DHA/day [54]. However, consumption of omega-3 fatty acids remains low particularly in pregnant and lactating women [55]. This is of relevance considering the vital roles of DHA in neurodevelopment, visual development, and neuroinflammation [56]. Moreover, pregnancy syndromes such as gestational diabetes and preeclampsia have also been associated with altered maternal omega-3 status and placental omega-3 metabolism [57–59].

The finding that there was a significant increase in calcidiol levels in supplemented women, but not in the non-supplemented control group, is also of interest. Vitamin D is essential for the health of both the developing fetus and the mother [60], and insufficient levels may have an adverse effect on skeletal homeostasis in the infant [61] and increase the maternal risk of preeclampsia [5].

In our study, no significant differences were observed between supplemented and control women regarding the markers of oxidative status. Oxidative stress has been implicated in many pathological processes during pregnancy [5]. However, this particular population of pregnant women was selectively chosen as a low-risk population, likely not at risk for decreased antioxidant status. Moreover, the sample size of the study was calculated based on the primary outcome; therefore, these results must be considered exploratory.

To our knowledge, this is the first randomized controlled trial evaluating the combination of MMS plus DHA in pregnant women. Our results indicate that in a high-income country setting, supplementation with micronutrients in combination with DHA can optimize maternal DHA status [49,62,63], despite the women in our supplemented group having a slightly lower intake of DHA from food. The timing of supplementation is important, and should occur in line with the development and growth of the embryonic brain, particularly during the later stages of pregnancy [17,21] when DHA rapidly begins to accumulate [18,22]. Furthermore, supplementation with MMS during pregnancy, as in our study, can improve maternal and infant outcomes, leading to reductions in the incidence of pre-eclampsia [64], neural-tube defects [64,65], low birthweight and small-for-gestational age babies [3], limb reduction defects, and congenital urinary tract abnormalities [64]. There may also be long-term benefits in children [4] (e.g., cognitive development [66,67]). Although many of these results have been reported from low- to middle-income countries, micronutrient levels in pregnant women are often insufficient even in industrialized countries, where dietary resources are more readily available [12]. However, the routine use of multivitamins during pregnancy has not yet been recommended in high-income countries, despite the benefits on clinical outcomes [68]. Currently, only folic acid and iron are recommended as standard interventions in pregnancy in industrialized countries [37].

Further research is necessary to better understand whether the improvements in maternal DHA status, as well as other improvements in omega-3 index and calcidiol levels, have a positive impact on maternal and infant clinical outcomes. Large, long-term randomized controlled trials on MMS supplementation including DHA are essential.

Our study has some limitations, including the lack of a placebo control group and the consequent unblinded nature of the study (which could have led to expectation bias [69]), the small sample size, and the fact that only Caucasian women were included (which limits the generalizability of the results). Adequately powered studies with a varied study population are necessary to better establish the impact of different baseline characteristics in pregnant women and to evaluate clinical outcomes.

5. Conclusions

Supplementation with MMS plus DHA in pregnant women can complement dietary intake and significantly improve maternal DHA and vitamin D status. This finding is important in light of the essential roles of DHA in the developing brain of the fetus, in visual development, and in immunomodulation.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/8/2432/s1, Table S1: Assessment schedule, Table S2: Full list of inclusion and exclusion criteria, Table S3: Composition of the multimicronutrient supplement (MMS) compared to the recommended dietary allowance (RDA) and upper tolerable limits (UL) for pregnant women, Table S4: Blood and plasma sampling for efficacy parameters in all pregnant women, Table S5: Change in primary and secondary maternal efficacy endpoints from Visit 1 to Visit 4 (gestational age week 34/36), Table S6: Infant assessments, Table S7: Daily macronutrient intakes during the study (per protocol population) compared with recommended allowances for pregnant women.

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References

1. Cazzola, R.; Russo-Volpe, S.; Miles, E.A.; Rees, D.; Banerjee, T.; Roynette, C.E.; Wells, S.J.; Goua, M.; Wahle, K.W.; Calder, P.C.; et al. Age-and dose-dependent effects of an eicosapentaenoic acid-rich oil on cardiovascular risk factors in healthy male subjects. *Atherosclerosis* **2007**, *193*, 159–167. [CrossRef] [PubMed]
2. Institute of Medicine. *Dietary Reference Intakes for Calcium and Vitamin D*; The National Academies Press: Washington, DC, USA, 2011.
3. Ramakrishnan, U.; Grant, F.K.; Goldenberg, T.; Bui, V.; Imdad, A.; Bhutta, Z.A. Effect of Multiple Micronutrient Supplementation on Pregnancy and Infant Outcomes: A Systematic Review. *Paediatr. Perinat. Epidemiol.* **2012**, *26*, 153–167. [CrossRef] [PubMed]
4. Cetin, I.; Bühling, K.; Demir, C.; Kortam, A.; Prescott, S.L.; Yamashiro, Y.; Yarmolinskaya, M.; Koletzko, B. Impact of Micronutrient Status during Pregnancy on Early Nutrition Programming. *Ann. Nutr. Metab.* **2019**, *74*, 269–278. [CrossRef]
5. Berti, C.; Cetin, I.; Agostoni, C.; Desoye, G.; Devlieger, R.; Emmett, P.M.; Ensenauer, R.; Hauner, H.; Herrera, E.; Hoesli, I.; et al. Pregnancy and Infants' Outcome: Nutritional and Metabolic Implications. *Crit. Rev. Food Sci. Nutr.* **2014**, *56*, 82–91. [CrossRef] [PubMed]
6. Cetin, I.; Berti, C.; Calabrese, S. Role of micronutrients in the periconceptional period. *Hum. Reprod. Updat.* **2009**, *16*, 80–95. [CrossRef]
7. Ramakrishnan, U.; Grant, F.; Goldenberg, T.; Zongrone, A.; Martorell, R. Effect of women's nutrition before and during early pregnancy on maternal and infant outcomes: A systematic review. *Paediatr. Perinat. Epidemiol.* **2012**, *26*, 285–301. [CrossRef]

8. Blumfield, M.L.; Hure, A.; Smith, R.; Collins, C.E.; MacDonald-Wicks, L. A systematic review and meta-analysis of micronutrient intakes during pregnancy in developed countries. *Nutr. Rev.* **2013**, *71*, 118–132. [CrossRef]
9. Elmadfa, I.; Meyer, A.; Nowak, V.; Hasenegger, V.; Putz, P.; Verstraeten, R.; Remaut-DeWinter, A.M. *European Nutrition and Health Report*; Karger Medical and Scientific Publishers: Basel, Switzerland, 2009; Volume 62, pp. 1–405.
10. Parisi, F.; Laoreti, A.; Cetin, I. Multiple Micronutrient Needs in Pregnancy in Industrialized Countries. *Ann. Nutr. Metab.* **2014**, *65*, 13–21. [CrossRef]
11. Gernand, A.D.; Schulze, K.J.; Stewart, C.P.; West, K.P.; Christian, P. Micronutrient deficiencies in pregnancy worldwide: Health effects and prevention. *Nat. Rev. Endocrinol.* **2016**, *12*, 274–289. [CrossRef]
12. Schaefer, E. Micronutrient Deficiency in Women Living in Industrialized Countries during the Reproductive Years: Is there a Basis for Supplementation with Multiple Micronutrients? *J. Nutr. Disord. Ther.* **2016**, *6*. [CrossRef]
13. World Health Organization; e-Library of Evidence for Nutrition Actions (eLENA). Daily Iron and Folic Acid Supplementation during Pregnancy. Available online: https://www.who.int/elena/titles/guidance_summaries/daily_iron_pregnancy/en/ (accessed on 31 July 2020).
14. Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. *Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. 7, Vitamin D*; National Academies Press: Washington, DC, USA, 1997. Available online: https://www.ncbi.nlm.nih.gov/books/NBK109831/. (accessed on 31 July 2020).
15. Mulligan, M.L.; Felton, S.K.; Riek, A.E.; Bernal-Mizrachi, C. Implications of vitamin D deficiency in pregnancy and lactation. *Am. J. Obstet. Gynecol.* **2010**, *202*, e421–e429. [CrossRef]
16. Dovnik, A.; Mujezinović, F. The Association of Vitamin D Levels with Common Pregnancy Complications. *Nutrients* **2018**, *10*, 867. [CrossRef] [PubMed]
17. Rees, A.; Sirois, S.; Wearden, A. Prenatal maternal docosahexaenoic acid intake and infant information processing at 4.5mo and 9mo: A longitudinal study. *PLoS ONE* **2019**, *14*, e0210984. [CrossRef] [PubMed]
18. Agostoni, C.; Nobile, M.; Ciappolino, V.; DelVecchio, G.; Tesei, A.; Turolo, S.; Crippa, A.; Mazzocchi, A.; Altamura, C.A.; Brambilla, P. The Role of Omega-3 Fatty Acids in Developmental Psychopathology: A Systematic Review on Early Psychosis, Autism, and ADHD. *Int. J. Mol. Sci.* **2017**, *18*, 2608. [CrossRef] [PubMed]
19. Echeverría, F.; Valenzuela, R.; Hernandez-Rodas, M.C.; Valenzuela, A. Docosahexaenoic acid (DHA), a fundamental fatty acid for the brain: New dietary sources. *Prostaglandins Leukot. Essent. Fat. Acids* **2017**, *124*, 1–10. [CrossRef] [PubMed]
20. Weiser, M.J.; Butt, C.M.; Mohajeri, M.H. Docosahexaenoic Acid and Cognition throughout the Lifespan. *Nutrients* **2016**, *8*, 99. [CrossRef]
21. Darnell, D.; Gilbert, S.F. Neuroembryology. *Wiley Interdiscip. Rev. Dev. Biol.* **2016**, *6*, e215. [CrossRef]
22. Delgado-Noguera, M.F.; Calvache, J.A.; Cosp, X.B.; Kotanidou, E.P.; Galli-Tsinopoulou, A. Supplementation with long chain polyunsaturated fatty acids (LCPUFA) to breastfeeding mothers for improving child growth and development. *Cochrane Database Syst. Rev.* **2015**, CD007901. [CrossRef]
23. Carlson, S.E.; Colombo, J. Docosahexaenoic Acid and Arachidonic Acid Nutrition in Early Development. *Adv. Pediatr.* **2016**, *63*, 453–471. [CrossRef]
24. Innis, S.M. The Role of Dietary n–6 and n–3 Fatty Acids in the Developing Brain. *Dev. Neurosci.* **2000**, *22*, 474–480. [CrossRef]
25. Al, M.D.M.; Van Houwelingen, A.C.; Kester, A.D.; Hasaart, T.H.; De Jong, A.E.P.; Hornstra, G. Maternal essential fatty acid patterns during normal pregnancy and their relationship to the neonatal essential fatty acid status. *Br. J. Nutr.* **1995**, *74*, 55–68. [CrossRef] [PubMed]
26. Grantham-McGregor, S.; Cheung, Y.B.; Cueto, S.; Glewwe, P.; Richter, L.; Strupp, B. Developmental potential in the first 5 years for children in developing countries. *Lancet* **2007**, *369*, 60–70. [CrossRef]
27. McCann, J.C.; Ames, B.N. Is docosahexaenoic acid, an n-3 long-chain polyunsaturated fatty acid, required for development of normal brain function? An overview of evidence from cognitive and behavioral tests in humans and animals. *Am. J. Clin. Nutr.* **2005**, *82*, 281–295. [CrossRef] [PubMed]
28. Innis, S.M. Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. *J. Pediatr.* **2003**, *143*, 1–8. [CrossRef]

29. Englund-Ögge, L.; Brantsæter, A.; Sengpiel, V.; Haugen, M.; Birgisdottir, B.E.; Myhre, R.; Meltzer, H.M.; Jacobsson, B. Maternal dietary patterns and preterm delivery: Results from large prospective cohort study. *BMJ* **2014**, *348*, g1446. [CrossRef]
30. Larqué, E.; Gil-Sánchez, A.; Prieto-Sánchez, M.T.; Koletzko, B. Omega 3 fatty acids, gestation and pregnancy outcomes. *Br. J. Nutr.* **2012**, *107* (Suppl. 2), S77–S84. [CrossRef]
31. Rogers, L.K.; Valentine, C.J.; Keim, S.A. DHA supplementation: Current implications in pregnancy and childhood. *Pharmacol. Res.* **2012**, *70*, 13–19. [CrossRef]
32. Larqué, E.; Krauss-Etschmann, S.; Campoy, C.; Hartl, D.; Linde, J.; Klingler, M.; Demmelmair, H.; Caño, A.; Gil, A.; Bondy, B.; et al. Docosahexaenoic acid supply in pregnancy affects placental expression of fatty acid transport proteins. *Am. J. Clin. Nutr.* **2006**, *84*, 853–861. [CrossRef]
33. Decsi, T.; Campoy, C.; Koletzko, B. Effect of N-3 Polyunsaturated Fatty Acid Supplementation in Pregnancy: The Nuheal Trial. *Pharm. Biotechnol.* **2005**, *569*, 109–113. [CrossRef]
34. Keats, E.C.; Haider, B.A.; Tam, E.; Bhutta, Z. A Multiple-micronutrient supplementation for women during pregnancy. *Cochrane Database Syst. Rev.* **2019**, *3*, CD004905. [CrossRef]
35. Middleton, P.; Gomersall, J.S.; Gould, J.F.; Shepherd, E.; Olsen, S.F.; Makrides, M. Omega-3 fatty acid addition during pregnancy. *Cochrane Database Syst. Rev.* **2018**, *11*, CD003402. [CrossRef] [PubMed]
36. FIGO Working Group on Good Clinical Practice in Maternal-Fetal Medicine Good clinical practice advice: Micronutrients in the periconceptional period and pregnancy. *Int. J. Gynecol. Obstet.* **2019**, *144*, 317–321. [CrossRef] [PubMed]
37. World Health Organization. Nutrition and Pregnancy. Available online: https://www.who.int/nutrition/publications/pregnant/en/ (accessed on 30 April 2020).
38. Food Nutrition Board of the Institute of Medicine. Nutrient Recommendations: Dietary Reference Intakes (DRI). National Institutes of Health, Office of Dietary Supplements. Available online: https://ods.od.nih.gov/Health_Information/Dietary_Reference_Intakes.aspx (accessed on 1 April 2020).
39. European Food Safety Authority. Dietary Reference Values for the EU: DRV Finder. Available online: https://www.efsa.europa.eu/en/interactive-pages/drvs (accessed on 25 March 2020).
40. Cazzola, R.; Cestaro, B. Red wine polyphenols protect n-3 more than n-6 polyunsaturated fatty acid from lipid peroxidation. *Food Res. Int.* **2011**, *44*, 3065–3071. [CrossRef]
41. Cazzola, R.; Rondanelli, M.; Faliva, M.; Cestaro, B. Effects of DHA-phospholipids, melatonin and tryptophan supplementation on erythrocyte membrane physico-chemical properties in elderly patients suffering from mild cognitive impairment. *Exp. Gerontol.* **2012**, *47*, 974–978. [CrossRef]
42. Cazzola, R.; Rondanelli, M.; Trotti, R.; Cestaro, B. Effects of weight loss on erythrocyte membrane composition and fluidity in overweight and moderately obese women. *J. Nutr. Biochem.* **2011**, *22*, 388–392. [CrossRef]
43. Harris, W.S.; Von Schacky, C. The Omega-3 Index: A new risk factor for death from coronary heart disease? *Prev. Med.* **2004**, *39*, 212–220. [CrossRef]
44. Wang, X.; Meng, L.; Su, C.; Shapses, S.A. Low free (but not total) 25-hydroxyvitamin d levels in subjects with normocalcemic hyperparathyroidism. *Endocr. Pract.* **2020**, *26*, 174–178. [CrossRef]
45. Cighetti, G.M.; Bamonti, F.; Aman, C.S.; Gregori, D.; De Giuseppe, R.; Novembrino, C.; De Liso, F.; Maiavacca, R.; Paroni, R. Oxidative status in different settings and with different methodological approaches compared by Receiver Operating Characteristic curve analysis. *Clin. Biochem.* **2015**, *48*, 73–78. [CrossRef]
46. Cazzola, R.; Rondanelli, M. N-Oleoyl-Phosphatidyl-Ethanolamine and Epigallo Catechin-3-Gallate Mitigate Oxidative Stress in Overweight and Class I Obese People on a Low-Calorie Diet. *J. Med. Food* **2020**, *23*, 319–325. [CrossRef]
47. Hsieh, T.-T.; Chen, S.-F.; Lo, L.-M.; Li, M.-J.; Yeh, Y.-L.; Hung, T.-H. The Association Between Maternal Oxidative Stress at Mid-Gestation and Subsequent Pregnancy Complications. *Reprod. Sci.* **2012**, *19*, 505–512. [CrossRef]
48. Vioque, J.; Navarrete-Muñoz, E.M.; Gimenez-Monzo, D.; García-de-la-Hera, M.; Granado-Lorencio, F.; Young, I.S.; Ramon, R.; Ballester, F.; Murcia, M.; Rebagliato, M.; et al. Reproducibility and validity of a food frequency questionnaire among pregnant women in a Mediterranean area. *Nutr. J.* **2013**, *12*, 26. [CrossRef] [PubMed]

49. Bergmann, R.L.; Haschke-Becher, E.; Klassen-Wigger, P.; Bergmann, K.E.; Richter, R.; Dudenhausen, J.W.; Grathwohl, D.; Haschke, F. Supplementation with 200 mg/day docosahexaenoic acid from mid-pregnancy through lactation improves the docosahexaenoic acid status of mothers with a habitually low fish intake and of their infants. *Ann. Nutr. Metab.* **2008**, *52*, 157–166. [CrossRef] [PubMed]
50. Jackson, K.H.; Harris, W.S. A Prenatal DHA Test to Help Identify Women at Increased Risk for Early Preterm Birth: A Proposal. *Nutrients* **2018**, *10*, 1933. [CrossRef] [PubMed]
51. Carlson, S.E.; Gajewski, B.J.; Valentine, C.J.; Rogers, L.K.; Weiner, C.P.; DeFranco, E.; Buhimschi, C.S. Assessment of DHA on reducing early preterm birth: The ADORE randomized controlled trial protocol. *BMC Pregnancy Childbirth* **2017**, *17*, 62. [CrossRef] [PubMed]
52. Von Schacky, C. Omega-3 Fatty Acids in Pregnancy—The Case for a Target Omega-3 Index. *Nutrients* **2020**, *12*, 898. [CrossRef]
53. Hoge, A.; Bernardy, F.; Donneau, A.-F.; Dardenne, N.; Degée, S.; Timmermans, M.; Nisolle, M.; Guillaume, M.; Castronovo, V. Low omega-3 index values and monounsaturated fatty acid levels in early pregnancy: An analysis of maternal erythrocytes fatty acids. *Lipids Health Dis.* **2018**, *17*, 63. [CrossRef]
54. Koletzko, B.; Cetin, I.; Brenna, J.T. Group Dietary fat intakes for pregnant and lactating women. *Br. J. Nutr.* **2007**, *98*, 873–877. [CrossRef]
55. Zhang, Z.; Fulgoni, V.L.; Kris-Etherton, P.M.; Mitmesser, S.H. Dietary Intakes of EPA and DHA Omega-3 Fatty Acids among US Childbearing-Age and Pregnant Women: An Analysis of NHANES 2001–2014. *Nutrients* **2018**, *10*, 416. [CrossRef]
56. Hubinont, C.; Savoye, T. Maternal and fetal benefits of DHA supplementation during pregnancy. *J. Pregnancy Reprod.* **2017**, *1*, 1. [CrossRef]
57. Cetin, I.; Alvino, G.; Cardellicchio, M. Long chain fatty acids and dietary fats in fetal nutrition. *J. Physiol.* **2009**, *587*, 3441–3451. [CrossRef]
58. Ramiro-Cortijo, D.; Herrera, T.; Rodríguez-Rodríguez, P.; De Pablo, Á.L.L.; De La Calle, M.; López-Giménez, M.R.; Mora-Urda, A.I.; Gutiérrez-Arzapalo, P.Y.; Gómez-Rioja, R.; Aguilera, Y.; et al. Maternal plasma antioxidant status in the first trimester of pregnancy and development of obstetric complications. *Placenta* **2016**, *47*, 37–45. [CrossRef] [PubMed]
59. Ramiro-Cortijo, D.; De La Calle, M.; Rodríguez-Rodríguez, P.; De Pablo, Á.L.L.; López-Giménez, M.R.; Aguilera, Y.; Martín-Cabrejas, M.A.; Gonzalez-Granado, J.M.; Arribas, S. Maternal Antioxidant Status in Early Pregnancy and Development of Fetal Complications in Twin Pregnancies: A Pilot Study. *Antioxidants* **2020**, *9*, 269. [CrossRef] [PubMed]
60. Mousa, A.; Naqash, A.; Lim, S.S. Macronutrient and Micronutrient Intake during Pregnancy: An Overview of Recent Evidence. *Nutrients* **2019**, *11*, 443. [CrossRef] [PubMed]
61. Pawley, N.; Bishop, N. Prenatal and infant predictors of bone health: The influence of vitamin D. *Am. J. Clin. Nutr.* **2004**, *80*, 1748S–1751S. [CrossRef]
62. Dunstan, J.A.; Mori, T.A.; Barden, A.; Beilin, L.J.; Holt, P.G.; Calder, P.C.; Taylor, A.L.; Prescott, S.L. Effects of n-3 polyunsaturated fatty acid supplementation in pregnancy on maternal and fetal erythrocyte fatty acid composition. *Eur. J. Clin. Nutr.* **2004**, *58*, 429–437. [CrossRef]
63. Krauss-Etschmann, S.; Shadid, R.; Campoy, C.; Hoster, E.; Demmelmair, H.; Jimenez, M.; Gil, A.; Rivero, M.; Veszprémi, B. Effects of fish-oil and folate supplementation of pregnant women on maternal and fetal plasma concentrations of docosahexaenoic acid and eicosapentaenoic acid: A European randomized multicenter trial. *Am. J. Clin. Nutr.* **2007**, *85*, 1392–1400. [CrossRef]
64. Dean, S.V.; Lassi, Z.S.; Imam, A.M.; Bhutta, Z.A. Preconception care: Nutritional risks and interventions. *Reprod. Heal.* **2014**, *11*, S3. [CrossRef]
65. Czeizel, A.E. The primary prevention of birth defects: Multivitamins or folic acid? *Int. J. Med Sci.* **2004**, *1*, 50–61. [CrossRef]
66. Supplementation with Multiple Micronutrients Intervention Trial (SUMMIT) Study Group. Effect of maternal multiple micronutrient supplementation on fetal loss and infant death in Indonesia: A double-blind cluster-randomised trial. *Lancet* **2008**, *371*, 215–227. [CrossRef]
67. Prado, E.L.; Sebayang, S.K.; Apriatni, M.; Adawiyah, S.R.; Hidayati, N.; Islamiyah, A.; Siddiq, S.; Harefa, B.; Lum, J.; Alcock, K.J.; et al. Maternal multiple micronutrient supplementation and other biomedical and socioenvironmental influences on children's cognition at age 9-12 years in Indonesia: Follow-up of the SUMMIT randomised trial. *Lancet Glob. Health* **2017**, *5*, e217–e228. [CrossRef]

68. Wolf, H.T.; Hegaard, H.; Huusom, L.D.; Pinborg, A. Multivitamin use and adverse birth outcomes in high-income countries: A systematic review and meta-analysis. *Am. J. Obstet. Gynecol.* **2017**, *217*, 404.e1–404.e30. [CrossRef] [PubMed]
69. Staudacher, H.M.; Irving, P.M.; Lomer, M.C.; Whelan, K. The challenges of control groups, placebos and blinding in clinical trials of dietary interventions. *Proc. Nutr. Soc.* **2017**, *76*, 203–212. [CrossRef] [PubMed]

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Concentration-Dependent Effects of N-3 Long-Chain Fatty Acids on Na,K-ATPase Activity in Human Endothelial Cells

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Abstract: N-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) seem to prevent endothelial dysfunction, a crucial step in atherogenesis, by modulating the levels of vasoactive molecules and by influencing Na,K-ATPase activity of vascular myocytes. The activity of endothelial Na,K-ATPase controls the ionic homeostasis of the neighboring cells, as well as cell function. However, controversy exists with respect to the vascular protective effect of EPA and DHA. We argue that this dispute might be due to the use of different concentrations of EPA and DHA in different studies. Therefore, this study was designed to define an optimal concentration of EPA and DHA to investigate endothelial function. For this purpose, human endothelial cells were exposed for 24 h to different concentrations of DHA or EPA (0–20 μM) to study membrane fluidity, peroxidation potential and Na,K-ATPase activity. EPA and DHA were linearly incorporated and this incorporation was mirrored by the linear increase of unsaturation index, membrane fluidity, and peroxidation potential. Na,K-ATPase activity peaked at 3.75 μM of EPA and DHA and then gradually decreased. It is noteworthy that DHA effects were always more pronounced than EPA. Concluding, low concentrations of EPA and DHA minimize peroxidation sensitivity and optimize Na,K-ATPase activity.

Keywords: endothelium; sodium pump; eicosapentaenoic acid; docosahexaenoic acid; membrane fluidity; lipid peroxidation

1. Introduction

The Na,K-ATPase or Na^+ pump (EC 3.6.37) is an ubiquitous membrane transport protein whose activity determines and maintains high K^+ and low Na^+ concentrations in the cytoplasm, generates a potential across the membrane and provides the driving force for secondary ion transport [1]. Na,K-ATPase consists of two subunits: A large ouabain-sensitive polypeptide (α) responsible for transporting ions and a smaller glycoprotein (β) needed for enzymatic activity. These subunits are closely associated with lipids that modulate the activity of the pump [2]. Ion homeostasis, that is maintained by Na,K-ATPase, is critical for numerous cellular functions and processes, including cell growth, differentiation, migration, contraction, secretion, and volume regulation [3]. At vascular level, Na,K-ATPase plays a key role in modulating blood pressure. Experimental evidence has shown that Na^+ pump inhibition causes the contraction of smooth muscle cells [4–6] by promoting Ca^{2+} entry into the smooth muscle cells through the inversion of the Na^+-Ca^{2+} exchange system. Endothelial cells modulate vascular tone by releasing vasoactive mediators [1] and by permitting active solute transport

between lumen and sub-endothelium. In particular, an asymmetrical localization of Na,K-ATPase between luminal and abluminal membranes of endothelial cells [4,5] determines trans-endothelial ionic gradients. These gradients control ionic homeostasis and the performance of the neighboring smooth muscle cells [7], while transendothelial K^+ and Na^+ transport regulates the activity of myocytal Na,K-ATPase [8], crucial for the regulation of vascular tone.

Controversy exists about the role of n-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in cardiovascular disease prevention and endothelial function [9–11]. Several human studies suggest that high levels of the n-3 long-chain (LC) polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid and docosahexaenoic acid in membrane phospholipids reduce cardiovascular risk [12,13] and improve endothelial function [14]. N-3 LC-PUFAs seem to regulate blood pressure and improve vascular integrity by preventing vascular inflammatory and adhesion cascades, and by enhancing the generation and bioavailability of nitric oxide through upregulation and the activation of endothelial nitric oxide synthase [15]. Surprisingly, incubation with high concentrations of n-3 LC-PUFAs has been shown to decrease Na, K-ATPase activity of human umbilical vein endothelial cells [16] and sheep pulmonary artery rings [17]. However, the underlying mechanisms of the effects of EPA and DHA have not yet been fully clarified. Na,K-ATPase activity is regulated by several factors, including the availability of substrates [18], hormones [19], circulating endogenous inhibitors, such as cardiotonic steroids (ouabain, digoxin, etc.) [3], as well as the chemical-physical features of the surrounding membrane lipid micro-environment, and in particular, the degree of membrane fluidity, the ratio between phospholipids and cholesterol [20]. It is widely believed that lipids that increase membrane fluidity promote Na,K-ATPase activation, and, vice versa, those that stiffen the membranes reduce pump activity [20].

Membrane lipids, such as phospholipid species, their fatty acyl chain length and degree of unsaturation, and cholesterol content all contribute to the fluidity of the membranes. In general, low cholesterol content and high degree of unsaturation of phospholipid fatty acyl chains are associated with fluid membranes [21–23]. The most unsaturated PUFAs in mammalian cell membranes are the long chain polyunsaturated FAs (LC-PUFAs) of the n-3 and n-6 families, such as EPA (C20:5 n-3), DHA (C22:6 n-3) and arachidonic acid (ARA, C20:4 n-6) derived from the nutritionally essential linolenic acid (C18:3 n-3), and linoleic acid (C18:2 n-6), respectively. The LC-PUFAs of these two families compete for the enzymes needed for their synthesis from the C18 precursors as well as for the enzymes that synthesize their derivatives. A diet rich in n-3 PUFA, or a dietary supplementation of EPA and/or DHA, increases the concentration of these FAs in membranes at the expense of ARA and, consequently, increases the degree of unsaturation of membrane lipids. It also reduces the levels of vasoconstrictor lipid mediators originating from ARA [24,25]. Moreover, recent studies on lipid bilayers and cellular systems have shown that EPA and DHA influence the size and order of membrane lipid microdomain. However, the complex effects of n-3 LC-PUFAs on membrane remodeling still need to be clarified [26].

The higher number of double bonds predispose LC-PUFAs to both enzymatic and non-enzymatic oxygenation. Enzymatic oxygenation gives rise to a plethora of metabolites that modulate receptor signaling and gene expression [27–29], whereas the non-enzymatic oxidation or peroxidation determines LC-PUFAs degradation to cytotoxic products, including peroxides and aldehydes that can greatly alter the physicochemical properties of membrane lipid bilayers [28,30,31], also through a reduction of their fluidity [32]. Moreover, lipid peroxidation-derived aldehydes react selectively with proteins or enzymes containing SH groups such as Na,K-ATPase, altering their functions [33]. Since peroxidation potential of PUFA increases with the degree of unsaturation, the enrichment in n-3 LC-PUFAs could enhance membrane lipid susceptibility to peroxidation. Therefore, high concentrations of LC-PUFA on one hand could improve Na,K-ATPase's activity through their fluidizing action on membranes, on the other hand, they could reduce pump activity by increasing peroxidation potential.

The controversy around the vascular protective effect of EPA and DHA might result from the different concentrations of these compounds used in different studies. Therefore, we designed the present study to investigate the effects of different concentrations of EPA or DHA on Na,K-ATPase

activity and membrane chemical-physical parameters that could affect the activity of this ATPase, such as the degree of membrane fluidity and the susceptibility to lipid peroxidation in human microvascular endothelial cells (HMEC).

2. Results and Discussion

2.1. Incorporation of N-3 LC-PUFA in Confluent HMEC

Membrane phospholipid (PL) fatty acid composition results from both *de novo* synthesis (Kennedy's pathway) and PL remodeling, through the deacylation-reacylation processes (Lands' pathway). The extent of the contribution of these two pathways varies from cell-to-cell, and depends on how fatty acids are supplied to cells. Human endothelial cells incorporate both free fatty acids and fatty acid-albumin complexes added to culture medium [34]. Since free FA form micelles in solution and act as detergents in the presence of lipid membranes [35], we have supplemented HMEC with EPA or DHA complexed with human albumin. PUFA supplied to human endothelial cells as albumin complexes are readily incorporated into the cellular PLs and, after 24 h of incubation, were found mainly (95%) in membranes where they modify fatty acid composition of PLs primarily via the Lands' pathway [34].

Since 18 h of incubation with lipids suffice to modulate Na,K-ATPase activity of human endothelial cells [16,36], we considered it reasonable to perform our experiments after 24 h exposure to EPA or DHA in HMEC.

As shown in Figure 1, 24 h of incubation with different concentrations of EPA or DHA did not affect cellular viability.

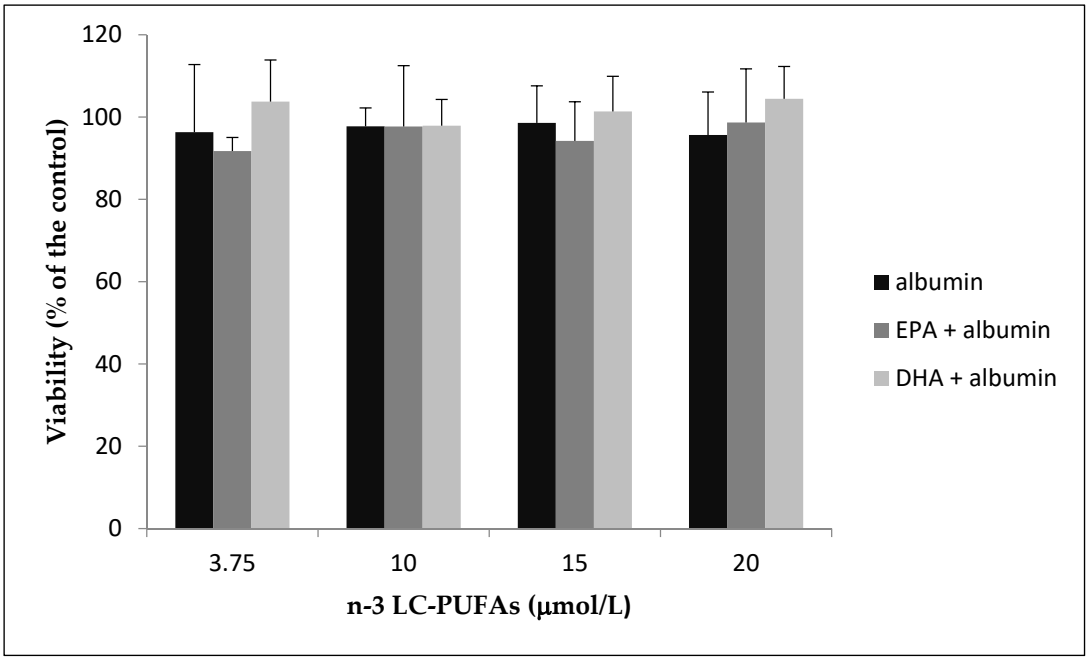

Figure 1. HMEC were incubated with increasing amounts of N-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) complexed with human defatted albumin or an equivalent amount of human defatted albumin (control) for 24 h. Viable cells were evaluated by MTT assay. Results from three separate experiments are shown as % of the control ± standard deviation.

Cell lipids were then extracted, and the levels of EPA and DHA measured by gas chromatography. The incorporation of both fatty acids after 24 h of incubation is reported in Figure 2. We found significantly different amounts of EPA and DHA in unstimulated cells. Indeed, the basal levels of EPA and DHA were 0.408 ± 0.019 nmol/10^6 cells and 3.8 ± 0.24 nmol/10^6 cells, respectively (Figure 2A).

The highest value incorporated of EPA and DHA were 18.0 ± 0.99 nmol/ 10^6 cells, and 16.9 ± 0.97 nmol/ 10^6 cells, respectively (Figure 2B).

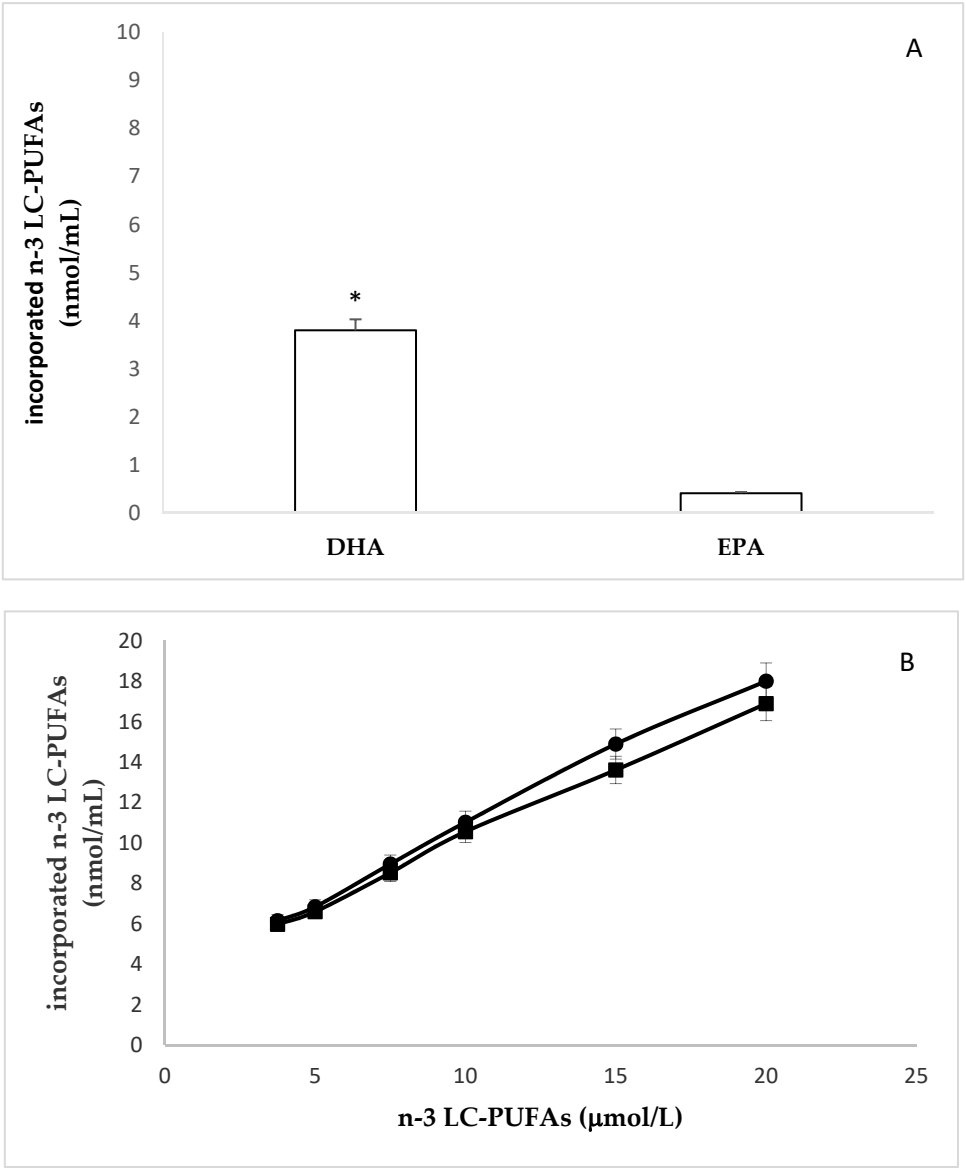

Figure 2. *Cont*.

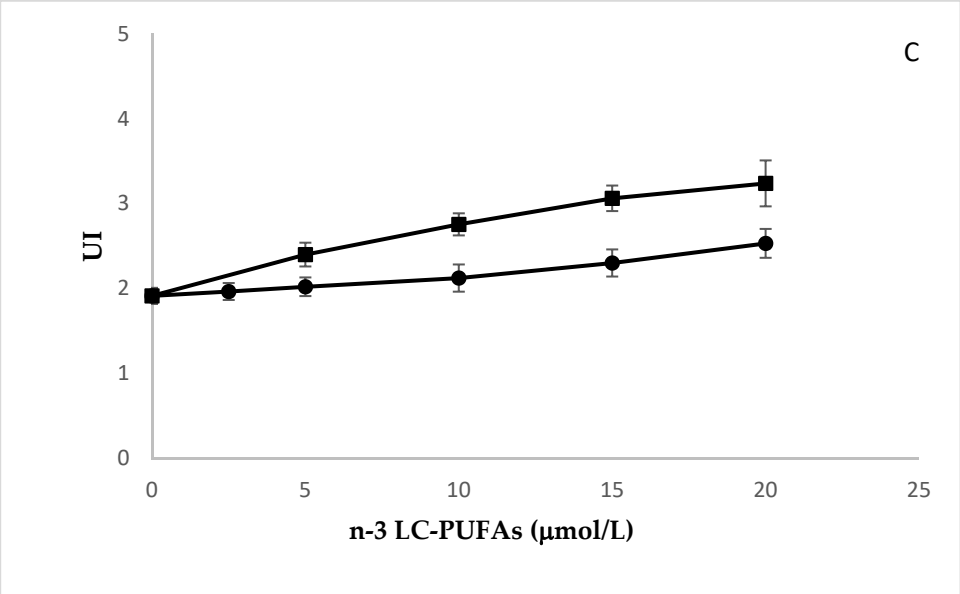

Figure 2. Incorporation of EPA or DHA in HMEC. (**A**): Basal levels of EPA and DHA. * EPA vs DHA $p \leq 0.001$ (**B**): incorporation of EPA (●) and DHA (■) after 24 h of cell incubation with increasing amounts of these n-3-LC-PUFAs. (**C**): Changes of unsaturation index (UI) after cell incubation with increasing amounts of EPA (●) and DHA (■). Pearson's r = 0.9999, $p < 0.0001$ and Pearson's r = 0.9996, $p < 0.0001$. Slope EPA vs. slope DHA $p \leq 0.0001$.

In agreement with previous studies [34], fatty acid analysis of HMEC, enriched with EPA and DHA, revealed an increase of the ratio between saturated and polyunsaturated fatty acids, and a consequent increase in the concentration of double bonds in membrane phospholipids (unsaturation index, UI). As expected and depicted in Figure 2C, at each dose of n-3 LC-PUFA added to cell culture medium, DHA (six double bonds) determined a greater increase of UI than EPA (five double bonds).

All the experiments were repeated at least three times. Data are expressed as mean ± SD of replicates.

2.2. Effects of N-3 LC-PUFA Incorporation on Membrane Fluidity in HMEC

Membrane fluidity after incubation with EPA and DHA was evaluated in HMEC by measuring fluorescence anisotropy (rs) of 1,6-diphenyl-1,3,5-hexatriene (DPH), a hydrophobic compound almost non-fluorescent in water. When supplied to the cells, it is readily absorbed into the membranes where, after intercalation with PL acyl chains, it becomes fluorescent. Rs is a measure of the rotational mobility of this fluorophore when it is excited with polarized light. The higher the rs, the lower the fluidity, and vice versa. We found that the incorporation of increasing amounts of EPA and DHA fluidized membranes, as indicated by the corresponding rs decrease (Figure 3). Interestingly, DHA increased membrane fluidity significantly more than EPA. The study of correlations showed that the concentration of these n-3 LC-PUFA significantly correlates with rs (Pearson's r = 0.9999, $p < 0.0001$ and Pearson's r = 0.9996, $p < 0.0001$; EPA and DHA, respectively). The comparison of linear regression curves by one-way ANOVA showed significant differences between the slopes ($p \leq 0.0001$). These results are in agreement with previous studies, showing that the unsaturation index (UI) of membrane phospholipids is one of the major factors influencing membrane fluidity [21–23]

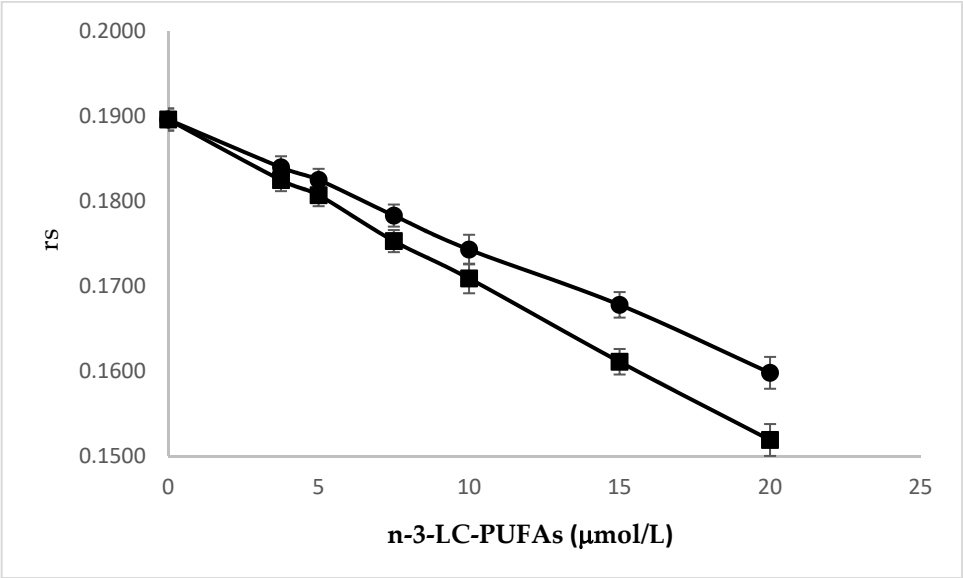

Figure 3. Effect of the incubation with increasing amounts of EPA (●) and DHA (■) on fluorescence anisotropy (rs) of DPH in HMEC membranes. Experiments were performed in triplicate. Data are expressed as mean ± SD of replicates. Pearson's r = 0.9999, $p < 0.0001$ and Pearson's r = 0.9996, $p < 0.0001$; EPA and DHA, respectively. Slope EPA vs. slope DHA $p \leq 0.0001$.

2.3. Effects of N-3 LC-PUFA Incorporation on Peroxidation Potential in HMEC

In confluent HMEC enriched with EPA or DHA, peroxidation was induced by a flux of aqueous peroxyl radicals derived from the thermal decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and monitored by measuring intracellular oxidation of dichlorofluorescin diacetate (DCFD). The increase of EPA and DHA contents in the cells was associated with an increase in susceptibility to peroxidation, as shown by the concentration-dependent increase of the propagation rate (slope) of the kinetic peroxidation curves (Figure 4). The study of correlations showed that the concentration of these n-3 LC-PUFA significantly correlated with rs (Pearson's r = 0.9921, $p \leq 0.0001$ and Pearson's r = 0.9973, $p \leq 0.0001$; EPA, and DHA, respectively). The comparison of linear regression curves by ANOVA showed significant differences between slopes ($p \leq 0.0001$). The concentration-dependent curves indicate that the effect of DHA was significantly higher than that of EPA.

AAPH is an extensively reported generator of free radicals that are physiologically relevant to biological systems [37,38]. The kinetic profile of peroxidation induced by AAPH is typically sigmoidal and characterized by a latency phase, a propagation phase, and termination [38]. In our experimental model, AAPH was added to culture medium, which contains antioxidants that protect the cells against peroxidation. Once the antioxidants are exhausted, AAPH radicals can attack the molecules of cell membranes. Given that PLs are more sensitive to these radical species than cholesterol and proteins [39], it is reasonable to propose that the PUFA of PLs are the first substrates to be oxidized, thereby promoting the initial formation of lipid peroxide in cell membranes. Lipid peroxidation of the membranes promotes the alteration of the redox homeostasis of cells and consequently the oxidation of DCFD. The compositional variation, due to the increased incorporation of n-3 LC-PUFAs, resulted in a higher concentration of carbon-carbon double bonds, which are the substrates of lipid peroxidation reactions. Moreover, the consequent increase in membrane fluidity renders PUFA easily accessible by the oxidants. As mentioned above, the incorporation of DHA increased IU and the fluidity of the membrane more than EPA.

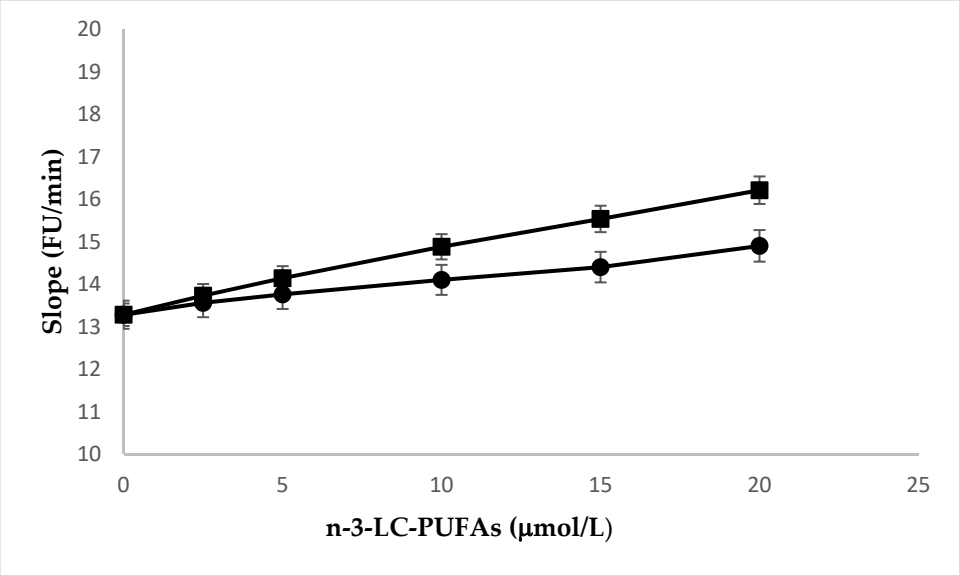

Figure 4. Effect of the incubation with increasing amounts of EPA (●) and DHA (■) enrichment on peroxidation kinetics of HMEC. Experiments were performed in triplicate. Data are expressed as mean ± SD of replicates. Pearson's r = 0.9921, $p < 0.0001$ and Pearson's r = 0.9973, $p < 0.0001$; EPA and DHA, respectively. Slope EPA vs. slope DHA $p \leq 0.0001$.

2.4. Effects of N-3 LC-PUFA Incorporation on Na,K-ATPase

Na,K-ATPase in the endothelium was the topic of several investigations. In cerebral endothelial cells, the inhibition of the pump increased Ca^{2+} release from the endoplasmic reticulum, thus leading to endothelial injury [40]. Accordingly, in the present study, we tested the effect of EPA or DHA exposure on Na,K-ATPase activity in HMEC. As reported in Figure 5, the activity of the pump peaked with the lowest concentration of EPA and DHA utilized (3.75 µM) and then gradually decreased as the concentration of these n-3 LC-PUFA increased. Interestingly, DHA-induced peak was significantly higher than EPA's ($p < 0.001$, Student's *t* test). It is complex to translate these results to human physiology, because serum levels of these nutritional essential fatty acids are influenced by several factors, including genetic factors, diet, and lipemia. A few studies report the serum levels of EPA, ranging from approximately 1 µmol/L [41], to 400 µmol/L [42] while the concentrations of DHA varied between 2.3 µmol/L [41] and 580 µmol/L [42]. In our experimental model, the lowest concentration of n-3 LC-PUFAs added to culture medium was 3.75 µM, which falls within the physiological range [41,42], and determined a 3.5-fold and 1.5-fold increase of EPA and DHA, respectively, if compared to the basal level ($p < 0.001$, Student's *t* test). Based on the concentration-dependent effects of n-3 LC-PUFAs, on all the parameters that we have measured, this seems to be the correct concentration to optimize the activity of Na, K-ATPase, since it exerts a fluidifying action without appreciably influencing peroxidability.

On the contrary, higher amounts of these PUFAs alter the chemical-physical properties of the membranes and inhibit the pump activity. Mayol et al. found that EPA and DHA inhibited Na,K-ATPase activity of macrovascular human endothelial cells by adding these n-3 LC-PUFAs to a culture medium at much higher concentrations (0.1 mM in form of emulsion with lecithin) than the ones we used in this study [16]. Similarly, Singh et al. found that 30 µM EPA inhibited pump activity in pulmonary vessel rings [17].

To the best of our knowledge, this paper is the first to describe a concentration dependent effect of n-3 LC-PUFA on Na^+ pump activity in endothelial cells and to individuate a concentration that minimizes detrimental effects and optimizes pump activity.

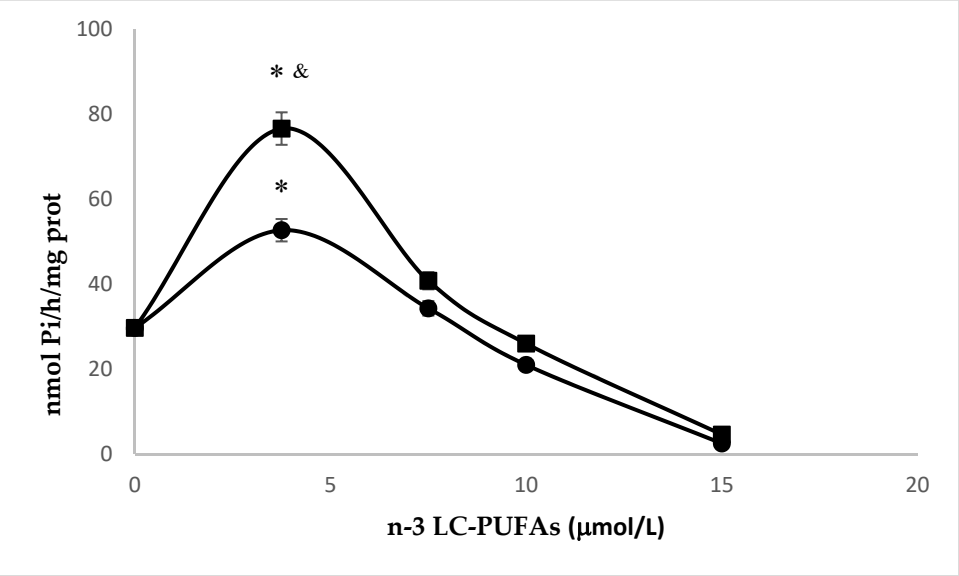

Figure 5. Effect of the incubation with increasing amounts of EPA (●) or DHA (■) on Na,K-ATPase activity of HMEC. Experiments were performed in triplicate. Data are expressed as mean ± SD of replicates. The incubation of HMEC for 24 h with 3.75 µM of n-3 LC-PUFAs significantly increased pump activity when compared to basal levels and the other concentrations (*, $p < 0.001$, Student's *t* test). DHA vs. EPA, $p < 0.001$, Student's *t* test ($^{\&}$).

3. Materials and Methods

3.1. Cell Culture

HMEC (LGC Standards-ATCC, Sesto S.G (MI), Italy) were grown in MCDB131 containing epidermal growth factor (EGF) (10 ng/mL), glutamine (2 mM) and 10% fetal bovine serum (FBS) on 2% gelatin-coated flasks. All culture reagents were from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). HMEC grown to confluence were incubated for 24 h in the presence of increasing amounts (from 3.75 to 20 µM) of DHA or EPA (Sigma-Aldrich, Milan, Italy) in complex with human defatted albumin (Sigma-Aldrich, Milan, Italy) or human defatted albumin (control). To test cell viability, the MTT assay was utilized as previously described [43]. In other experiments, the cells were harvested for lipid extraction and subsequent determination of membrane fluidity, peroxidation potential and Na,K-ATPase activity.

3.2. Fatty Acids Analysis

To determine cell fatty acid composition, lipids were extracted in 2:1 chloroform/methanol (Sigma-Aldrich, Milan, Italy) containing 0.2% butylated hydroxytoluene (Merck, Darmstadt, Germany), according to the method by Folch et al. [44]. The lipid extracts were evaporated under nitrogen stream and trans-methylated with methanol/BF_3 (Sigma-Aldrich, Milan, Italy) at 90 °C for 2 h. Fatty acid methyl esters were extracted 3-fold with hexane, concentrated under nitrogen stream and analyzed using capillary gas chromatography as previously described [45]. The amount of each considered fatty acid was calculated as nmol/10^6 cells. The degree of unsaturation (unsaturation index, U.I.) was calculated as the sum of each unsaturated fatty acid concentration, multiplied by its double bond number and divided by the total unsaturated fatty acid concentration.

3.3. Membrane Fluidity and Peroxidation Potential

The effects of the enrichment of HMEC with n-3 LC-PUFA on both peroxidation potential and membrane properties were determined after 24 hours of incubation performed, as described above. Membrane fluidity status was determined measuring the anisotropy of the fluorescent probe

1,6-diphenyl-1,3,5-hexatriene (DPH) [32,46]. The DPH probe was excited at a wavelength of 340 nm, and the emission wavelength was set at 420 nm. Samples were then excited with vertically polarized light and the intensity of the emitted light, vertically (I_v) and horizontally (I_h) polarized, were measured. Anisotropy (rs) was calculated with the equation: $r_s = I_v - I_h/I_v + 2I_h$

Membrane fluidity is a biophysical property of membranes that quantitatively expresses the mobility and the rate of membrane lipid molecule rotational motion. The anisotropy of DPH is inversely related to membrane fluidity when inserted between the outermost portions of the fatty acyl chains.

The sensitivity to peroxidation was determined by detecting the kinetics of intracellular reactive oxygen species formation after adding 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C, using dichlorofluorescin diacetate as probe [47]. AAPH is a thermolabile compound that can give rise to a flux of hydro-soluble free radicals. The slope of the kinetics of the peroxidation curves was used as an index of peroxidation sensitivity.

3.4. Na,K-ATPase Activity

The effects of n-3 LC-PUFA enrichment on the activity of the membrane enzyme Na,K-ATPase were measured by determining its inhibition by oubain (1 mM), as previously described [48]. This concentration guarantees the inhibition of both the isoforms of human endothelial cell sodium pump [16]. Briefly, after 24 h of incubation performed as described above, the cells were trypsinized, centrifuged (Megafuge 8 series, Heraeus™, Thermo Scientific, Monza, Italy) at 120× *g* for 5 min and washed twice with PBS. To lyze the cells, HMEC were resuspended in 25 volumes of cold hypotonic buffer (10 mM Tris-HCl, pH 7.4) and incubated on ice for 5 min. Lysates were then centrifuged for 30 min at 100,000× *g*, 4 °C (Optima Max, Beckman Coulter, Cassina De' Pecchi, Milan, Italy). The membrane pellet was then re-suspended in cold hypotonic buffer and the centrifugation step was repeated 3 times. Protein concentrations were determined by the method of Lowry et al. [49] using bovine serum albumin (Sigma-Aldrich, Milan, Italy) as a standard. ATP-ase activity was measured by preincubating the cells at 37 °C for 10 with 92 mM tris-HCl pH 7.4 containing 100 mM NaCl, 20 mM KCl, 5 mM $MgSO_4$,1 mM EDTA (Sigma-Aldrich, Milan, Italy), with or without 1 mM oubain (Sigma-Aldrich, Milan, Italy). After incubation at 37 °C for 10 min with 4 mM vanadate-free ATP (Sigma-Aldrich, Milan, Italy), the reaction was stopped by adding ice-cold trichloroacetic acid (final concentration 5%). Cells were then centrifuged for 10 min at 5,500× *g* at 4 °C and the supernatant was used for the determination of inorganic phosphate (Pi) [50]. Pump activity was calculated as the difference between the Pi concentrations, obtained in the presence or in the absence of oubain, and expressed as nmol Pi/hour/mg protein.

3.5. Statistical Analysis

All the measurements were repeated at least three times in triplicate. Data are expressed as the mean ± SD. The linear relationships between n-3 LC-PUFA concentration and EPA and DHA incorporation, membrane fluidity and peroxidation potential were assessed using Pearson's correlations. Linear regression curves were compared by one-way ANOVA. The significance of the difference between the means was assessed by unpaired *t*-test. A p-value ≤ 0.05 was considered statistically significant. Statistical analysis was performed by using StatistiXL software (version 1.5; StatistiXL, Nedlands, Australia).

4. Conclusions

Increased dietary intake of long-chain n-3 polyunsaturated fatty acids has been shown to be beneficial for the vascular tree [51]. However, controversies exist on this topic [10]. Recently, EPA was shown to improve endothelial function [52]. This is crucial since endothelial cells are considered the gatekeeper of vascular health. Whereas high concentrations alter membrane properties and inhibit Na,K-ATPase pump activity, our study highlights that a low concentration, namely 3.75 μM/mL, of EPA and DHA minimizes peroxidation potential and optimizes activity. While this concentration will

be useful for designing new *in vitro* and *in vivo* studies that might reconcile the contrasting reports available in the literature, it should be noted that our results are complex to translate into clinical observation because the normal range of EPA and DHA fall within a very wide range.

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References

1. Marín, J.; Redondo, J. Vascular sodium pump: Endothelial modulation and alterations in some pathological processes and aging. *Pharmacol. Ther.* **1999**, *84*, 249–271. [CrossRef]
2. Cornelius, F.; Habeck, M.; Kanai, R.; Toyoshima, C.; Karlish, S.J.D. General and specific lipid–protein interactions in Na,K-ATPase. *Biochim. Biophys. Acta Biomembr.* **2015**, *1848*, 1729–1743. [CrossRef] [PubMed]
3. Matchkov, V.V.; Krivoi, I.I. Specialized Functional Diversity and Interactions of the Na,K-ATPase. *Front. Physiol.* **2016**, *7*, 179. [CrossRef] [PubMed]
4. Maigaard, S.; Forman, A.; Andersson, K.-E. Digoxin inhibition of relaxation induced by prostacyclin and vasoactive intestinal polypeptide in small human placental arteries. *Placenta* **1985**, *6*, 435–443. [CrossRef]
5. Marín, J.; Sánchez-Ferrer, C.F.; Salaices, M. Effects of ouabain on isolated cerebral and femoral arteries of the cat: a functional and biochemical study. *Br. J. Pharmacol.* **1988**, *93*, 43–52. [CrossRef]
6. Vassalle, M. Contribution of the Na+/K+-pump to the membrane potential. *Experientia* **1987**, *43*, 1135–1140. [CrossRef]
7. Fransen, P.; Hendrickx, J.; Brutsaert, D.L.; Sys, S.U. Distribution and role of Na+/K+-ATPase in endocardial endothelium. *Cardiovasc. Res.* **2001**, *52*, 487–499. [CrossRef]
8. Zhang, J.; Lee, M.Y.; Cavalli, M.; Chen, L.; Berra-Romani, R.; Balke, C.W.; Bianchi, G.; Ferrari, P.; Hamlyn, J.M.; Iwamoto, T.; et al. Sodium pump α2 subunits control myogenic tone and blood pressure in mice. *J. Physiol.* **2005**, *569*, 243–256. [CrossRef]
9. Colussi, G.; Catena, C.; Novello, M.; Bertin, N.; Sechi, L.A. Impact of omega-3 polyunsaturated fatty acids on vascular function and blood pressure: Relevance for cardiovascular outcomes. *Nutr. Metab. Cardiovasc. Dis.* **2017**, *27*, 191–200. [CrossRef]
10. Maki, K.C.; Dicklin, M.R. Omega-3 fatty acid supplementation and cardiovascular disease risk: Glass half full or time to nail the coffin shut? *Nutrients* **2018**, 10. [CrossRef]
11. Bowen, K.J.; Harris, W.S.; Kris-Etherton, P.M. Omega-3 Fatty Acids and Cardiovascular Disease: Are There Benefits? *Curr. Treat. Options Cardiovasc. Med.* **2016**, 18. [CrossRef] [PubMed]
12. Endo, J.; Arita, M. Cardioprotective mechanism of omega-3 polyunsaturated fatty acids. *J. Cardiol.* **2016**, *67*, 22–27. [CrossRef] [PubMed]
13. Jain, A.P.; Aggarwal, K.K.; Zhang, P.-Y. Omega-3 fatty acids and cardiovascular disease. *Eur. Rev. Med. Pharmacol. Sci.* **2015**, *19*, 441–445. [PubMed]
14. Zehr, K.R.; Walker, M.K. Omega-3 polyunsaturated fatty acids improve endothelial function in humans at risk for atherosclerosis: A review. *Prostaglandins Other Lipid Mediat.* **2018**, *134*, 131–140. [CrossRef]
15. Balakumar, P.; Taneja, G. Fish oil and vascular endothelial protection: Bench to bedside. *Free Radic. Biol. Med.* **2012**, *53*, 271–279. [CrossRef]
16. Mayol, V.; Duran, M.J.; Gerbi, A.; Dignat-George, F.; Lévy, S.; Sampol, J.; Maixent, J.M. Cholesterol and omega-3 fatty acids inhibit Na, K-ATPase activity in human endothelial cells. *Atherosclerosis* **1999**, *142*, 327–333. [CrossRef]
17. Singh, T.; Garg, S.; Mishra, S. Evaluation of effects of eicosapentaenoic acid on Na^+-K^+-ATPase in sheep pulmonary artery. *Hum. Exp. Toxicol.* **2012**, *31*, 579–587. [CrossRef]
18. Soltoff, S.P.; Mandel, L.J. Active ion transport in the renal proximal tubule. II. Ionic dependence of the Na pump. *J. Gen. Physiol.* **1984**, *84*, 623–642. [CrossRef]
19. Ewart, H.S.; Klip, A. Hormonal regulation of the Na + -K +-ATPase: mechanisms underlying rapid and sustained changes in pump activity. *Am. J. Physiol. Physiol.* **1995**, *269*, C295–C311. [CrossRef]

20. Therien, A.G.; Blostein, R. Mechanisms of sodium pump regulation. *Am. J. Physiol. Physiol.* **2000**, *279*, C541–C566. [CrossRef]
21. Cazzola, R.; Rondanelli, M.; Russo-Volpe, S.; Ferrari, E.; Cestaro, B. Decreased membrane fluidity and altered susceptibility to peroxidation and lipid composition in overweight and obese female erythrocytes. *J. Lipid Res.* **2004**, *45*, 1846–1851. [CrossRef] [PubMed]
22. Rodrigo, R.; Miranda-Merchak, A.; Valenzuela Grau, R.; Bachler, J.P.; Vergara, L. Modulation of (Na,K)-ATPase activity by membrane fatty acid composition: therapeutic implications in human hypertension. *Clin. Exp. Hypertens.* **2014**, *36*, 17–26. [CrossRef] [PubMed]
23. Hashimoto, M.; Hossain, S.; Yamasaki, H.; Yazawa, K.; Masumura, S. Effects of eicosapentaenoic acid and docosahexaenoic acid on plasma membrane fluidity of aortic endothelial cells. *Lipids* **1999**, *34*, 1297–1304. [CrossRef] [PubMed]
24. Simopoulos, A.P. Omega-3 Fatty Acids in Inflammation and Autoimmune Diseases. *J. Am. Coll. Nutr.* **2002**, *21*, 495–505. [CrossRef]
25. Simonetto, M.; Infante, M.; Sacco, R.L.; Rundek, T.; Della-Morte, D. A Novel Anti-Inflammatory Role of Omega-3 PUFAs in Prevention and Treatment of Atherosclerosis and Vascular Cognitive Impairment and Dementia. *Nutrients* **2019**, *11*, 2279. [CrossRef]
26. Shaikh, S.R.; Kinnun, J.J.; Leng, X.; Williams, J.A.; Wassall, S.R. How polyunsaturated fatty acids modify molecular organization in membranes: Insight from NMR studies of model systems. *Biochim. Biophys. Acta Biomembr.* **2015**, *1848*, 211–219. [CrossRef]
27. Schmitz, G.; Ecker, J. The opposing effects of n−3 and n−6 fatty acids. *Prog. Lipid Res.* **2008**, *47*, 147–155. [CrossRef]
28. Ayala, A.; Muñoz, M.F.; Argüelles, S. Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid. Med. Cell. Longev.* **2014**, *2014*, 360438. [CrossRef]
29. Bonafini, S.; Fava, C. Omega-3 fatty acids and cytochrome P450-derived eicosanoids in cardiovascular diseases: Which actions and interactions modulate hemodynamics? *Prostaglandins Other Lipid Mediat.* **2017**, *128–129*, 34–42. [CrossRef]
30. Cholewski, M.; Tomczykowa, M.; Tomczyk, M. A Comprehensive Review of Chemistry, Sources and Bioavailability of Omega-3 Fatty Acids. *Nutrients* **2018**, *10*. [CrossRef]
31. Catalá, A. Five decades with polyunsaturated Fatty acids: chemical synthesis, enzymatic formation, lipid peroxidation and its biological effects. *J. Lipids* **2013**, *2013*, 710290. [CrossRef] [PubMed]
32. Cazzola, R.; Rondanelli, M.; Trotti, R.; Cestaro, B. Effects of weight loss on erythrocyte membrane composition and fluidity in overweight and moderately obese women. *J. Nutr. Biochem.* **2011**, *22*, 388–392. [CrossRef] [PubMed]
33. Thomas, C.E.; Reed, D.J. Radical-induced inactivation of kidney Na+,K+-ATPase: Sensitivity to membrane lipid peroxidation and the protective effect of vitamin E. *Arch. Biochem. Biophys.* **1990**, *281*, 96–105. [CrossRef]
34. Héliès-Toussaint, C.; Gambert, S.; Roller, P.; Tricot, S.; Lacour, B.; Grynberg, A. Lipid metabolism in human endothelial cells. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2006**, *1761*, 765–774. [CrossRef] [PubMed]
35. Henriksen, J.R.; Andresen, T.L.; Feldborg, L.N.; Duelund, L.; Ipsen, J.H. Understanding detergent effects on lipid membranes: a model study of lysolipids. *Biophys. J.* **2010**, *98*, 2199–2205. [CrossRef] [PubMed]
36. Lau, Y.T. Cholesterol enrichment inhibits Na+/K+ pump in endothelial cells. *Atherosclerosis* **1994**, *110*, 251–257. [CrossRef]
37. Niki, E. Free radical initiators as source of water- or lipid-soluble peroxyl radicals. *Methods Enzymol.* **1990**, *186*, 100–108.
38. Pinchuk, I.; Lichtenberg, D. Analysis of the kinetics of lipid peroxidation in terms of characteristic time-points. *Chem. Phys. Lipids* **2014**, *178*, 63–76. [CrossRef]
39. Cazzola, R.; Cassani, E.; Barichella, M.; Cestaro, B. Impaired fluidity and oxidizability of HDL hydrophobic core and amphipathic surface in dyslipidemic men. *Metabolism.* **2013**, *62*. [CrossRef]
40. Kurauchi, Y.; Hisatsune, A.; Seki, T.; Katsuki, H. Na+, K+-ATPase dysfunction causes cerebrovascular endothelial cell degeneration in rat prefrontal cortex slice cultures. *Brain Res.* **2016**, *1644*, 249–257. [CrossRef]
41. Liu, W.; Xie, X.; Liu, M.; Zhang, J.; Liang, W.; Chen, X. Serum ω-3 Polyunsaturated Fatty Acids and Potential Influence Factors in Elderly Patients with Multiple Cardiovascular Risk Factors. *Sci. Rep.* **2018**, *8*. [CrossRef] [PubMed]

42. Stark, K.D.; Van Elswyk, M.E.; Higgins, M.R.; Weatherford, C.A.; Salem, N. Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults. *Prog. Lipid Res.* **2016**, *63*, 132–152. [CrossRef] [PubMed]
43. Castiglioni, S.; Casati, S.; Ottria, R.; Ciuffreda, P.; Jeanette, A.M.M. N6-Isopentenyladenosine and its Analogue N6-Benzyladenosine Induce Cell Cycle Arrest and Apoptosis in Bladder Carcinoma T24 Cells. *Anticancer. Agents Med. Chem.* **2013**, *13*, 672–678. [CrossRef] [PubMed]
44. Folch, J.; Lees, M.; Sloane Stanley, G.H. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509.
45. Cazzola, R.; Cestaro, B. Red wine polyphenols protect n-3 more than n-6 polyunsaturated fatty acid from lipid peroxidation. *Food Res. Int.* **2011**, *44*, 3065–3071. [CrossRef]
46. Cazzola, R.; Viani, P.; Allevi, P.; Cighetti, G.; Cestaro, B. pH sensitivity and plasma stability of liposomes containing N-stearoylcysteamine. *Biochim. Biophys. Acta Biomembr.* **1997**, *1329*. [CrossRef]
47. Royall, J.A.; Ischiropoulos, H. Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. *Arch Biochem. Biophys.* **1993**, *302*, 348–355. [CrossRef]
48. Raccah, D.; Dadoun, F.; Coste, T.; Vague, P. Decreased Na/K ATPase ouabain binding sites in red blood cells of patients with insulin-dependent diabetes and healthy north African control subjects: relationship with diabetic neuropathy. *Horm. Metab. Res.* **1996**, *28*, 128–132. [CrossRef]
49. LOWRY, O.H.; ROSEBROUGH, N.J.; FARR, A.L.; RANDALL, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
50. Bartlett, G.R. Phosphorus assay in column chromatography. *J. Biol. Chem.* **1959**, *234*, 466–468.
51. Rimm, E.B.; Appel, L.J.; Chiuve, S.E.; Djoussé, L.; Engler, M.B.; Kris-Etherton, P.M.; Mozaffarian, D.; Siscovick, D.S.; Lichtenstein, A.H. Seafood Long-Chain n-3 Polyunsaturated Fatty Acids and Cardiovascular Disease: A Science Advisory From the American Heart Association. *Circulation* **2018**, *138*. [CrossRef] [PubMed]
52. Preston Mason, R. New Insights into Mechanisms of Action for Omega-3 Fatty Acids in Atherothrombotic Cardiovascular Disease. *Curr. Atheroscler. Rep.* **2019**, *21*. [CrossRef] [PubMed]

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ORIGINAL ARTICLE

Anti-inflammatory effects of diet and caloric restriction in metabolic syndrome

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Abstract

Background Weight loss in patients with metabolic syndrome has positive effects on cardiovascular and type 2 diabetes risks, but its effects on peripheral cytokines and lipid profiles in patients are still unclear.

Aim To determine the effects of diet-induced weight loss on metabolic parameters, lipids and cytokine profiles.

Methods Eighteen adult males with metabolic syndrome (defined according to IDF 2009) and Body Mass Index (BMI) between 25 and 35 kg/m^2 were subjected to a balanced hypocaloric diet for 6 months to reach at least a 5% body weight loss.

Results After weight loss, a significant improvement in BMI, waist circumference, insulin, fasting blood glucose and HOMA-IR (homeostasis model assessment of insulin resistance) was observed. The analysis of LDL (low-density lipoprotein cholesterol) and HDL (high-density lipoprotein cholesterol) lipoproteins showed a change in their composition with a massive transfer of triacylglycerols from HDL to LDL. This was associated with a significant reduction in peripheral pro-inflammatory cytokines such as IL-6, TNF-α, IL-8 and MIP-1β, leading to an overall decreased inflammatory score. An interesting positive correlation was also observed among peripheral cytokines levels after diet and peripheral levels of CETP (cholesteryl ester transfer protein), an enzyme with a key role in lipid change.

L. Montefusco, F. D'Addio and C. Loretelli are Co-first-authors.

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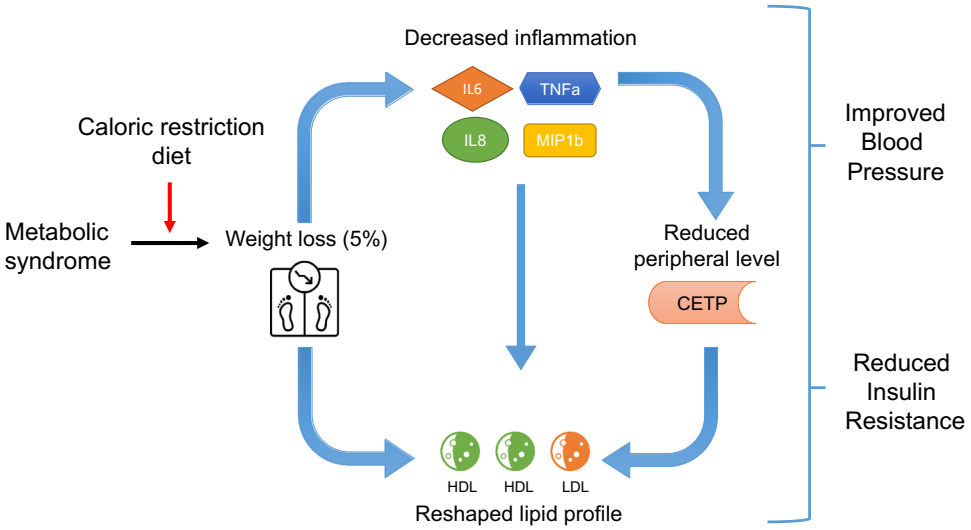
Conclusion Weight loss through caloric restriction is associated with an improvement in peripheral lipid and cytokine profiles that may play a major role in improving cardiovascular risk.

Graphic abstract



Keywords Metabolic syndrome · Caloric restriction · Pro-inflammatory cytokines · Cholesteryl ester transfer protein · Lipids

Introduction

Prevalence of obesity and metabolic syndrome (MetS) are worldwide increasing to epidemic proportions, leading to increased risk of cardiovascular disease (CVD) and type 2 diabetes. The prevention of these diseases is a public health challenge. Diet, particularly Mediterranean diet, is one of the most important instruments to reduce obesity, MetS, cardiovascular diseases [1–3] and the risk of developing type 2 diabetes [4–7]. To this end, mechanisms whereby diet controls metabolic parameters are still poorly understood; however,, it seems that the pro-atherogenic inflammatory system may be involved [8]. It has been already demonstrated that diet may reduce circulating markers of inflammation in obese non-diabetic subjects [8, 9]. Moreover, this reduction seems to be directly linked to kind of food consumed (cereals, fruits, nuts, virgin oil) [5]. Conversely, some pro-inflammatory markers, such as IL-6 and C-reactive protein, may predict the risk of future type 2 diabetes and those are increased in MetS [9]. Interestingly, while robust association has been found among pro-inflammatory markers' changes and glucose metabolism or insulin resistance changes, an association has not been equally demonstrated with lipid changes [8]. A major player in driving lipid changes is cholesteryl ester transfer protein (CETP), which enables the exchange of cholesteryl esters and triglycerides between high-density lipoprotein cholesterol (HDL) and triglyceride-rich lipoproteins. Reduced CETP activity leads to reduction of low-density lipoprotein cholesterol (LDL) and increased HDL [11] and consequently to significantly lower risk of atherosclerotic cardiovascular disease mainly in genetic studies [12], but not in pharmacological study aimed at targeting CETP activity [11]. Patients affected by type 2 diabetes showed increased CETP activity [13, 14]; whether CETP levels are associated with changes of lipid profile during weight loss has not been described yet. The aim of this study was to determine the effects of a diet-induced weight loss achieved with a balanced low-calorie diet on physical and biochemical metabolic parameters, on the pro-inflammatory profile and on the chemical composition of lipoproteins in patients suffering from overweight/mild obesity and metabolic syndrome.

Subjects and methods

Patients

The subjects included in the present study were originally part of the "Oxidative Stress, Inflammation, and Lipoprotein in Metabolic Syndrome" study (ClinicalTrials.gov Identifier: NCT03553381). From this cohort we defined 18 overweight and moderately obese male Caucasian subjects with Body Mass Index (BMI) comprised between 25 and 35 kg/m^2,

who on enrolment had metabolic syndrome (MetS) defined according to International Diabetes Federation 2009 [15] and who after following a balanced hypo-caloric diet had lost at least 5% of their initial weight. Subjects were told and trained to reduce their daily energy intake of 800 kcal/day for 8 weeks with dietary counseling performed by a registered dietician. Macronutrient content of hypocaloric diet, expressed as percentage of ingested energy, was 25% fat, 60% carbohydrate and 15% protein. All subjects were non-smokers. Inclusion criteria also included the following: (i) alcohol consumption < 25 g/die, (ii) no use of antioxidant-based supplements and (iii) absence of hormonal treatments. Patients receiving hypoglycemic treatment, treatments that alter lipoprotein metabolism and pregnant women were also excluded (Fig. 1). The study was approved by the ethics committees of the Istituti Clinici di Perfezionamento of Milan and of L. Sacco Hospital of Milan and was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. Subjects gave their written consent to the study. The primary endpoint of the study was to evaluate the change in the lipoprotein profile and the reduction in inflammation in patients who underwent a balanced hypo-caloric diet and lost at least 5% of their initial weight. Sample size was set at 18, as it would provide the study with 80% power to detect a reduction of at least 0.4 pg/ml in the cytokine levels after weight loss, with a significance level of $\alpha = 0.05$, given that an increase of 0.4 pg/ml in IL-6 plasma level has been observed in patients with glucose intolerance [16].

Blood collection and analyses

As previously described [17], overnight fast blood was drawn from subjects (12 h without food) in the morning at study entry and at the end of treatment after body weight loss. Blood collection and handling were carried out under strictly standardized conditions and in line with manufacturer recommendations. C-reactive protein level (CRP) was measured with the high-sensitivity assay. Plasma samples (EDTA as anticoagulant) were stored at − 80 °C until use. Lipoproteins were isolated from plasma as previously described [18] by adapting the procedure to "Optima Max" tabletop ultracentrifuge (Beckman Coulter). For a complete removal of albumin, the HDL fraction (density, 1.063–1.210 g/ml) was subjected to a second centrifugation [procedure 15 in ref. [18]]. After separation, lipoproteins were dialyzed and their levels of proteins, cholesterol (total and free), phospholipid and triacylglycerols were determined [18, 20].

Lipopolysaccharide and lipopolysaccharide binding protein

Plasma lipopolysaccharide (LPS) levels were measured by Limulus amebocyte lysate test according to manufacturer instructions (Euroclone S.p.A, Milan, Italy). Plasma lipopolysaccharide binding protein (LBP) concentration was measured using an enzyme-linked immunosorbent assay kit (BioSource, Milan, Italy).

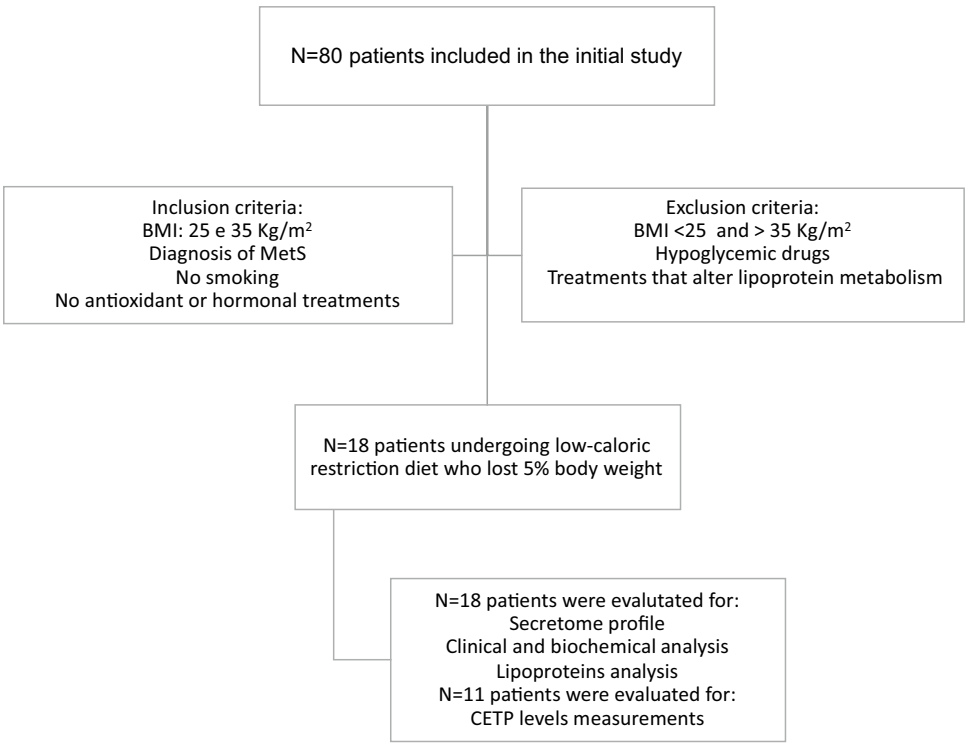


Fig. 1 Flow-chart describing the study population

Measurement of plasma cytokines

Levels of cytokines were assessed in plasma of patients enrolled in the study using the Bio-Plex Pro human cytokine 17-plex panel (M5000031YV, Bio-Rad) according to the manufacturer's protocol [21]. The secretome profile was assessed at baseline (T0) and after diet/weight loss (T1) and a delta (T1–T0) of plasma cytokine level has been also calculated.

Inflammatory score

Each plasma cytokine value was stratified into quartiles to determine cutoff points and assign a score ranging from 0, which was assigned to the lowest quartile, to 4, which was assigned to the highest quartile [22].

Measurement of CETP

CETP levels were assessed using commercially available ELISA kit, according to the manufacturer's instructions (MyBioSource, MBS266702, Milan, Italy). Human-derived hepatoma cell line (Huh7) was cultured for 5 days in Dulbecco's Modified Eagle's (DMEM) containing 10% Fetal Bovine Serum (FBS) at different glucose concentrations: 5.5, 20 and 35.5 mM as already described [23]. Culturing supernatant was collected, and CETP levels were assessed by ELISA.

Statistical analysis

Data are expressed as median ± standard error of mean (SEM) unless otherwise reported. Since the Kolmogorov–Smirnov normality test revealed non-normal distribution, the results were analyzed by non-parametric tests. The effects of the hypo-caloric diet were analyzed by paired comparison (values before vs. after the intervention) using Wilcoxon tests. Two-tailed p-values ≤ 0.05 were considered significant. All statistical analyses were performed by using StatistiXL software (version 1.5; StatistiXL, Western Australia) and Prism Graphpad 7. A Pearson/Spearman correlation analysis was used as appropriate to define correlations between each cytokine level and CETP quantification, and also relations among improvement in glucose metabolism (glycemia reduction, insulin reduction, HOMA-IR reduction) and CEPT levels.

Results

Clinical characteristic at baseline and at the end of follow up

The anthropometric characteristics of patients, including blood pressure and blood parameter values before and after weight loss are summarized in Table 1. The mean age of the enrolled subjects was 47.5 ± 8.7 years and a 5% body weight loss was achieved in the study patients after a mean of 191.0 ± 46.2 days. All variables included in the metabolic syndrome improved significantly after diet and all patients reversed MetS. In particular, the diet treatment significantly improved BMI, waist circumference, systolic and diastolic blood pressure, fasting glycaemia, fasting insulin and HOMA-IR (homeostasis model assessment of insulin resistance). With regard to other biochemical parameters measured (serum protein, electrolyte, iron, uric acid, creatinine, thyroid hormone, white and red blood cells) no difference was observed, except for a slight reduction of CRP values although not reaching statistically significant difference (Table 1).

Lipid profile

While a change in total LDL and HDL levels was not evident, the analysis of extracted lipoproteins demonstrated that diet-induced weight loss also substantially reshaped lipoprotein chemical compositions. In fact, as shown in Table 2, a significant change in the concentration of triacylglycerols that increased in LDL while decreased in HDL ($p < 0.01$) was found. A significant increase in HDL Apo concentration has been observed after diet. Dietary treatment did not significantly influence LPS and LBP plasma levels (data not shown).

Table 1 Anthropometric characteristics, blood pressure and blood parameters of subjects before (T0) and after (T1) weight loss

	T0	T1
BMI (kg/m^2)	34.7 ± 3.4	31.6 ± 2.9**
Waist circumference (cm)	113.0 ± 10.7	106.0 ± 8.4**
Systolic blood pressure (mmHg)	140.0 ± 15.5	128.0 ± 11.2**
Diastolic blood pressure (mmHg)	88.0 ± 9.4	79.0 ± 8.8**
Glycemia (mg/dL)	103.0 ± 22.5	97.0 ± 14.**
Insulin (μU/L)	16.1 ± 11.3	10.8 ± 5.4*
HOMA-IR	4.1 ± 3.0	2.5 ± 1.4**
HbA1c (mmol/mol)	5.6 ± 0.7	5.8 ± 0.2
Total cholesterol (mg/dL)	198.0 ± 30.6	198.0 ± 35.3
Triacylglycerols (mg/dL)	125.0 ± 66.4	135.0 ± 81.6
LDL cholesterol (mg/dL)	123 ± 30.0	123 ± 29.6
HDL cholesterol (mg/dL)	48 ± 12.8	48 ± 15.3
CRP (mg/L)	0.5 ± 0.35	0.4 ± 0.6

BMI body mass index, *HOMA-IR* homeostasis model assessment of insulin resistance, *CRP* C-reactive protein

*$p \leq 0.05$

**$p \leq 0.01$

Table 2 Protein (Apo) and lipid concentrations of lipoproteins before (T0) and after (T1) weight loss

	VLDL		LDL		HDL	
	T0	T1	T0	T1	T0	T1
Apo (mg/dL)	9.8 ± 5.7	9.7 ± 5.4	12.3 ± 2.8	11.8 ± 2.4	9.0 ± 2.8	11.1 ± 2.8**
TC (mg/dL)	37.3 ± 20.7	34.4 ± 19.6	103.2 ± 29.9	108.6 ± 32.4	43.6 ± 11.7	42.8 ± 16.4
TAG (mg/dL)	60.0 ± 33.2	67.1 ± 42.5	45.3 ± 12.5	61.2 ± 16.2**	29.3 ± 7.8	21.9 ± 8.1**
PL (mg/dL)	18.7 ± 9.9	19.7 ± 11.6	72.2 ± 19.8	76.4 ± 19.7	47.8 ± 12.4	45.0 ± 14.2
TL (mg/dL)	116.3 ± 61.5	121.6 ± 76.1	220.7 ± 59.6	246.2 ± 66.7*	121.0 ± 31.5	110.3 ± 37.5*

Apo apolipoprotein; *TC* Total Cholesterol; *TAG* Triacylglycerols; *PL* Phospholipids; *TL* Total Lipids

*$p \leq 0.05$

**$p \leq 0.01$

Table 3 Peripheral cytokine and CETP levels before (T0) and after (T1) weight loss

	T0	T1
IL-6 (pg/mL)	1.9 ± 0.7	1.5 ± 0.6*
TNFα (pg/mL)	0.8 ± 0.3	0.3 ± 0.8*
IL-8 (pg/mL)	2.7 ± 0.6	1.6 ± 0.3*
IL-10 (pg/mL)	3.5 ± 1.2	2.5 ± 0.6
IL-12 (pg/mL)	8.1 ± 2.2	6.2 ± 1.8
IL-7 (pg/mL)	1.3 ± 0.4	1.0 ± 0.2
IL-17 (pg/mL)	8.0 ± 2.7	6.9 ± 2.4
G-CSF (pg/mL)	17.9 ± 3.8	12.1 ± 2.6
MIP-1β (pg/mL)	20.2 ± 1.4	18.4 ± 1.4*
MCP-1 (pg/mL)	31.4 ± 4.5	31.6 ± 4.5
CETP (ng/mL)	356.2 ± 57.4	405.8 ± 97.9

Data are expressed as mean ± SEM (standard error of the mean)

IL-2 interleukine 2; *IL-6* interleukine 6; *IL-7* interleukine 7; *IL-8* interleukine 8; *IL-10* interleukine 10; *IL-12* interleukine 12; *IL-17* interleukine 17; *GM-CSF* Granulocye-Macrophage Colony Stimulating Factor; *MCP1* Monocyte Chemoattractant Protein 1; *MIP1b* Macrophage Inflammatory Protein 1b.; *CETP* cholesterol ester transferase protein

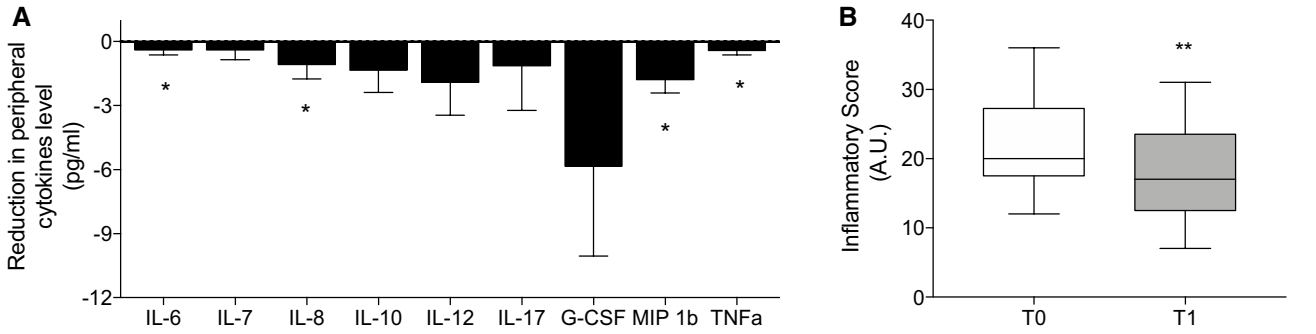
*$p \leq 0.05$

Peripheral pro-inflammatory cytokines profile

The quantification of cytokines is reported in Table 3, with data showing a significant decrease of the peripheral levels of IL-6, TNF-α, IL-8 and MIP-1β (Table 3). Moreover, for other cytokines, such as IL-17, GM-CSF and MCP-1, a trend toward a downregulation, albeit non statistically significant, was observed (Fig. 2a). We next stratified cytokines levels based on quartiles and calculated an inflammatory score, which summarizes the overall inflammatory state detected in the periphery. Our results further confirmed that a higher inflammatory state was evident in patients before weight loss and that dietary treatment was able to reshape it (Fig. 2c, d). Finally, unchanged CETP peripheral blood levels have been observed after weight loss (Table 3). However, an interesting correlation between CETP levels and different peripheral cytokine levels, in particular MIP-1b and IL-8, has been observed after weight loss (Fig. 3a, b), while that between reduction of cytokines levels and CETP did not result statistically significantly different. Spearman's correlation analysis was also performed among CEPT level and glycemia levels, insulin levels and HOMA-IR index, but no significant correlations have been found (data not shown). In order to understand the basis for influence of cytokine changes on CEPT production, which is primarily released by the liver, we cultured a human hepatocytes-derived cell line (Huh7) in vitro with sera obtained from patients with MetS before and after diet and did not demonstrate a significant change in supernatant secretion of CETP. Similarly, no differences have been observed among supernatant CETP levels from human hepatocyte-derived cell line (Huh7) cultured in vitro with sera obtained from patients with MetS as compared to those obtained from healthy controls (data not shown).

Discussion

Our study showed the efficacy of body weight loss obtained through caloric restriction in reducing inflammatory cytokine levels and in reshaping lipid composition. In previous studies adherence to Mediterranean diet led to an improvement in clinical cardiovascular outcomes with a reduction 10–30% in relative risk of CVD [6], to a protection against recurrent coronary heart disease [3], to a reduction of 18–40% in incident diabetes and to a reduction of 14% in the prevalence of MetS [6, 7]. The results of present study show as expected [1, 5], an efficacy of balanced caloric restrictions in reverting MetS by reducing body weight, associated with a reduction in blood pressure, glycaemia levels and insulin resistance. Interestingly in this cohort, balanced caloric restriction leads also to an improvement in lipid composition, with an increase in the triacylglycerol concentration in LDL and with a decrease in HDL, and an increase in Apo concentration in HDL. It is also known that obesity is associated with a chronic low-grade inflammatory state of adipose



C

Parameters T0	IL-2	IL-6	IL-7	IL-8	IL-10	IL-12	IL-17	G-CSF	MIP-1b	TNF-a	Blood glucose	HOMA-I
IL-2	1											
IL-6	0,456	1										
IL-7	0,803	0,835	1									
IL-8	0,576	0,284	0,452	1								
IL-10	0,384	0,934	0,801	0,246	1							
IL-12	0,665	0,802	0,879	0,616	0,848	1						
IL-17	0,794	0,493	0,738	0,735	0,549	0,871	1					
G-CSF	0,421	0,251	0,370	0,795	0,235	0,639	0,732	1				
MIP-1b	0,286	0,490	0,432	0,428	0,422	0,553	0,395	0,604	1			
TNF-a	0,484	0,520	0,560	0,230	0,402	0,326	0,257	-0,003	-0,067	1		
Blood glucose	-0,091	-0,127	-0,230	-0,044	-0,267	-0,211	-0,155	0,160	0,162	-0,351	1	
HOMA-I	-0,360	-0,143	-0,504	-0,229	-0,255	-0,421	-0,435	-0,196	0,100	-0,358	0,524	1

D

Parameters T1	IL-2	IL-6	IL-7	IL-8	IL-10	IL-12	IL-17	G-CSF	MIP-1b	TNF-a	Blood glucose	HOMA-I
IL-2	1											
IL-6	0,209	1										
IL-7	-0,050	0,045	1									
IL-8	0,420	0,296	0,618	1								
IL-10	0,691	0,378	-0,051	0,478	1							
IL-12	0,788	0,496	0,203	0,661	0,883	1						
IL-17	0,533	0,719	0,401	0,659	0,452	0,741	1					
G-CSF	0,384	0,296	0,719	0,640	0,290	0,624	0,614	1				
MIP-1b	0,221	0,385	0,171	0,535	0,452	0,629	0,505	0,582	1			
TNF-a	0,255	0,184	-0,049	0,049	0,382	0,249	0,013	0,022	-0,186	1		
Blood glucose	0,366	-0,134	-0,065	0,173	0,088	0,102	0,125	-0,251	-0,150	-0,119	1	
HOMA-I	-0,260	-0,202	-0,330	0,037	-0,249	-0,316	-0,343	-0,473	0,060	-0,140	0,337	1

Fig. 2 **a** Relative variation in peripheral cytokine levels of patients included in the study. Cytokines levels are expressed in pg/mL. **b** Inflammatory score assessed in patients before (T0) and after weight loss (T1). **c, d** Correlation matrix between cytokines level and clinical parameters before (T0) and after weight loss (T1). *IL* interleukin; *TNF* tumor necrosis factor; *G-CSF* Granulocyte-Colony Stimulating Factor; *MCP1* Monocyte Chemoattractant Protein 1; *MIP1b* Macrophage Inflammatory Protein 1b; *A.U.* Arbitrary Unit; *HOMA-IR* homeostasis model assessment of insulin resistance. $*p \leq 0.05$; $**p < 0.01$

tissue and that this independently increases the risk of adverse cardiovascular outcomes [24, 25]. Previous diet intervention studies showed that weight loss has positive effect on pro-inflammatory cytokines levels, particularly it is effective in reducing IL-6 and CRP levels, but this cytokine reduction was not related to significant lipids levels and composition improvement [8, 26]. Interestingly, in our study we described a significant reduction of not only several inflammatory cytokines such as IL-6 but also IL-8, TNF-α and MIP-1b after diet, which was ultimately summarized in a reduction of the inflammatory score upon dietary treatment. The aforementioned cytokines have been mainly linked to the innate immunity response [27] and we may suggest that the metabolic syndrome itself may promote an increased release of cytokines by circulating monocytes. Given the beneficial effect obtained in reducing those cytokines' level through the weight loss in our study, in which a reduction of the waist was evident, we may further speculate that adipocytes residing in the visceral adipose tissue may also be responsible for the cytokine production. Moreover, we have found a correlation between the levels of different inflammatory cytokines and CETP levels after diet, in particular lower IL-8 and MIP-1b levels were associated with lower CETP level, which has been associated with the formation of HDL and a reduced the risk of atherosclerosis [28]. Beyond the role in the lipid exchanges among lipoprotein classes, CETP

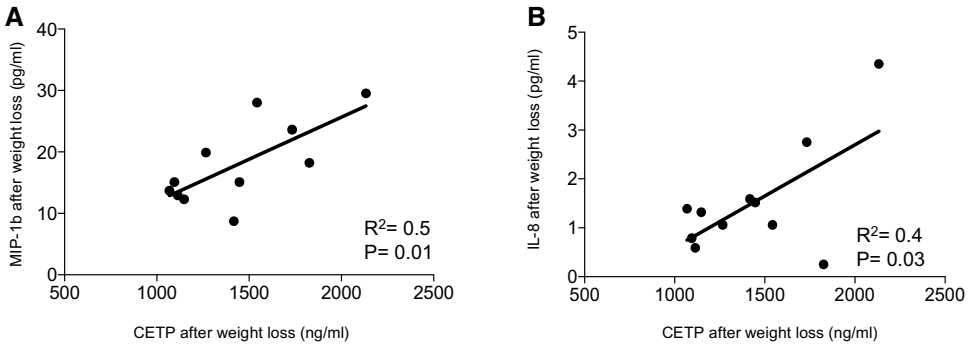


Fig. 3 Correlation between MIP-1b (**a**), IL8 (**b**) and IL10 (**c**) with CETP level after weight loss in the 18 patients. **a** Correlation between MIP1b (Macrophage Inflammatory Protein 1b expressed in pg/mL) and CETP (cholesterol ester transfer protein expressed in ng/mL) after weight loss. **b** Correlation between IL-8 (Interleukin 8 expressed in pg/mL) and CETP (cholesterol ester transfer protein expressed in ng/mL) after weight loss

has also postulated roles in inflammatory processes and in the immunological defenses of the organism [29]. This has been also suggested by our study, which showed a link between peripheral CETP level and that of some pro-inflammatory cytokines, such as IL-8 and MIP-1b, with an emerging key-role in the field of metabolic disease and diabetes. Further studies are needed to better unveil the relationship between lipid changes, pro-inflammatory cytokine profile modifications and CEPT, both level and activity. Finally, we acknowledge that the lack of a control group and the small sample size are probably a limitation of this study and further larger case–control studies would be required to confirm our results. However, our study demonstrates that a link exists between lipids and pro-atherogenic inflammatory cytokines and that it can be strongly modulated by diet. In conclusion, body weight loss through balanced caloric restriction in patients with metabolic syndrome leads to a protective anti-atherogenic lipid profile and a reduced peripheral inflammatory environment, which are both associated with a decrease in cardiovascular risk.

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Author contributions L.M. designed the study, performed experiments, analyzed data, and wrote the paper; F.D performed experiments, analyzed data and wrote the paper; C.L. and M.B.N. analyzed data and wrote the paper; M.G., A.R., L.P., M.E.L., A.M.B., C.L., M.D.P., A.A., E.A., M.B., A.M., V.U., I.P., F.M., G.V.Z., C.R. participated to the research and edited the paper; P.F. conceived the idea, designed the study and wrote and edited the paper. P.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors have given their permission for submission of this manuscript.

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Declarations

Conflict of interest No potential conflicts of interest relevant to this article were reported.

Research involving human participants and/or animals All procedures were approved by local institutional review boards.

Informed consent Written informed consent was obtained from all the legal guardians, and from the patients when applicable, prior to inclusion.

References

1. Keane D, Kelly S, Healy NP, McArdle MA, Holohan K, Roche HM (2013) Diet and metabolic syndrome: an overview. Curr Vasc Pharmacol 11:842
2. Kastorini CM, Milionis HJ, Esposito K, Giugliano D, Goudevenos JA, Panagiotakos DB (2011) The effect of Mediterranean diet on

metabolic syndrome and its components. A meta-analysis of 50 studies and 534,906 individuals. J Am Coll Cardiol 57:1299
3. de Lorgeril M, Salen P, Martin JL, Monjaud I, Delaye J, Mamelle N (1999) Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. Circulation 99:779
4. Koloverou E, Esposito K, Giugliano D, Panagiotakos D (2014) The effect of Mediterranean diet on the development of type 2 diabetes mellitus: a meta-analysis of 10 prospective studies and 136,846 participants. Metabolism 63:903
5. Cespedes EM, Hu FB, Tinker L, Rosner B, Redline S, Garcia L, Hingle M, Van Horn L, Howard BV, Levitan EB, Li W, Manson JE, Phillips LS, Rhee JJ, Waring ME, Neuhouser ML (2016) Multiple healthful dietary patterns and type 2 diabetes in the women's health initiative. Am J Epidemiol 183:622
6. Babio N, Toledo E, Estruch R, Ros E, MartínezGonzález MA, Castañer O, Bulló M, Corella D, Arós F, GómezGracia E, Ruiz-Gutiérrez V, Fiol M, Lapetra J, LamuelaRaventos RM, Serra-Majem L, Pintó X, Basora J, Sorlí JV, SalasSalvadó J, PREDIMED study investigators (2014) Mediterranean diets and metabolic syndrome status in the PREDIMED randomized trial. Can Med Assoc J 186:E649
7. Salas-Salvado J, Diaz-Lopez A, Ruiz-Canela M, Basora J, Fito M, Corella D, Serra-Majem L, Romaguera D, Estruch R, Vidal J, Martınez JA, Aros F, Vazquez C, Ros E, Vioque J, Lopez-Miranda J, Bueno-Cavanillas A, Tur JA, Tinahones FJ, Martın V, Lapetra J, Pinto X, Daimiel L, Delgado-Rodrıguez M, Matıa P, Gomez-Garcia E, Dıez-Espino J, Babio N, Castaner O, Sorlı JV, Fiol M, Zulet MA, Bullo M, Goday A, Martınez-Gonzalez MA, for the PREDIMED-Plus investigators (2019) Effect of a lifestyle intervention program with energy-restricted mediterranean diet and exercise on weight loss and cardiovascular risk factors: one-year results of the PREDIMED-plus trial. Diabetes Care 42:777–788
8. Herder C, Peltonen M, Koenig W, Sutfels K, Lindstrom J, Martin S, Ilanne-Parikka P, Eriksson JG, Aunola S, Keinanen-Kiukaanniemi S, Valle TT, Uusitupa M, Kolb H, Tuomilehto J (2009) Anti-inflammatory effect of lifestyle changes in the Finnish Diabetes Prevention Study. Diabetologia 52:433
9. Schwingshackl L (2014) Hoffman, Mediterranean dietary pattern, inflammation and endothelial function: a systematic review and meta-analysis of intervention trials. Nutr Metab Cardiovasc Dis 24:929
10. Wang X, Bao W, Liu J, Ouyang YY, Wang D, Rong S, Xiao X, Shan ZL, Zhang Y, Yao P, Liu LG (2013) Inflammatory markers and risk of type 2 diabetes. A systematic review and meta-analysis. Diabetes Care 36:166
11. Taheri H, Filion KB, Windle SB, Reynier P, Eisenberg MJ (2020) Cholesteryl Ester Transfer Protein Inhibitors and Cardiovascular Outcomes: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Cardiology 145:236
12. Barter PJ, Nicholls SJ, Kastelein JJP, Rye KA (2015) Is cholesteryl ester transfer protein inhibition an effective strategy to reduce cardiovascular risk? CETP inhibition as a strategy to reduce cardiovascular risk: the pro case. Circulation 132(5):423
13. Kahri J, Syvänne M, Taskinen MR (1994) Plasma cholesteryl ester transfer protein activity in non-insulin-dependent diabetic patients with and without coronary artery disease. Metabolism 43(12):1498
14. Riemens SC, van Tol A, Sluiter WJ, Dullaart RP (1999) Plasma phospholipid transfer protein activity is lowered by 24-h insulin and acipimox administration: blunted response to insulin in type 2 diabetic patients. Diabetes 48(8):1631
15. Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WPT, Loria CM, Smith SC Jr (2009) Harmonizing themetabolic syndrome: a joint interim statement of the international diabetes federation task force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for theStudy of Obesity. Circulation 120(16):1640–1645
16. Esposito K, Nappo F, Marfella R, Giugliano G, Giugliano F, Ciotola M, Quagliaro L, Ceriello A, Giugliano D (2002) Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. Circulation 106(16):2067–2072
17. Cazzola R, Cassani E, Barichella M, Cestaro B (2013) Impaired fluidity and oxidizability of HDL hydrophobic core and amphipathic surface in dyslipidemic men. Metabolism 62(7):986–991
18. Cazzola R, Cervato G, Cestaro B (1999) Variability in alpha-tocopherol antioxidant activity in the core and surface layers of low- and high-density lipoproteins. J Nutr Sci Vitaminol (Tokyo) 45(1):39
19. Chung BH, Segrest JP, Ray MJ, Brunzell JD, Hokanson JE, Krauss RM, Beaudrie K, Cone JT (1986) Single vertical spin density gradient ultracentrifugation. Methods Enzym 128:181
20. Viani P, Cazzola R, Cervato G, Gatti P, Cestaro B (1996) Pyrene lipids as markers of peroxidative processes in different regions of low and high density lipoproteins. Biochim. Biophys Acta Mol Basis Dis. 1315(2):78
21. D'Addio F, Vergani A, Potena L, Maestroni A, Usuelli V, Ben NM, Bassi R, Tezza S, Dellepiane S, El Essawy B, Iascone M, Iacovoni A, Borgese L, Liu K, Visner G, Dhe-Paganon S, Corradi D, Abdi R, Starling RC, Folli F, Zuccotti GV, Sayegh MH, Heeger PS, Chandraker A, Grigioni F, Fiorina P (2018) P2X7R mutation disrupts the NLRP3-mediated Th program and predicts poor cardiac allograft outcomes. J Clin Invest 128(8):3490
22. Daniele G, Guardado MR, Winnier D, Fiorentino TV, Pengou Z, Cornell J, Andreozzi F, Jenkinson C, Cersosimo E, Federici M, Tripathy D, Folliet F (2014) The inflammatory status score including IL-6, TNF-alpha, osteopontin, fractalkine, MCP-1 and adiponectin underlies whole-body insulin resistance and hyperglycemia in type 2 diabetes mellitus. Acta Diabetol 51:123–131
23. D'Addio F, La Rosa S, Maestroni A, Jung P, Orsenigo E, Ben NM, Tezza S, Bassi R, Finzi G, Marando A, Vergani A, Frego R, Albarello L, Andolfo A, Manuguerra R, Viale E, Staudacher C, Corradi D, Batlle E, Breault D, Secchi A, Folli F, Fiorina P (2015) Circulating IGF-I and IGFBP3 levels control human colonic stem cell function and are disrupted in diabetic enteropathy. Cell Stem Cell 17(4):486–498
24. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr (2003) Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112:1796–1808
25. Bullo M, Garcia-Lorda P, Megias I, Salas-Salvado J (2003) Systemic inflammation, adipose tissue tumor necrosis factor, and leptin expression. Obes Res 11:525
26. Casas R, Urpi-Sardà M, Sacanella E, Arranz S, Corella D, Castañer O, Lamuela-Raventós RM, Salas-Salvadó J, Lapetra J, Portillo MP, Estruch R (2017) Anti-inflammatory effects of the mediterranean diet in the early and late stages of atheroma plaque development. Mediators Inflamm 2017:3674390
27. Solerte SB, D'Addio F, Trevisan R, Lovati E, Rossi A, Pastore I, Dell'Acqua M, Ippolito E, Scaranna C, Bellante R, Galliani S, Dodesini AR, Lepore G, Geni F, Fiorina RM, Catena E, Corsico A, Colombo R, Mirani M, De Riva C, Oleandri SE, Abdi R, Bonventre JV, Rusconi S, Folli F, Di Sabatino A, Zuccotti G, Galli M, Fiorina P (2020) Sitagliptin treatment at the time of hospitalization was associated with reduced mortality in patients with type 2 diabetes and COVID-19: a multicenter,

case-control, retrospective, observational study. Diabetes Care 43(12):2999–3006
28. Fisher EA, Feig JE, Hewing B, Hazen SL, Smith JD (2012) High-density lipoprotein function, dysfunction, and reverse cholesterol transport. Arterioscler Thromb Vasc Biol 32(12):2813
29. Venancio TM, Machado RM, Castoldi A, Amano MT, Nunes VS, Quintao EC, Camara NO, Soriano FG, Cazita PM (2016) CETP lowers TLR4 expression which attenuates the inflammatory response induced by LPS and polymicrobial sepsis. Mediat Inflamm 2016:1784014

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Raman spectroscopy characterization of the major classes of plasma lipoproteins

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ABSTRACT

Raman spectroscopy has been vastly employed for the characterization of different bio-molecular species spanning from single protein to the in-vivo analysis of tissues. However, despite the huge work done, a detailed description of the Raman spectra acquired from the main classes of plasma lipoproteins is still missing.

In this work, we extracted, the major classes of lipoproteins: the triacylglycerol-rich very low density lipoproteins (VLDL); the more cholesterol-rich low density lipoproteins (LDL); and the high density lipoproteins (HDL); from human plasma of six fasting healthy volunteers. The extracted lipoproteins were dried on CaF_2 slides and analysed using a 633 nm laser line non-in resonance with the carotenoids present in the sample.

The obtained spectra showed peaks relative to the different biomolecules composing lipoproteins: cholesterol, triglycerides, membrane lipids, carotenoids, and apolipoproteins (proteins). The intensity of the peaks from lipids and proteins are well in accordance with the measured composition of lipoproteins; but the information is acquired in a much faster way by Raman spectroscopy. Besides, Raman spectroscopy provides easily information on the levels of carotenoids and unsaturated fatty acid present in the samples.

Overall, our data provide a clear comprehension of the Raman spectra from lipoproteins ad suggest that Raman spectroscopy could be a viable approach for the fast characterization of lipoproteins.

1. Introduction

Raman spectroscopy (RS) has been extensively used for the characterization of many biochemical species and extensive literature is now available proving that RS can be used for the characterization of cells, tissues, and biofluids [1–3]. More recently, the coupling of RS with multivariate statistical analysis was successfully applied for the analysis and classification of patients affected by several diseases [4–6]. Until now, most of the studies seem to focus on the analysis of proteins and other small metabolites (such as carotenoids) present in the samples, while the analysis of biological lipids by RS remained a slightly less explored field of study, even if they have very clear spectra [7,8].

Due to their hydrophobic nature, lipids travel in blood inside lipoproteins (LPs); complex particles responsible for the transport of fatty acids, cholesterol, carotenes, and vitamin E. LPs fractionation and quantification are a matter of primary interest in clinical medicine since several large-scale epidemiological studies showed that elevated concentrations of fasting cholesterol and triacylglycerols, stored in specific LPs, are associated with an increased incidence of cardiovascular events [9,10]. Therefore, the quantification of cholesterol and triglycerides transported within LPs is a standard clinical practice commonly applied to subjects at risk of cardiovascular events and to those undergoing cardiac rehabilitation [11]. More recently, new researches suggest that LPs are likely related with neurological diseases, such as Alzheimer's disease and Amyotrophic Lateral Sclerosis [12,13].

In the blood of a fasting subject, three major lipoprotein classes are present: the triacylglycerol-rich very low density lipoproteins (VLDL); the more cholesterol-rich low density lipoproteins (LDL); and the high density lipoproteins (HDL).

The main aim of the present work is to demonstrate the suitability of RS as a valuable tool to extract, easily and with a reduced sample preparation, valuable information about the lipid content of the main classes of LPs present in blood: VLDL, LDL and HDL.

Although RS was previously used to characterize LPs used in

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functional studies on macrophages, [14] most of the works published until now were focused on the attempt to extract by RS information on LPs content in serum or plasma [15,16]. To the best of our knowledge, a systematic description of the Raman spectra of the main classes of LPs extracted from human plasma of the same subjects is still lacking.

On the contrary, RS is a well-suited tool for this task. In fact, RS was previously used for the characterization of edible oils, such as olive oil or sunflower oil, [17] and as a rapid method to unveil oil adulterations [18]. Equally important, most modern Raman instruments are coupled with microscopy (micro-Raman) and this makes them suitable for the analysis of very small amount of samples such as the volumes of LPs extracted from the blood plasma.

In this work, we extracted VLDL, LDL and HDL by ultracentrifugation from the plasma sample of six healthy volunteers in fasting conditions. For all the subjects, Raman spectra from lipoproteins were collected in the regions between 400 cm^{-1} and 1800 cm^{-1} (low frequency region) and between 2600 and 3200 cm^{-1} (high frequency region). The data here presented show the main bands that are observed on the different LPs and how the intensity ratio of the peaks reflect their different biochemical composition.

2. Material and methods

2.1. Subjects and methods

Six healthy volunteers were recruited for the study. The study participants comprised three women and three men, age 30 ± 3 years. Blood was collected from fasting subjects (at least 12 h after food intake). Blood samples were obtained by venepuncture and were collected in EDTA-containing Vacutainer tubes. Plasma was prepared by centrifugation at 3000 rpm for 20 min at 4 °C, then centrifuged again under the same conditions to completely remove the erythrocytes. Plasma samples were aliquoted and stored at −80 °C until used. Subjects gave their written consent to the study.

2.2. Lipoprotein isolation and characterization

Major lipoproteins classes (VLDL, LDL and HDL) were isolated from plasma by ultracentrifugation in discontinuous KBr density gradient adapting procedure 16 in ref. [19] to “Optima Max” tabletop ultracentrifuge (Beckman Coulter) as previously described [20]. To remove albumin completely, the HDL fraction (density, 1.063–1.210 g/mL) was subjected to a second centrifugation [procedure 15 in ref. [19]. After separation, lipoproteins were dialyzed overnight in 10 mM phosphate buffer pH 7.4 containing 154 mM NaCl. The concentration of proteins was determined by the Lowry method using bovine serum albumin as standard. Lipids were extracted by the Folch procedure [21]. The levels of cholesterol, phospholipid, and triacylglycerols in lipid extracts were determined as previously described [22]. Ultrastructural characterization of VLDL, LDL and HDL has been performed by Transmission Electron microscopy, as follows: a drop of VLDL, LDL or HDL suspension was dried on the Formvar net at RT, stained with uranil-acetate 1% for 30 s at RT and dried overnight at RT. Samples were evaluated by Transmission Electron Microscopy (Tecnai Spirit, FEI).

2.3. Raman spectra acquisition and analysis

Raman spectra were acquired using an InVia Reflex confocal Raman microscope (Renishaw plc, Wotton-under-Edge, UK) equipped with a He-Ne laser light source operating at 633 nm. The Raman spectrometer was calibrated daily using the band at 520.7 cm^{-1} of a silicon wafer.

In a typical experiment, a 3.5 μL drop of LPs was dropped on the surface of Raman-compatible CaF_2 discs (Crystran, UK) and dried for 20 min at room temperature. The Raman study was performed using a 633 nm excitation laser with 100 % power (around 125 mW at source), a 1200 L/mm grating and a 100× objective (Leica). The diameter of the laser spot on the surface was ≈ 1 μm. Spectra were acquired independently in the region between 400 and 1800 cm^{-1} (low frequency region) and between 2600 and 3200 cm^{-1} (high frequency region) as the sum of five acquisitions of 30 s. Spectra resolution was about 1.1 cm^{-1}. For each sample, five different spectra were collected on different positions of the drop. The software package WIRE 5 (Renishaw, UK) was used for the spectral acquisition and to remove cosmic rays. Background fluorescence was removed by Asymmetric Least Square Smoothing Baseline using OriginPro software. The spectra acquired for each sample were normalized by dividing each value for the intensity of the peak at 1439 cm^{-1} in the low frequency region. For the intensity of the peak at 2894 cm^{-1} in the high frequency region. The average of the five spectra was considered as the final spectrum representative of each subject. Baseline subtraction, spectrum normalization and data analysis were performed using OriginPro, Version 2019 (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Isolation of lipoprotein

The chemical composition of LPs extracted by ultracentrifugation is reported in Table 1. Each lipoprotein class showed its expected distinctive chemical composition [23]. VLDL contained approx. 11 ± 1 % protein and 89 ± 5 % total lipids of which cholesterol, triacylglycerol and phospholipid concentrations were approx. 21.7 ± 0.6 %, 49.5 ± 4.2 % and 17.9 ± 0.1 %, respectively. LDL contained approx. 32 ± 2 % protein and 68 ± 4 % total lipids of which cholesterol, triacylglycerol and phospholipid concentrations were approx. 37.5 ± 0.8 %, 6.7 ± 0.5 % and 23.8 ± 0.2 %, respectively. Finally, HDL contained approx. 49 ± 2% protein and 51 ± 2 % total lipids of which cholesterol, triacylglycerol and phospholipid concentrations were approx. 20.2 ± 0.7 %, 4.5 ± 0.4 % and 26.4 ± 0.1 %, respectively. Extracted LPs were also characterized by Transmission Electron Microscopy to confirm, through the morphological analysis, the nature of LPs collected in each fraction. As expected, VLDL, LDL and HDL images reported in Fig. 1 resumes typical features of different classes of LPs.

3.2. Raman Spectra in the high frequency region

As first step, we acquired spectra from LPs in the high frequency region between 2600 and 3200 cm^{-1}. This region of the spectrum is dominated by three main peaks related to CH_2 bonds at 2851, 2894 and 2930 cm^{-1} in all the samples analysed. Besides, the fourth peak at 3012 cm^{-1} refers to the −CH moieties present in the samples. Interestingly, while the position of the peak was constant in VLDL, LDL and HDL their relative intensity was strongly dependent on the analysed class (Fig. 2 A–C). In particular, we observed a drastic variation of the intensity of the peaks at 2851 cm^{-1}, which mostly refers to CH_2 in lipids, and at 2930 cm^{-1}, that refers to CH_2 in proteins [24]. As expected, the peak relative to lipids decreased its intensity from VLDL to LDL and HDL, while the one relative to the protein content had an opposite behaviour. Notably, the ratio between the two peaks, when calculated for each class of LPs on all the six subjects included, was univocally able to

Table 1
Lipoprotein chemical composition. Data are expressed as mean ± standard deviation.

% Dry weight	VLDL	LDL	HDL
Protein	10.8 ± 1	31.8 ± 2	48.9 ± 2
Lipids:			
Total	89.1 ± 5	68.2 ± 4	51.1 ± 2
Cholesterol	21.7 ± 0.6	37.5 ± 0.8	20.2 ± 0.7
Triacylglycerols	49.5 ± 4.2	6.7 ± 0.5	4.5 ± 0.4
Phospholipids	17.9 ± 0.1	23.8 ± 0.2	26.4 ± 0.1

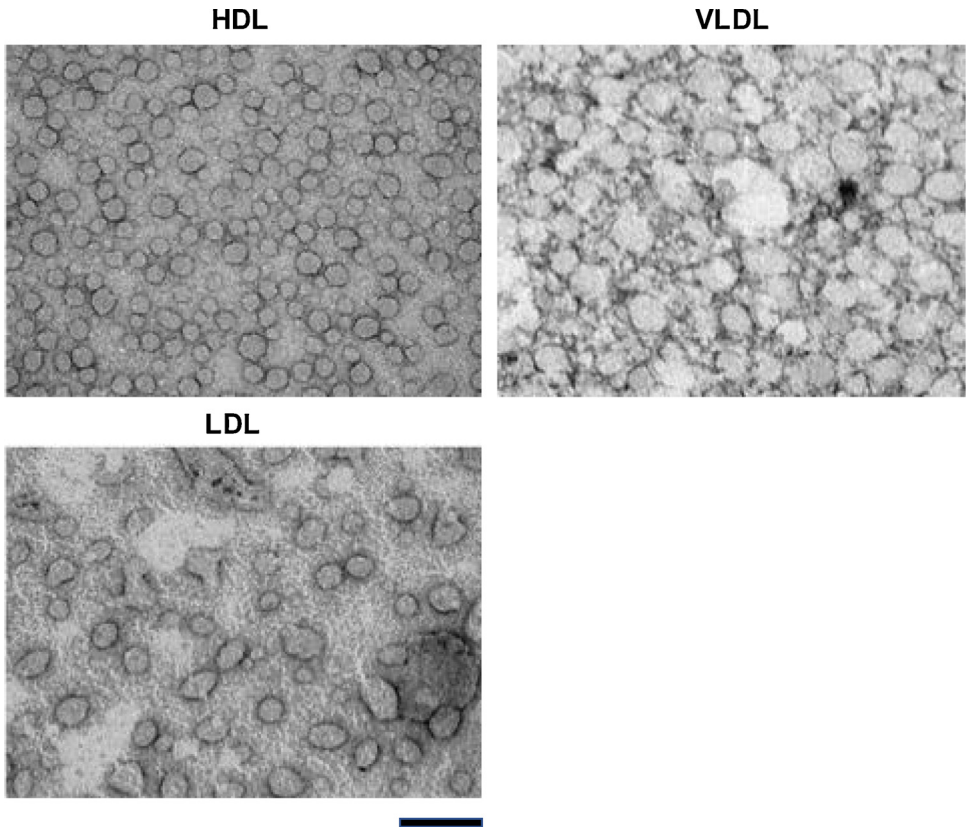

Fig. 1. Transmission electron microscopy images of VLDL, LDL and HDL extracted from a single subject. Scale bar = 100 nm.

discriminate between the three major classes of LPs (Fig. 2 D). However, because of the limited number of subjects included in the study, these results need to be further validated. On the contrary, the peak at 3012 cm^{-1} remained constant in both its Raman shift and intensity, regardless of the subject and of the class of LPs analysed.

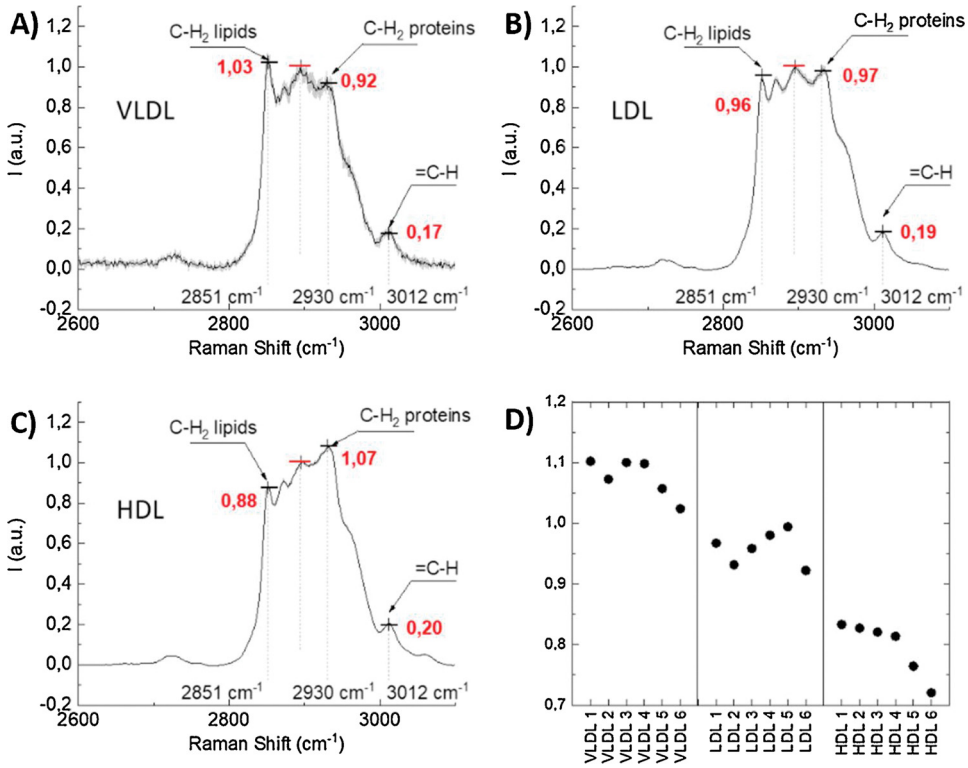

Fig. 2. Raman spectra acquired in the high frequency region on VLDL (A), LDL (B) and HDL (C) extracted from a single subject. The black line is the mean value of the five spectra acquired from each sample. The grey shadow represents the standard deviation of the five measures. D) Ratio between the peaks at 2851 and 2930 cm^{-1} calculated on VLDL, LDL and HDL forthe six subjects included in the study.

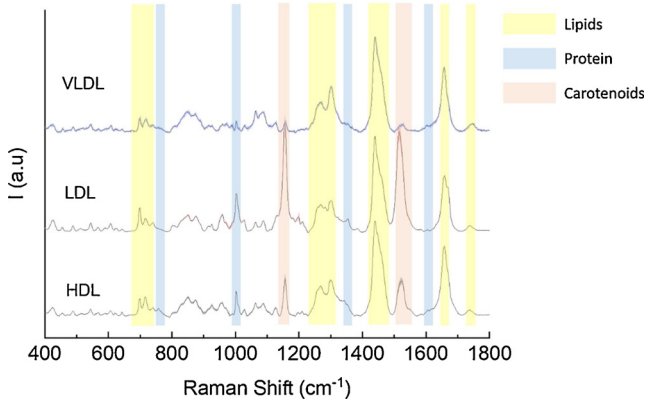

Fig. 3. Raman spectra acquired in the low frequency region on VLDL (top), LDL (middle) and HDL (bottom) extracted from a single subject. The black line is the mean value of the five spectra acquired from each sample. The grey shadow represents the standard deviation of the five measures. Color bands highlight the classes of a biomolecules that are represented by the peaks in the spectra. Yellow bands highlight peaks relative to the lipid content; light blue bands highlight peaks relative to proteins, while the pink band highlight peaks relative to carotenoids.

3.3. Raman spectra in the low frequency region

A second series of Raman spectra were acquired in the low frequency region between 400 and 1800 cm^{-1}. For all the classes of LPs, we were able to acquire very clear spectra (Fig. 3) presenting several peaks clearly related with the major classes of biomolecules present: cholesterol, lipids, proteins and antioxidants (carotenoids). Even in these spectra the shift of the peaks was the same in all the different classes of LPs reflecting the fact that their basic constituents remained constant. This region of the spectrum allowed us to clearly evidence the presence of cholesterol, presumably in its esterified form as part of the hydrophobic core of the LPs, as peaks at 700 and 740 cm^{-1} and as a shoulder at 1668 cm^{-1}, [25]. Other peaks at 1061, 1127, 1300 and 1439 cm^{-1} were related with the hydrophobic chains of lipids. Other important peaks, that were present in all the spectra and that referred to lipids, were the one at 716 cm^{-1}, very characteristic for the asymmetric stretching of choline $N^{+}(CH_3)_3$ groups present in the hydrophilic head of phosphatidylcholine, and the one at 1656 cm^{-1} of trans C=C groups [26]. The first peak, can be used as an indicator of the ratio between the lipids in the hydrophobic core and the ones in the hydrophilic membrane; the latter could represent a way to measure the unsaturated lipids within LPs. The last peak typical of lipid was observed in the region between 1730 and 1745 cm^{-1} due to the C=O stretching bands of the ester bonds [25].

The peaks at 1154 and 1520 cm^{-1} were well known to be peaks relative to the presence of carotenoids and, in general, of antioxidants micronutrients [27]. Other, less intense peaks are typical of aromatic amino acids: 760 and 1355 cm^{-1} for tryptophan; 1003 and 1603 cm^{-1} for phenylalanine [28].

Even in the low frequency region, the intensity of the peaks observed changed between the different classes of LPs and, in some cases, even between the single subjects included in the study. Io order to better understand the extent of the variations, we selected three portions of the spectra, particularly relevant and informative, that were analysed more in detail: the region between 650 and 800 cm^{-1}; the region between 1200 and 1400 cm^{-1} and the region between 1400 and 1800 cm^{-1}.

3.4. Region between 650 and 800 cm^{-1}

The region of the spectra between 650 and 800 cm^{-1} is characterized by the presence of three main peaks at 700, 717 and 740 cm^{-1}. As previously introduced however the intensity of these peaks was not the same in all the classes of LPs studied. In particular the peak of the in-plane deformations of the B ring of cholesterol at 700 cm^{-1} was more intense in LDL spectra (Fig. 4B,D), than in HDL (Fig. 4C,D) and VLDL (Fig. 4A,D). This trend is in good accordance with the composition of LPs measured and with the composition of LPs reported in literature. In fact, LDL are the main vector for the transport of

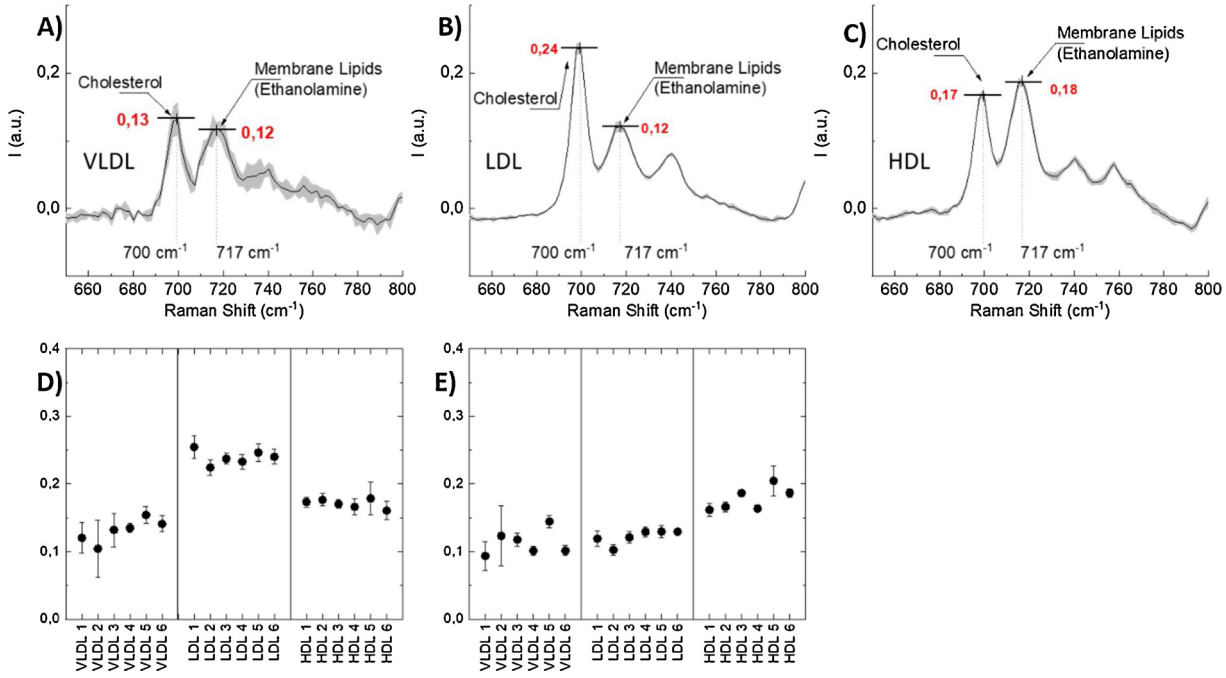

Fig. 4. Raman spectra acquired between 650 and 800 cm^{-1} on VLDL (A), LDL (B) and HDL (C) extracted from a single subject. The black line is the mean value of the five spectra acquired from each sample. The grey shadow represents the standard deviation of the five measures. D) Intensity of the peaks at 700 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. E) Intensity of the peaks at 717 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. In Fig. 4D-E black dots are the mean of the five spectra acquired on each sample, brackets are the standard deviations.

cholesterol and are composed of 35–40 % of their weight by esterified cholesterol and by free cholesterol (see Table 1). On the contrary, the peaks at 717 cm^{-1} of the polar head of phosphatidylcholine were more pronounced in HDL than in VLDL and LDL, (Fig. 4E). Even these data were in close accordance with the literature. HDL are very small particles (5 – 20 nm) and, in fasting subjects, their main constituents are the membrane lipids (as phosphatidylcholine) and apolipoproteins. The percentage of membrane lipids is higher in HDL than in other LPs as also appears in Table 1. The good accordance of RS with the composition of LPs was also proven by the presence of the peak of tryptophan at 760 cm^{-1} in HDL spectra where proteins are much more concentrated (about 50 % of dry weight according to our measures). The same peak was almost undetectable in LDL and VLDL, where proteins are significantly diluted.

By comparing the intensity of the two peaks at 700 and 717 cm^{-1} in the different subjects, it was also remarkable to observe how values remained rather constant and did not have a large inter-subjects variability.

3.5. Region between 1200 and 1400 cm^{-1}

The region of the spectra between 1200 and 1400 cm^{-1} is characterized by the presence of three main peaks at 1270, 1300 and 1355 cm^{-1} (Fig. 5A–C). The peak at 1270 cm^{-1} of = C–H in-plane deformation was very constant in all samples studied and did not provide any significant information to the analysis except to the presence of unsaturated lipids. The peak at 1300 cm^{-1} of CH_2 twisting mode is a marker of the presence of the hydrophobic chains of fatty acids [25]. It is notable that even if cholesterol esters are also characterized by the presence of CH_2 chains, they do not have a peak at 1300 cm^{-1} (or it has a very reduced intensity). This was previously reported in the literature [25] and can be observed in the Raman spectrum of cholesteryl palmitate reported in the supplementary materials (Figure S1). For this reason, the peak at 1300 cm^{-1} can be used as a marker of the amount of triglycerides in LPs and was more pronounced in the triglycerides rich VLDL (Fig. 5D).

Moreover, the shoulder observed at 1355 cm^{-1} is a band of the indole ring from tryptophan. The different intensity observed in VLDL compared to LDL and HDL might be due to a different exposure of the indole ring to the surface of the particles [29].

3.6. Region between 1400 and 1800 cm^{-1}

The last region of the spectra identified as particularly informative was the one between 1400 and 1800 cm^{-1}. Here, LPs have four main peaks at 1439, 1524, 1656 and 1738 cm^{-1}. The one at 1439 cm^{-1} of CH_2 scissoring vibrations appeared to be rather constant in all the acquired spectra and was selected as reference peak for the normalization of data. The band at around 1524 cm^{-1} of carotenoids, on the opposite, was highly variable between the classes of LPs and subjects. VLDL consistently had a very low, or absent, band of carotenoids, while LDL and HDL had bands with drastically different intensity (Fig. 6D). It is also notable to observe that also the position of the band of carotenoids changed between the subjects. This reflect the fact that the composition of anti-oxidants in LPs is not constant. For most of the analysed sample, the maximum of the carotenoids band was recorded at 1525 cm^{-1}, suggesting that beta-carotene is the most concentrated carotenoid in accordance with the scientific literature on the topic [30]. However, the shape of the band was not constant and, in some spectra, the maximum was shifted to 1530 cm^{-1} or 1515 cm^{-1}. The band at 1655 cm^{-1} of trans C=C stretching vibrations allowed to detect the presence of unsaturated lipids. In fact, the ratio between the peaks at 1655 and the one at 1439 cm^{-1} was reported as a method to measure the level of instauration in living cells [31]. This might be a particularly interesting finding as numerous studies focus on the different nutritional values of lipids with different level of unsaturation. A rapid and simple method to study how different nutritional lipids reflects on the unsaturation level of LPs could thus be of potential clinical interest. Even if our data are preliminary, we must notice that no clear difference emerged in the intensity of this peak at 1655 cm^{-1} between LVDL, LDL and HDL. Otherwise, it was highly variable between subjects.

The band at 1735 – 1750 cm^{-1} of C=O stretching in esters was

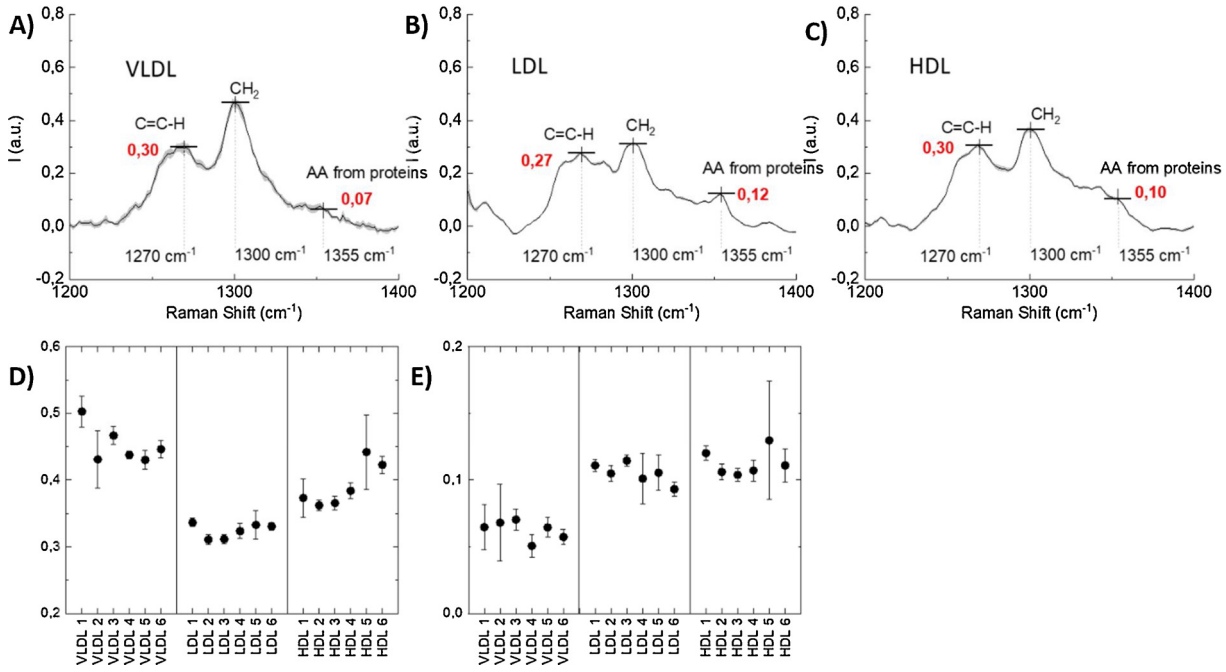

Fig. 5. Raman spectra acquired between 1200 and 1400 cm^{-1} on VLDL (A), LDL (B) and HDL (C) extracted from a single subject. The black line is the mean value of the five spectra acquired from each sample. The grey shadow represents the standard deviation of the five measures. D) Intensity of the peak at 1300 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. E) Intensity of the peaks at 1355 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. In Fig. 4D-E black dots are the mean of the five spectra acquired on each sample, brackets are the standard deviations.

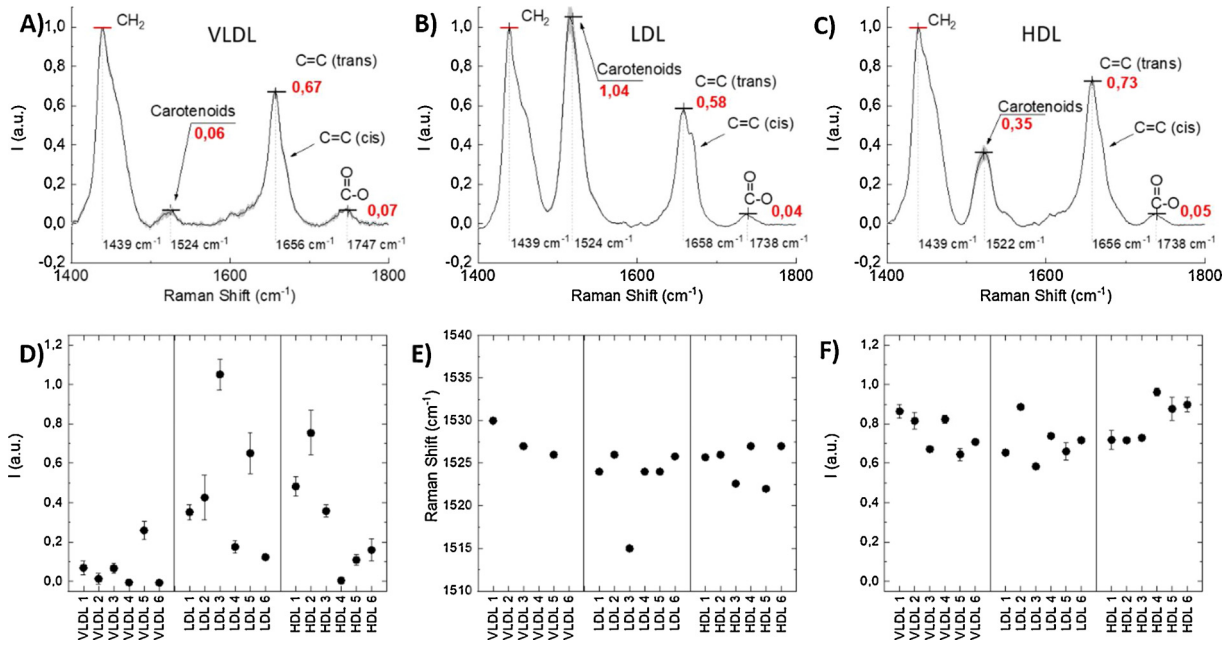

Fig. 6. Raman spectra acquired between 1400 and 1800 cm^{-1} on VLDL (A), LDL (B) and HDL (C) extracted from a single subject. The black line is the mean value of the five spectra acquired from each sample. The grey shadow represents the standard deviation of the five measures. D) Intensity of the bands of carotenoids at about 1524 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. E) Exact peak position of the band of carotenoids on VLDL, LDL and HDL of the six subjects included in the study. F) Intensity of the peaks at 1656 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. In Fig. 4D-E black dots are the mean of the five spectra acquired on each sample, brackets are the standard deviations.

found in all classes of LPs and its intensity remained rather stable in all subjects. However, the position of this peak is shifted at 1750 cm^{-1} in LDL remarking the fact that they were mostly cholesterol esters, while HDL are constituted mostly by triglycerides and membrane lipids.

4. Conclusion

The aim of this study, performed on a limited number of healthy subjects, was to demonstrate how it is possible to obtain clear and reproducible Raman spectra from LPs extracted from plasma by ultracentrifugation in discontinuous KBr density gradient. The spectra obtained showed several peaks relative to the different biomolecules composing LPs and their intensity well reflects their relative composition. However, the Raman characterization of LPs is faster and requires significantly less work if compared with the traditional approaches used in lipidomic.

Since the composition of LPs is known to be altered in many pathological conditions, such as dyslipidaemias, this study paves the way for the application of RS in clinical nutritional studies on a better understanding of several metabolic and pathologic conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vibspec.2020.103073.

References

[1] H.J. Butler, L. Ashton, B. Bird, et al., Using Raman spectroscopy to characterize biological materials, Nat. Protoc. 11 (2016) 664–687.
[2] C. Krafft, M. Schmitt, I.W. Schie, et al., Label-free molecular imaging of biological cells and tissues by linear and nonlinear raman spectroscopic approaches, Angew. Chem. Int. Ed. Engl. 56 (16) (2017) 4392–4430.
[3] T.J.E. Hubbard, A. Shore, N. Stone, Raman spectroscopy for rapid intra-operative margin analysis of surgically excised tumour specimens, Analyst 144 (2019) 6479–6496.
[4] S. Cervo, E. Mansutti, G. Del Mistro, et al., SERS analysis of serum for detection of early and locally advanced breast cancer, Anal. Bioanal. Chem. 407 (24) (2015) 7503–7509.
[5] M. Paraskevaidi, C.L.M. Morais, D.E. Halliwell, et al., Raman spectroscopy to diagnose alzheimer's disease and dementia with Lewy Bodies in blood, ACS Chem. Neurosci. 9 (11) (2018) 2786–2794.
[6] H.F. Nargis, H. Nawaz, A. Ditta, et al., Raman spectroscopy of blood plasma samples from breast cancer patients at different stages, Spectrochim. Acta A. Mol. Biomol. Spectrosc. 222 (2019) 117210.
[7] E. Szafraniec, E. Kus, A. Wislocka, et al., Raman spectroscopy-based insight into lipid droplets presence and contents in liver sinusoidal endothelial cells and hepatocytes, J. Biophotonics 12 (4) (2019) e201800290.
[8] J. O'Malley, R. Kumar, A.N. Kuzmin, et al., Lipid quantification by Raman microspectroscopy as a potential biomarker in prostate cancer, Cancer Lett. 397 (2017) 52–60.
[9] E.D. Michos, J.W. McEvoy, R.S. Blumenthal, Lipid management for the prevention of atherosclerotic cardiovascular disease, N. Engl. J. Med. 381 (16) (2019) 1557–1567.
[10] B.A. Ference, J.J.P. Kastelein, K.K. Ray, Association of triglyceride-lowering LPL variants and LDL-C-Lowering LDLR variants with risk of coronary heart disease, JAMA. 321 (4) (2019) 364–373.
[11] P.A. Ades, P.D. Savage, E.T. Poehlman, et al., Lipid lowering in the cardiac rehabilitation setting, J. Cardiopulm. Rehabil. 19 (4) (1999) 255–260.
[12] Y. Yamazaki, N. Zhao, T.R. Caulfield, et al., Apolipoprotein E and Alzheimer disease: pathobiology and targeting strategies, Nat. Rev. Neurol. 15 (9) (2019) 501–518.
[13] D.A. Hottman, D. Chernick, S. Cheng, HDL and cognition in neurodegenerative disorders, Neurobiol. Dis. 72 (Pt A) (2014) 22–36.
[14] C. Stiebing, L. Schmölz, M. Wallert, et al., Raman imaging of macrophages incubated with triglyceride-enriched oxLDL visualizes translocation of lipids between endocytic vesicles and lipid droplets, J. Lipid Res. 58 (5) (2017) 876–883.
[15] A. Oleszko, J. Hartwich, A. Wójtowicz, et al., Comparison of FTIR-ATR and Raman spectroscopy in determination of VLDL triglycerides in blood serum with PLS regression" *Spectrochim*, Acta A Mol. Biomol. Spectrosc 183 (2017) 239–246.
[16] L. Silveira Jr., R.C.F. Borges, R.S. Navarro, et al., Quantifying glucose and lipid components in human serum by Raman spectroscopy and multivariate statistics, Lasers Med. Sci. 32 (4) (2017) 787–795.

[17] P. Wang, J. Sun, T. Zhang, et al., Vibrational spectroscopic approaches for the quality evaluation and authentication of virgin olive oil, Appl. Spectrosc. Rev. 51 (10) (2016) 763–790.
[18] X.F. Zhang, M.Q. Zou, X.H. Qi, et al., Quantitative detection of adulterated olive oil by Raman spectroscopy and chemometrics, J. Raman Spectrosc. 42 (9) (2011) 1784–1788.
[19] B.H. Chung, J.P. Segrest, M.J. Ray, et al., Single vertical spin density gradient ultracentrifugation, Methods Enzym. 128 (1986) 181–209.
[20] R. Cazzola, G. Cervato, B. Cestaro, Variability in alpha-tocopherol antioxidant activity in the core and surface layers of low- and high-density lipoproteins, J. Nutr. Sci. Vitaminol. (Tokyo). 45 (1999) 39–48.
[21] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[22] R. Cazzola, E. Cassani, M. Barichella, et al., Impaired Fluidity and Oxidizability of HDL Hydrophobic Core and Amphipathic Surface in Dyslipidemic Men 62 (2013), pp. 986–991.
[23] A.V. Nichols, Functions and interrelationships of different classes of plasma lipoproteins, Proc. Natl. Acad. Sci. U.S A. 64 (1969) 1128–1137.
[24] W. Lee, A. Nanou, L. Rikkert, et al., Label-free prostate Cancer detection by characterization of extracellular vesicles using raman spectroscopy, Anal. Chem. 90 (19) (2018) 11290–11296.
[25] K. Czamara, K. Majzner, M.Z. Pacia, et al., Raman spectroscopy of lipids: a review, J. Raman Spectrosc. 46 (2015) 4–20.
[26] H. Sadeghi-Jorabchi, R.H. Wilson, P.S. Belton, et al., Quantitative analysis of oils and fats by Fourier transform Raman spectroscopy, Spectrochim. Acta A. Mol. Biomol. Spectrosc. 47 (1991) 1449–1458.
[27] M. Casella, A. Lucotti, M. Tommasini, et al., Raman and SERS recognition of β-carotene and haemoglobin fingerprints in human whole blood, Spectrochim. Acta A. Mol. Biomol. Spectrosc. 79 (5) (2011) 915–919.
[28] A. Mahadevan-Jansen, R. Richards-Kortum, Raman spectroscopy for the detection of cancers and precancers, J. Biomed. Opt. 1 (1) (1996) 31–70.
[29] T. Miura, H. Takeuchi, I. Harada, Characterization of individual tryptophan side chains in proteins using raman spectroscopy and hydrogen-deuterium exchange kinetics, Biochemistry 27 (1) (1988) 88–94.
[30] L.K. Bjornson, H.J. Kayden, E. Miller, et al., The transport of α-tocopherol and β-carotene in human blood, J. Lipid Res. 17 (1976) 343–352.
[31] O. Samek, Z. Pilat, A. Jonas, et al., Raman microspectroscopy based sensor of algal lipid unsaturation, Proceedings of SPIE Optics + Optoelectronics 8073 (2011) 807300.

Bibliography

1. Calder, P.C. Functional Roles of Fatty Acids and Their Effects on Human Health. *J. Parenter. Enter. Nutr.* 2015, *39*, 18S-32S.
2. Vallim, T.; Salter, A.M. Regulation of hepatic gene expression by saturated fatty acids. *Prostaglandins Leukot. Essent. Fat. Acids* **2010**, *82*, 211–218.
3. Tobin, K.A.R.; Steineger, H.H.; Alberti, S.; Spydevold, Ø.; Auwerx, J.; Gustafsson, J.-Å.; Nebb, H.I. Cross-Talk between Fatty Acid and Cholesterol Metabolism Mediated by Liver X Receptor-α. *Mol. Endocrinol.* **2000**, *14*, 741–752.
4. Suganami, T.; Tanimoto-Koyama, K.; Nishida, J.; Itoh, M.; Yuan, X.; Mizuarai, S.; Kotani, H.; Yamaoka, S.; Miyake, K.; Aoe, S.; et al. Role of the Toll-like receptor 4/NF-κB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. *Arterioscler. Thromb. Vasc. Biol.* **2007**, *27*, 84–91.
5. Palomer, X.; Pizarro-Delgado, J.; Barroso, E.; Vázquez-Carrera, M. Palmitic and Oleic Acid: The Yin and Yang of Fatty Acids in Type 2 Diabetes Mellitus. *Trends Endocrinol. Metab.* 2018, *29*, 178–190.

6. Cao, H.; Gerhold, K.; Mayers, J.R.; Wiest, M.M.; Watkins, S.M.; Hotamisligil, G.S. Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. *Cell* **2008**, *134*, 933–944.

7. Guo, X.; Li, H.; Xu, H.; Halim, V.; Zhang, W.; Wang, H.; Ong, K.T.; Woo, S.L.; Walzem, R.L.; Mashek, D.G.; et al. Palmitoleate induces hepatic steatosis but suppresses liver inflammatory response in mice. *PLoS One* **2012**, *7*, e39286.

8. Hussein, N.; Ah-Sing, E.; Wilkinson, P.; Leach, C.; Griffin, B.A.; Millward, D.J. Long-chain conversion of [13C]linoleic acid and α-linolenic acid in response to marked changes in their dietary intake in men. *J. Lipid Res.* **2005**, *46*, 269–280.

9. Harris, W.S.; Mozaffarian, D.; Rimm, E.; Kris-Etherton, P.; Rudel, L.L.; Appel, L.J.; Engler, M.M.; Engler, M.B.; Sacks, F. Omega-6 fatty acids and risk for cardiovascular disease: A science advisory from the American Heart Association nutrition subcommittee of the council on nutrition, physical activity, and metabolism; council on cardiovascular nursing; and council on epidemiology and prevention. *Circulation* **2009**, *119*, 902–907.

10. Zou, S.; Kumar, U.; Zou, S.; Kumar, U. Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. *Int. J. Mol. Sci.* **2018**, *19*, 833.

11. Russo, G.L. Dietary n - 6 and n - 3 polyunsaturated fatty acids: From biochemistry to clinical implications in

cardiovascular prevention. *Biochem. Pharmacol.* 2009, *77*, 937–946.

12. Li, D.; Wahlqvist, M.L.; Sinclair, A.J. Advances in n-3 polyunsaturated fatty acid nutrition. *Asia Pac. J. Clin. Nutr.* 2019, *28*, 1–5.

13. Kim, H.Y.; Spector, A.A. Synaptamide, endocannabinoid-like derivative of docosahexaenoic acid with cannabinoid-independent function. *Prostaglandins Leukot. Essent. Fat. Acids* **2013**, *88*, 121–125.

14. Rees, A.; Sirois, S.; Wearden, A. Prenatal maternal docosahexaenoic acid intake and infant information processing at 4.5mo and 9mo: A longitudinal study. *PLoS One* **2019**, *14*, e0210984.

15. Calder, P.C. Polyunsaturated fatty acids and inflammation. *Prostaglandins Leukot. Essent. Fat. Acids* **2006**, *75*, 197–202.

16. Brouwers, H.; Jónasdóttir, H.S.; Kuipers, M.E.; Kwekkeboom, J.C.; Auger, J.L.; Gonzalez-Torres, M.; López-Vicario, C.; Clària, J.; Freysdottir, J.; Hardardottir, I.; et al. Anti-Inflammatory and Proresolving Effects of the Omega-6 Polyunsaturated Fatty Acid Adrenic Acid. *J. Immunol.* **2020**, *205*, 2840–2849.

17. Ntambi, J.M.; Bené, H. Polyunsaturated fatty acid regulation of gene expression. *J. Mol. Neurosci.* **2001**, *16*, 273–278.

18. Jump, D.B. The biochemistry of n-3 polyunsaturated fatty acids. *J. Biol. Chem.* 2002, *277*, 8755–8758.

19. Schmitz, G.; Ecker, J. The opposing effects of n−3 and n−6 fatty acids. *Prog. Lipid Res.* **2008**, *47*, 147–155.

20. Nakamura, M.T.; Yudell, B.E.; Loor, J.J. Regulation of energy metabolism by long-chain fatty acids. *Prog. Lipid Res.* 2014, *53*, 124–144.

21. Calder, P.C. Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 2015, *1851*, 469–484.

22. Cazzola, R.; Russo-Volpe, S.; Miles, E.A.; Rees, D.; Banerjee, T.; Roynette, C.E.; Wells, S.J.; Goua, M.; Wahle, K.W.J.; Calder, P.C.; et al. Age- and dose-dependent effects of an eicosapentaenoic acid-rich oil on cardiovascular risk factors in healthy male subjects. *Atherosclerosis* **2007**, *193*, 159–167.

23. Shearer, G.C.; Savinova, O. V.; Harris, W.S. Fish oil - How does it reduce plasma triglycerides? *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 2012, *1821*, 843–851.

24. Geleijnse, J.M.; Giltay, E.J.; Grobbee, D.E.; Donders, A.R.T.; Kok, F.J. Blood pressure response to fish oil supplementation: Metaregression analysis of randomized trials. *J. Hypertens.* **2002**, *20*, 1493–1499.

25. Simopoulos, A.P. An increase in the Omega-6/Omega-3 fatty acid ratio increases the risk for obesity. *Nutrients* 2016, *8*.

26. Peterson, L.D.; Jeffery, N.M.; Thies, F.; Sanderson, P.; Newsholme, E.A.; Calder, P.C. Eicosapentaenoic and

docosahexaenoic acids alter rat spleen leukocyte fatty acid composition and prostaglandin E2 production but have different effects on lymphocyte functions and cell-mediated immunity. *Lipids* **1998**, *33*, 171–180.

27. Rondanelli, M.; Opizzi, A.; Faliva, M.; Mozzoni, M.; Antoniello, N.; Cazzola, R.; Savarè, R.; Cerutti, R.; Grossi, E.; Cestaro, B. Effects of a diet integration with an oily emulsion of DHA-phospholipids containing melatonin and tryptophan in elderly patients suffering from mild cognitive impairment. *Nutr. Neurosci.* **2012**, *15*.

28. Endo, J.; Arita, M. Cardioprotective mechanism of omega-3 polyunsaturated fatty acids. *J. Cardiol.* **2016**, *67*, 22–27.

29. Kitajka, K.; Sinclair, A.J.; Weisinger, R.S.; Weisinger, H.S.; Mathai, M.; Jayasooriya, A.P.; Halver, J.E.; Puskás, L.G. Effects of dietary omega-3 polyunsaturated fatty acids on brain gene expression. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 10931–10936.

30. Singh, P.; Kesharwani, R.K.; Keservani, R.K. Antioxidants and Vitamins: Roles in Cellular Function and Metabolism. . In *Sustained Energy for Enhanced Human Functions and Activity*; Elsevier Inc., 2017; pp. 385–407 ISBN 9780128093320.

31. Yadav, U.C.S. Oxidative stress-induced lipid peroxidation: Role in inflammation. In *Free Radicals in Human Health and Disease*; Springer India, 2015; pp. 119–129 ISBN 9788132220350.

32. Lauritzen, L.; Brambilla, P.; Mazzocchi, A.; Harsløf, L.B.S.; Ciappolino, V.; Agostoni, C. DHA effects in brain development and function. *Nutrients* **2016**, *8*, 1–6.

33. Jasani, B.; Simmer, K.; Patole, S.K.; Rao, S.C. Long chain polyunsaturated fatty acid supplementation in infants born at term. *Cochrane Database Syst. Rev.* 2017, *2017*.

34. Delgado-Noguera, M.F.; Calvache, J.A.; Bonfill Cosp, X.; Kotanidou, E.P.; Galli-Tsinopoulou, A. Supplementation with long chain polyunsaturated fatty acids (LCPUFA) to breastfeeding mothers for improving child growth and development. *Cochrane Database Syst. Rev.* **2015**, *2015*, 113–114.

35. Middleton, P.; Gomersall, J.C.; Gould, J.F.; Shepherd, E.; Olsen, S.F.; Makrides, M. Omega-3 fatty acid addition during pregnancy. *Cochrane Database Syst. Rev.* 2018, *2018*.

36. Hamazaki, K.; Matsumura, K.; Tsuchida, A.; Kasamatsu, H.; Tanaka, T.; Ito, M.; Inadera, H.; Kamijima, M.; Yamazaki, S.; Ohya, Y.; et al. Maternal dietary intake of fish and PUFAs and child neurodevelopment at 6 months and 1 year of age: a nationwide birth cohort—the Japan Environment and Children's Study (JECS). *Am. J. Clin. Nutr.* **2020**, *112*, 1295–1303.

37. Lain, K.Y.; Catalano, P.M. Metabolic changes in pregnancy. *Clin. Obstet. Gynecol.* **2007**, *50*, 938–948.

38. Ryan, E.A. Hormones and insulin resistance during pregnancy. *Lancet* 2003, *362*, 1777–1778.

39. Sonagra, Amit D.; Biradar Shivaleela M., D.K..

M.J.D.S. Normal Pregnancy- A State of Insulin Resistance. *J. Clin. DIAGNOSTIC Res.* **2014**, *8*, CC01.

40. Stanley, K.; Fraser, R.; Bruce, C. Physiological changes in insulin resistance in human pregnancy: Longitudinal study with the hyperinsulinaemic euglycaemic clamp technique. *Br. J. Obstet. Gynaecol.* **1998**, *105*, 756–759.

41. Buchanan, T.A.; Metzger, B.E.; Freinkel, N.; Bergman, R.N. Insulin sensitivity and B-cell responsiveness to glucose during late pregnancy in lean and moderately obese women with normal glucose tolerance or mild gestational diabetes. *Am. J. Obstet. Gynecol.* **1990**, *162*, 1008–1014.

42. Catalano, P.M.; Roman-Drago, N.M.; Amini, S.B.; Sims, E.A.H. Longitudinal changes in body composition and energy balance in lean women with normal and abnormal glucose tolerance during pregnancy. *Am. J. Obstet. Gynecol.* **1998**, *179*, 156–165.

43. Akash, M.S.H.; Rehman, K.; Liaqat, A. Tumor Necrosis Factor-Alpha: Role in Development of Insulin Resistance and Pathogenesis of Type 2 Diabetes Mellitus. *J. Cell. Biochem.* **2018**, *119*, 105–110.

44. Kirwan, J.P.; Hauguel-De Mouzon, S.; Lepercq, J.; Challier, J.C.; Huston-Presley, L.; Friedman, J.E.; Kalhan, S.C.; Catalano, P.M. TNF-α is a predictor of insulin resistance in human pregnancy. *Diabetes* **2002**, *51*, 2207–2213.

45. Sakowicz, A. The role of NFκB in the three stages of pregnancy – implantation, maintenance, and labour: a

review article. *BJOG An Int. J. Obstet. Gynaecol.* **2018**, *125*, 1379–1387.

46. Moore, T.A.; Ahmad, I.M.; Schmid, K.K.; Berger, A.M.; Ruiz, R.J.; Pickler, R.H.; Zimmerman, M.C. Oxidative Stress Levels Throughout Pregnancy, at Birth, and in the Neonate. *Biol. Res. Nurs.* **2019**, *21*, 485–494.

47. Malti, N.; Merzouk, H.; Merzouk, S.A.; Loukidi, B.; Karaouzene, N.; Malti, A.; Narce, M. Oxidative stress and maternal obesity: Feto-placental unit interaction. *Placenta* **2014**, *35*, 411–416.

48. Dsouza, V.; Rani, A.; Patil, V.; Pisal, H.; Randhir, K.; Mehendale, S.; Wagh, G.; Gupte, S.; Joshi, S. Increased oxidative stress from early pregnancy in women who develop preeclampsia. *Clin. Exp. Hypertens.* **2016**, *38*, 225–232.

49. Dan Dunn, J.; Alvarez, L.A.J.; Zhang, X.; Soldati, T. Reactive oxygen species and mitochondria: A nexus of cellular homeostasis. *Redox Biol.* **2015**, *6*, 472–485.

50. Muñoz, A.; Costa, M. Nutritionally mediated oxidative stress and inflammation. *Oxid. Med. Cell. Longev.* **2013**, *2013*.

51. Jones, M.L.; Mark, P.J.; Waddell, B.J. Maternal dietary omega-3 fatty acids and placental function. *Reproduction* **2014**, *147*.

52. Konukoglu, D.; Uzun, H. Endothelial dysfunction and hypertension. In *Advances in Experimental Medicine and Biology*; Springer New York LLC, 2016; Vol. 956, pp. 511–540.

53. Zehr, K.R.; Walker, M.K. Omega-3 polyunsaturated

fatty acids improve endothelial function in humans at risk for atherosclerosis: A review. *Prostaglandins Other Lipid Mediat.* 2018, *134*, 131–140.

54. Zanetti, M.; Grillo, A.; Losurdo, P.; Panizon, E.; Mearelli, F.; Cattin, L.; Barazzoni, R.; Carretta, R. Omega-3 polyunsaturated fatty acids: Structural and functional effects on the vascular wall. *Biomed Res. Int.* 2015, *2015*.

55. Félétou, M. Endothelium-dependent hyperpolarization and endothelial dysfunction. *J. Cardiovasc. Pharmacol.* **2016**, *67*, 373–387.

56. Therien, A.G.; Blostein, R. Mechanisms of sodium pump regulation. *Am. J. Physiol. Physiol.* **2000**, *279*, C541–C566.

57. Namazi, G.; Asa, P.; Sarrafzadegan, N.; Pourfarzam, M. Decreased Na+/K+-ATPase Activity and Altered Susceptibility to Peroxidation and Lipid Composition in the Erythrocytes of Metabolic Syndrome Patients with Coronary Artery Disease. *Ann. Nutr. Metab.* **2019**, *74*, 140–148.

58. Minihane, A.M.; Vinoy, S.; Russell, W.R.; Baka, A.; Roche, H.M.; Tuohy, K.M.; Teeling, J.L.; Blaak, E.E.; Fenech, M.; Vauzour, D.; et al. Low-grade inflammation, diet composition and health: Current research evidence and its translation. *Br. J. Nutr.* **2015**, *114*, 999–1012.

59. Golia, E.; Limongelli, G.; Natale, F.; Fimiani, F.; Maddaloni, V.; Pariggiano, I.; Bianchi, R.; Crisci, M.; D'Acierno, L.; Giordano, R.; et al. Inflammation and cardiovascular disease: From pathogenesis to therapeutic

target. *Curr. Atheroscler. Rep.* **2014**, *16*, 1–7.

60. Donath, M.Y.; Shoelson, S.E. Type 2 diabetes as an inflammatory disease. *Nat. Rev. Immunol.* 2011, *11*, 98–107.

61. Ruiz-Núñez, B.; Pruimboom, L.; Dijck-Brouwer, D.A.J.; Muskiet, F.A.J. Lifestyle and nutritional imbalances associated with Western diseases: Causes and consequences of chronic systemic low-grade inflammation in an evolutionary context. *J. Nutr. Biochem.* **2013**, *24*, 1183–1201.

62. Egger, G.; Dixon, J. Non-nutrient causes of low-grade, systemic inflammation: Support for a "canary in the mineshaft" view of obesity in chronic disease. *Obes. Rev.* 2011, *12*, 339–345.

63. Obesity.

64. Obesity Update - OECD.

65. Tilg, H.; Moschen, A.R. Adipocytokines: Mediators linking adipose tissue, inflammation and immunity. *Nat. Rev. Immunol.* **2006**, *6*, 772–783.

66. Longo, M.; Zatterale, F.; Naderi, J.; Parrillo, L.; Formisano, P.; Raciti, G.A.; Beguinot, F.; Miele, C. Adipose Tissue Dysfunction as Determinant of Obesity-Associated Metabolic Complications. *Int. J. Mol. Sci.* **2019**, *20*, 2358.

67. Stienstra, R.; Stefan, N. Tipping the inflammatory balance: Inflammasome activation distinguishes metabolically unhealthy from healthy obesity. *Diabetologia* **2013**, *56*, 2343–2346.

68. Cotillard, A.; Poitou, C.; Torcivia, A.; Bouillot, J.-L.; Dietrich, A.; Klöting, N.; Grégoire, C.; Lolmede, K.; Blüher,

M.; Clément, K. Adipocyte size threshold matters: link with risk of type 2 diabetes and improved insulin resistance after gastric bypass. *J. Clin. Endocrinol. Metab.* **2014**, *99*, E1466-70.

69. Kim, J.I.; Huh, J.Y.; Sohn, J.H.; Choe, S.S.; Lee, Y.S.; Lim, C.Y.; Jo, A.; Park, S.B.; Han, W.; Kim, J.B. Lipid-Overloaded Enlarged Adipocytes Provoke Insulin Resistance Independent of Inflammation. *Mol. Cell. Biol.* **2015**, *35*, 1686–1699.

70. Wueest, S.; Rapold, R.A.; Rytka, J.M.; Schoenle, E.J.; Konrad, D. Basal lipolysis, not the degree of insulin resistance, differentiates large from small isolated adipocytes in high-fat fed mice. *Diabetologia* **2009**, *52*, 541–546.

71. Arner, E.; Westermark, P.O.; Spalding, K.L.; Britton, T.; Rydén, M.; Frisén, J.; Bernard, S.; Arner, P. Adipocyte turnover: Relevance to human adipose tissue morphology. *Diabetes* **2010**, *59*, 105–109.

72. Rutkowski, J.M.; Stern, J.H.; Scherer, P.E. The cell biology of fat expansion. *J. Cell Biol.* 2015, *208*, 501–512.

73. Haczeyni, F.; Bell-Anderson, K.S.; Farrell, G.C. Causes and mechanisms of adipocyte enlargement and adipose expansion. *Obes. Rev.* 2018, *19*, 406–420.

74. Reilly, S.M.; Saltiel, A.R. Adapting to obesity with adipose tissue inflammation. *Nat. Rev. Endocrinol.* **2017**, *13*, 633–643.

75. Ringseis, R.; Eder, K.; Mooren, F.C.; Krüger, K. Metabolic signals and innate immune activation in obesity and exercise. *Exerc. Immunol. Rev.* 2015, *21*, 58–68.

76. Wang, X.; Wang, Y.; Antony, V.; Sun, H.; Liang, G. Metabolism-Associated Molecular Patterns (MAMPs). *Trends Endocrinol. Metab.* **2020**, *31*, 712–724.

77. Lee, J.Y.; Sohn, K.H.; Rhee, S.H.; Hwang, D. Saturated Fatty Acids, but Not Unsaturated Fatty Acids, Induce the Expression of Cyclooxygenase-2 Mediated through Toll-like Receptor 4. *J. Biol. Chem.* **2001**, *276*, 16683–16689.

78. Huang, S.; Rutkowsky, J.M.; Snodgrass, R.G.; Ono-Moore, K.D.; Schneider, D.A.; Newman, J.W.; Adams, S.H.; Hwang, D.H. Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. *J. Lipid Res.* **2012**, *53*, 2002–2013.

79. Cao, X. Self-regulation and cross-regulation of pattern-recognition receptor signalling in health and disease. *Nat. Rev. Immunol.* 2016, *16*, 35–50.

80. Rogero, M.M.; Calder, P.C. Obesity, inflammation, toll-like receptor 4 and fatty acids. *Nutrients* 2018, *10*.

81. Hotamisligil, G.S.; Shargill, N.S.; Spiegelman, B.M. Adipose expression of tumor necrosis factor-α: Direct role in obesity-linked insulin resistance. *Science (80-.).* **1993**, *259*, 87–91.

82. Shoelson, S.E.; Lee, J.; Yuan, M. Inflammation and the IKKβ/IκB/NF-κB axis in obesity- and diet-induced insulin resistance. *Int. J. Obes.* **2003**, *27*, S49–S52.

83. Wisse, B.E. The inflammatory syndrome: The role of adipose tissue cytokines in metabolic disorders linked to

obesity. *J. Am. Soc. Nephrol.* 2004, *15*, 2792–2800.

84. Asghar, A.; Sheikh, N. Role of immune cells in obesity induced low grade inflammation and insulin resistance. *Cell. Immunol.* **2017**, *315*, 18–26.

85. Meessen, E.C.E.; Warmbrunn, M. V.; Nieuwdorp, M.; Soeters, M.R. Human postprandial nutrient metabolism and low-grade inflammation: A narrative review. *Nutrients* **2019**, *11*, 1–21.

86. Connaughton, R.M.; McMorrow, A.M.; McGillicuddy, F.C.; Lithander, F.E.; Roche, H.M. Impact of anti-inflammatory nutrients on obesity-associated metabolic-inflammation from childhood through to adulthood. *Proc. Nutr. Soc.* **2016**, *75*, 115–124.

87. Lyte, J.M.; Gabler, N.K.; Hollis, J.H. Postprandial serum endotoxin in healthy humans is modulated by dietary fat in a randomized, controlled, cross-over study. *Lipids Health Dis.* **2016**, *15*, 1–10.

88. López-Moreno, J.; García-Carpintero, S.; Jimenez-Lucena, R.; Haro, C.; Rangel-Zúñiga, O.A.; Blanco-Rojo, R.; Yubero-Serrano, E.M.; Tinahones, F.J.; Delgado-Lista, J.; Pérez-Martínez, P.; et al. Effect of Dietary Lipids on Endotoxemia Influences Postprandial Inflammatory Response. *J. Agric. Food Chem.* **2017**, *65*, 7756–7763.

89. Nakarai, H.; Yamashita, A.; Nagayasu, S.; Iwashita, M.; Kumamoto, S.; Ohyama, H.; Hata, M.; Soga, Y.; Kushiyama, A.; Asano, T.; et al. Adipocyte-macrophage interaction may mediate LPS-induced low-grade

inflammation: Potential link with metabolic complications. *Innate Immun.* **2012**, *18*, 164–170.

90. Massiera, F.; Barbry, P.; Guesnet, P.; Joly, A.; Luquet, S.; Moreilhon-Brest, C.; Mohsen-Kanson, T.; Amri, E.-Z.; Ailhaud, G. A Western-like fat diet is sufficient to induce a gradual enhancement in fat mass over generations. *J. Lipid Res.* **2010**, *51*, 2352–2361.

91. Zeyda, M.; Stulnig, T.M. Obesity, inflammation, and insulin resistance - A mini-review. *Gerontology* 2009, *55*, 379–386.

92. Anderson, E.J.; Lustig, M.E.; Boyle, K.E.; Woodlief, T.L.; Kane, D.A.; Lin, C. Te; Price, J.W.; Kang, L.; Rabinovitch, P.S.; Szeto, H.H.; et al. Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. *J. Clin. Invest.* **2009**, *119*, 573–581.

93. Tan, B.L.; Norhaizan, M.E.; Liew, W.P.P. Nutrients and oxidative stress: Friend or foe? *Oxid. Med. Cell. Longev.* 2018, *2018*.

94. Mutlu-Türkoğlu, Ü.; Öztezcan, S.; Telci, A.; Orhan, Y.; Aykaç-Toker, G.; Sivas, A.; Uysal, M. An increase in lipoprotein oxidation and endogenous lipid peroxides in serum of obese women. *Clin. Exp. Med.* **2003**, *2*, 171–174.

95. Arca, M. Alterations of intestinal lipoprotein metabolism in diabetes mellitus and metabolic syndrome. *Atheroscler. Suppl.* **2015**, *17*, 12–16.

96. Adiels, M.; Borén, J.; Caslake, M.J.; Stewart, P.; Soro,

A.; Westerbacka, J.; Wennberg, B.; Olofsson, S.O.; Packard, C.; Taskinen, M.R. Overproduction of VLDL1 driven by hyperglycemia is a dominant feature of diabetic dyslipidemia. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 1697–1703.

97. Borggreve, S.E.; De Vries, R.; Dullaart, R.P. Alterations in high-density lipoprotein metabolism and reverse cholesterol transport in insulin resistance and type 2 diabetes mellitus: role of lipolytic enzymes, lecithin:cholesterol acyltransferase and lipid transfer proteins. *Eur J Clin Invest* **2003**, *33*, 1051–1069.

98. Cazzola, R.; Cassani, E.; Barichella, M.; Cestaro, B. Impaired fluidity and oxidizability of HDL hydrophobic core and amphipathic surface in dyslipidemic men. **2013**, *62*, 986–991.

99. Wilson, P.W.F.; Abbott, R.D.; Castelli, W.P. High density lipoprotein cholesterol and mortality. The Framingham heart study. *Arteriosclerosis* **1988**, *8*, 737–741.

100. High-density lipoprotein cholesterol and risk of cardiovascular disease Available online: https://www.escardio.org/Journals/E-Journal-of-Cardiology-Practice/Volume-19/high-density-lipoprotein-cholesterol-and-risk-of-cardiovascular-disease (accessed on Nov 30, 2020).

101. Ricciardi, A.; Piuri, G.; Porta, M. Della; Mazzucchelli, S.; Bonizzi, A.; Truffi, M.; Sevieri, M.; Allevi, R.; Corsi, F.; Cazzola, R.; et al. Raman spectroscopy characterization of the

major classes of plasma lipoproteins. *Vib. Spectrosc.* **2020**, *109*.

102. Wu, Z.; Zhang, Q.; Li, N.; Pu, Y.; Wang, B.; Zhang, T. Comparison of critical methods developed for fatty acid analysis: A review. *J. Sep. Sci.* **2017**, *40*, 288–298.

103. Wolf, C.; Quinn, P.J. Lipidomics: Practical aspects and applications. *Prog. Lipid Res.* **2008**, *47*, 15–36.

104. Arab, L. Biomarkers of Nutritional Exposure and Nutritional Status Biomarkers of Fat and Fatty Acid Intake 1.

105. Sun, Q.; Ma, J.; Campos, H.; Hankinson, S.E.; Hu, F.B. Comparison between plasma and erythrocyte fatty acid content as biomarkers of fatty acid intake in US women. *Am. J. Clin. Nutr.* **2007**, *86*, 74–81.

106. Katerji, M.; Filippova, M.; Duerksen-Hughes, P. Approaches and methods to measure oxidative stress in clinical samples: Research applications in the cancer field. *Oxid. Med. Cell. Longev.* 2019, *2019*.

107. Dotan, Y.; Lichtenberg, D.; Pinchuk, I. Lipid peroxidation cannot be used as a universal criterion of oxidative stress. *Prog. Lipid Res.* 2004, *43*, 200–227.

108. Cighetti, G.; Bamonti, F.; Aman, C.S.; Gregori, D.; De Giuseppe, R.; Novembrino, C.; de Liso, F.; Maiavacca, R.; Paroni, R. Oxidative status in different settings and with different methodological approaches compared by Receiver Operating Characteristic curve analysis. *Clin. Biochem.* **2015**, *48*, 73–78.

109. Vassalle, C.; Boni, C.; Di Cecco, P.; Ndreu, R.; Zucchelli, G.C. Automation and validation of a fast method

for the assessment of in vivo oxidative stress levels. *Clin. Chem. Lab. Med.* **2006**, *44*, 1372–1375.

110. Ito, F.; Ito, T.; Suzuki, C.; Yahata, T.; Ikeda, K.; Hamaoka, K. The application of a modified d-ROMs test for measurement of oxidative stress and oxidized high-density lipoprotein. *Int. J. Mol. Sci.* **2017**, *18*, 454.

111. Cazzola, R.; Rondanelli, M. N-Oleoyl-Phosphatidyl-Ethanolamine and Epigallo Catechin-3-Gallate Mitigate Oxidative Stress in Overweight and Class i Obese People on a Low-Calorie Diet. *J. Med. Food* **2020**, *23*, 319–325.

112. Xuan, Y.; Bobak, M.; Anusruti, A.; Jansen, E.H.J.M.; Pająk, A.; Tamosiunas, A.; Saum, K.-U.; Holleczek, B.; Gao, X.; Brenner, H.; et al. Association of serum markers of oxidative stress with myocardial infarction and stroke: pooled results from four large European cohort studies. *Eur. J. Epidemiol.* **2019**, *34*, 471–481.

113. Schöttker, B.; Brenner, H.; Jansen, E.H.J.M.; Gardiner, J.; Peasey, A.; Kubínová, R.; Pajak, A.; Topor-Madry, R.; Tamosiunas, A.; Saum, K.U.; et al. Evidence for the free radical/oxidative stress theory of ageing from the CHANCES consortium: A meta-analysis of individual participant data. *BMC Med.* **2015**, *13*.

114. Dickinson, D.A.; Forman, H.J. Cellular glutathione and thiols metabolism. *Biochem. Pharmacol.* **2002**, *64*, 1019–1026.

115. Wu, G.; Fang, Y.Z.; Yang, S.; Lupton, J.R.; Turner, N.D. Glutathione Metabolism and Its Implications for

Health. *J. Nutr.* 2004, *134*, 489–492.

116. Pinchuk, I.; Lichtenberg, D. Analysis of the kinetics of lipid peroxidation in terms of characteristic time-points. *Chem. Phys. Lipids* 2014, *178*, 63–76.

117. Cervato, G.; Viani, P.; Cazzola, R.; Cestaro, B. A fluorescence method for the determination of plasma susceptibility to lipid peroxidation. *Clin. Biochem.* **1999**, *32*, 171–177.

118. Viani, P.; Cazzola, R.; Cervato, G.; Gatti, P.; Cestaro, B. Pyrene lipids as markers of peroxidative processes in different regions of low and high density lipoproteins. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1996**, *1315*, 78–86.

119. Cazzola, R.; Cestaro, B. Red wine polyphenols protect n-3 more than n-6 polyunsaturated fatty acid from lipid peroxidation. *Food Res. Int.* **2011**, *44*, 3065–3071.

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