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Aspetti biochimico-molecolari della regolazione nutrizionale della biosintesi del colesterolo

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INTRODUCTION

1. Cholesterol

1.1 Cholesterol structure

Cholesterol (Chol) was discovered in 1815 by the French chemist M.E. Chevreul (Figure 1a) but its structure was determined only in 1932 by Heinrich Wieland (figure 1b). The molecule consists of three main functional elements (figure 2):

I. The steroid ring
II. The hydrophilic 3β-hydroxyl group
III. The hydrocarbon chain

Any modification of these structural elements decreases effects of cholesterol on lipid bilayers.

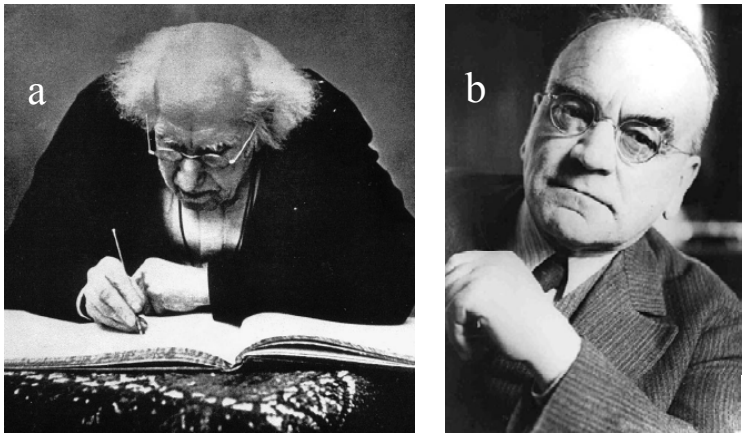

Figure 1: a) M.E. Chevreul the French chemist that discovered cholesterol in 1815 picture from *Vance and Bosch 2000* **b)** Heinrich Wieland Nobel Prize 1927 picture from *Vaupel 2007*

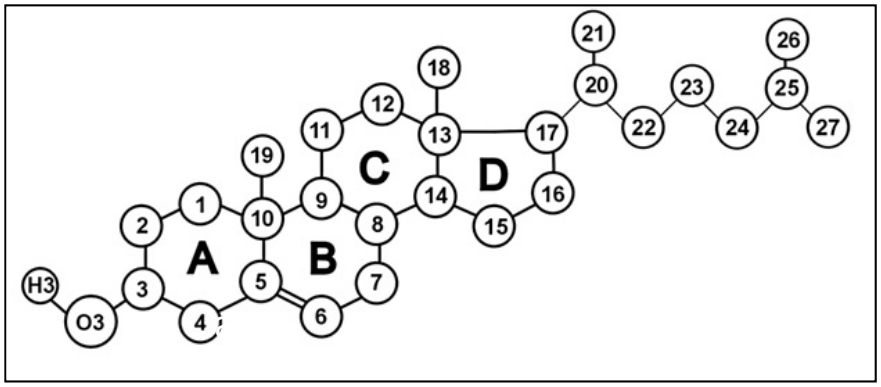

Figure 2: Cholesterol molecule: chemical structure with numbering of carbon atoms and rings (labeled A, B, C, and D). Image from *Róg et al. 2009*

I. <u>The steroid ring</u>: Cholesterol steroid ring system consist in four rings of which three have six carbons and one has five. These rings are trans connected and create a flat and rigid structure which characterizes cholesterol and most of its analogues. Two

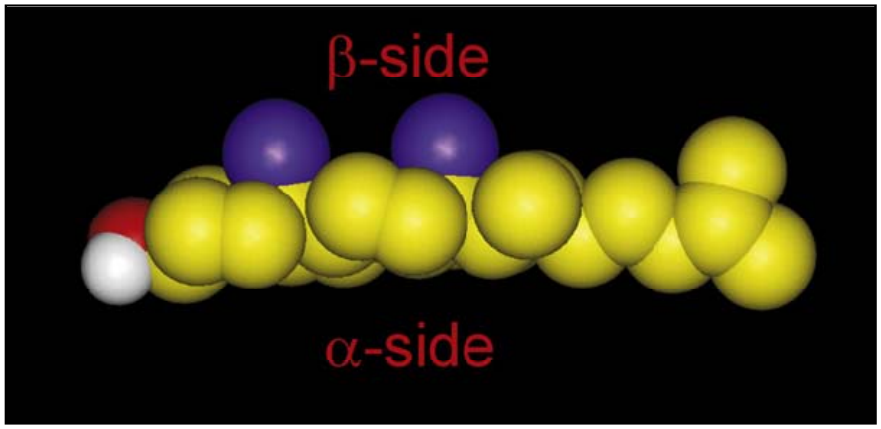

Figure 3 Cholesterol CPK representation, the smooth α-face and rough β-face are labeled. Image from Róg et al 2009.

methyl substituents, C18 and C19, are attached at positions 10 and 13, in relative cis orientations. Due to the above, the cholesterol ring system is asymmetric: one side is flat without any substituents, while the other is rough characterized by the presence of the two methyl substituents. The flat face of cholesterol is called the α-face, and all substituents located on this face (in trans conformation relative to C19) are called α, while the substituents located on the rough β-face (in cis conformation relative to C19) are called β (figure 3). In cholesterol there is only one double bond between C5 and C6 in ring B, in other sterols the number and positions of the double bonds vary to some extent.

II. The hydrophilic 3β-hydroxyl group:

The only additional group in cholesterol molecule is the polar 3β-hydroxyl group, while in other sterols there are many possible α and β substituents. Due to the presence of this hydroxyl group cholesterol is a steroid alcohol.

III. The hydrocarbon chain:

The hydrocarbon chain is composed by 8 C molecule and is attached to the steroid ring at the position 17.

[1-3]

1.2 Double origin of cholesterol:

Human Chol concentration mainly depends on *de novo* endogenous biosynthesis and on dietary intake, with a ratio estimated as ~70:30.

Such as fatty acid (FA), cholesterol is a primary component of cellular membranes. Cells, according to their growth demand, need to synthesize both cholesterol and FA. Chol biosynthetic pathway is linked to synthesis of bile acids and steroid hormones in liver and endocrine organs respectively.

The synthesis for fatty acids and triglycerides (TG), lipogenesis, is an energy storage system specialized to lipogenic organs such as liver and adipose tissues. In contrast to cholesterol synthesis, which is tightly regulated by a feedback system to maintain cellular cholesterol levels, fatty acid synthesis is driven primarily by the availability of carbohydrates and the actions of hormones such as insulin. Both pathways are nutritionally controlled at the transcriptional level.

[4, 5]

1.2.1 Cholesterol adsorption by the diet.

Cholesterol dietary intake is not a complete adsorption, but only the 40–60% is assimilated. Most of the cholesterol mass escape intestinal absorption, is degraded to coprostanol through reduction of the double bond at C:5 by colonic bacteria, and is excreted in feces.

Early lipid digestion, from the oral cavity to the duodenum sub-layer of the intestine, produce crude emulsions consisting of free cholesterol, triglycerides, free fatty acids, and phospholipids. Ones in the intestine these emulsions are mixed with bile salt micelles emulsified triglycerides and cholesteryl esters; then they are hydrolyzed by pancreatic lipase (PL) and carboxyl ester lipase respectively. It is important to note that only non-esterified cholesterol can be incorporated into bile acid micelles and absorbed by enterocytes. Most dietary cholesterol exists in the form of the free sterol, with only 10–15% existing as the cholesteryl ester (Chol-E). The latter must be hydrolyzed by cholesterol esterase to release free-Chol for absorption. So micelle acts as carrier and solubilizer for cholesterol, assisting in overcoming the resistance of the unstirred water layer adjacent to the brush border of enterocytes, and maintaining a high concentration gradient for the passage of cholesterol into the aqueous phase, from which uptake occurs into the cellular membrane. In particular uptake is facilitated by the NPC1L1 (Niemann–Pick C1-like-1) protein located at the apical membrane of enterocytes. Once dissociated from mixed micelles at the aqueous phase, and by virtue of their insolubility, cholesterol monomers are taken up by intestinal cells at a slower rate than that of the more soluble

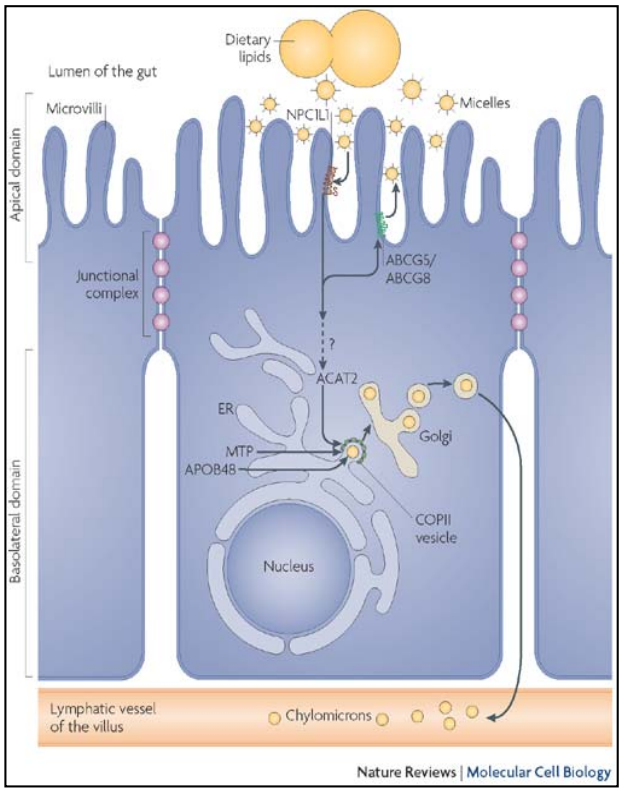

Figure 4: Micellar solubilization of dietary sterols by bile acids allows them to move through the diffusion barrier overlying the luminal surface of enterocytes. The NPC1L1 (Niemann–Pick C1-like-1) protein (dark red) is located at the apical membrane of enterocytes and facilitates the uptake of cholesterol across the brush border membrane. This is blocked by the cholesterol absorption inhibitor ezetimibe. Acyl CoA cholesterol acyltransferase isoform-2 (ACAT2) esterifies the absorbed cholesterol, which becomes incorporated into nascent chylomicron particles. How the absorbed cholesterol reaches ACAT2 is not known. Chylomicrons are synthesized around the APOB48 apoprotein in the endoplasmic reticulum (ER). Dietary fatty acids are used for triglyceride synthesis in the smooth ER and MTP (microsomal triglyceride transfer protein) transfers triglycerides and cholesteryl esters to APOB48. The nascent chylomicrons leave the ER in COPII-coated vesicles, are secreted through the Golgi complex to the basolateral side of the enterocyte, and reach the venous circulation through lymphatic vessels. Image from *Ikonen Nature 2008.*

protonated fatty acids. This explains in part the inefficiency of intestinal cholesterol absorption (figure 4).

Once inside enterocytes, dietary cholesterol is packaged into chylomicrons (CM) and placed into circulation. This process starts with the esterification of large amounts of free cholesterol by the acylCoA-cholesterol acyltransferase isoform-2 (ACAT2). In the endoplasmic reticulum (ER), cholesteryl esters, phospholipids and triglycerides are amalgamated together with ApoB-48 by the microsomal triglyceride transfer protein (MTP). The action of MTP[1] is not localized to enterocytes, or even to chylomicron synthesis (§2.1), instead it is nearly ubiquitously expressed and a crucial element of very low density lipoprotein (VLDL) synthesis(§2.1). As well as nascent chylomicron synthesis nears completion, the particles are transported to the Golgi apparatus where additional triglycerides are recruited, and then the particles are transported via vesicular structures to clatherin-coated pits and exocytosed.

Apolipoprotein B (ApoB) is the major protein component of all lipoproteins except high density lipoprotein (HDL). During chylomicron synthesis in the intestine, *ApoB* mRNA sequence is altered by (APOBEC1)[2] (apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 protein), so that the 6666th nucleotide is changed from a cytosine to a uracil . This unique process leads to a premature UAA stop codon and production of a truncated ApoB peptide only 48% the length of full ApoB. Considering that ApoB is a direct measurement of potentially atherogenic particles, the ApoB levels in circulation are considered a more appropriate trait to measure risk of cardiovascular disease than low density lipoprotein (LDL). However LDL level remains more clinically utilized.

While the digestion and packaging of dietary lipids into chylomicrons takes about one hour, lipids half-life in chylomicrons is only 4.5 minutes. Upon exiting enterocytes, the only protein component of chylomicrons is ApoB-48. After passage through the thoracic duct and into the bloodstream, nascent chylomicrons accept ApoC2 and ApoE from HDL, a process that yields mature chylomicrons. As mature chylomicrons circulate, the newly acquired ApoC2 on the particle surface activates lipoprotein lipase (LPL), which is bound to epithelial surfaces of capillaries in adipose and muscle tissue, where it is differentially expressed according to fed/fasting conditions. Into chylomicrons, LPL[3] catalyzes the hydrolysis of triglycerides, a crucial process that distributes fatty acids to tissues, generates non-esterified FA in plasma, and remodels chylomicrons into

[1] Inactivating mutations in *MTP* result in abetalipoproteinemia, in which chylomicrons and VLDL are not synthesized, ApoB containing particles in general are absent, and lipids accumulate in the intestine.

[2] *APOBEC1* inactivated mice demonstrate a complete lack of ApoB-48, 178% more ApoB-100, and decreased HDL cholesterol. Mutations in the *ApoB* gene itself are an important source of phenotypic variation in humans. In a recent review, 132 genetic variants in the *ApoB* gene are listed including one in the promoter region, one in the 5' untranslated region, 85 in the coding region (22 synonymous), 44 in the various introns, and one in the 3' UTR. Notably, genotypes of the very common T2488T and E4154K mutations have crucial implications to LDL homeostasis.

[3] The absence of *LPL* causes familial LPL deficiency, which is characterized by hypertriglyceridemia, decreased HDL and LDL, and massive accumulation of chylomicrons in plasma.

chylomicrons remnant (CRs). As CM shed triglycerides to epithelial cells, ApoC2 is lost (ApoE is retained). As a result, CR loses further lipase activation.

As CRs lose TG, they become enriched in ApoE and consequently are destined for the liver. ApoE peptides are essential for particle uptake into hepatocytes, as demonstrated by *ApoE* inactivated mice, which demonstrate tiny CR clearance. The model for hepatic clearance involves ApoE interaction with hepatocytes cell surface molecules followed by an endocytosis step mediated by a complex of low density lipoprotein receptor (LDLR) and the low density lipoprotein related 1 protein (LRP1). Another factor influencing CR uptake is cholesterol content of the macromolecule. Chylomicron-like particles, created without cholesterol, undergo triglyceride hydrolysis by LPL but are not taken up by hepatocytes. This result has been interpreted as a failure of ApoE to attain the necessary conformation to achieve receptor binding with LRP1. After delivery to the liver, CR lipids are hydrolyzed once again into free fatty acids and free cholesterol for eventual synthesis of VLDL.
[4-7]

1.2.2 Cholesterol biosynthesis

Cholesterol biosynthesis could be divided in 4 step. The first one take place with two acetyl coenzyme A (Ac-CoA) molecules condensation and finish with the mevalonate generation. The second part is the activated isoprenoid production. The third reaction set consist in squalene assembly and the last one generate cholesterol molecule by squalene cyclization.

I. Mevalonate generation

The first part of cholesterol biosynthesis (Figure 5) take place in cell cytoplasm and reactions starts when two acetyl-CoA molecules are condensed to acetoacetyl-CoA by a thiolase (Acetyl-CoA C-acetyltransferase). The acetoacetyl-CoA is further combined by hydroxymethylglutaryl-CoA synthase with a third acetyl-CoA to form the six carbon (C6)

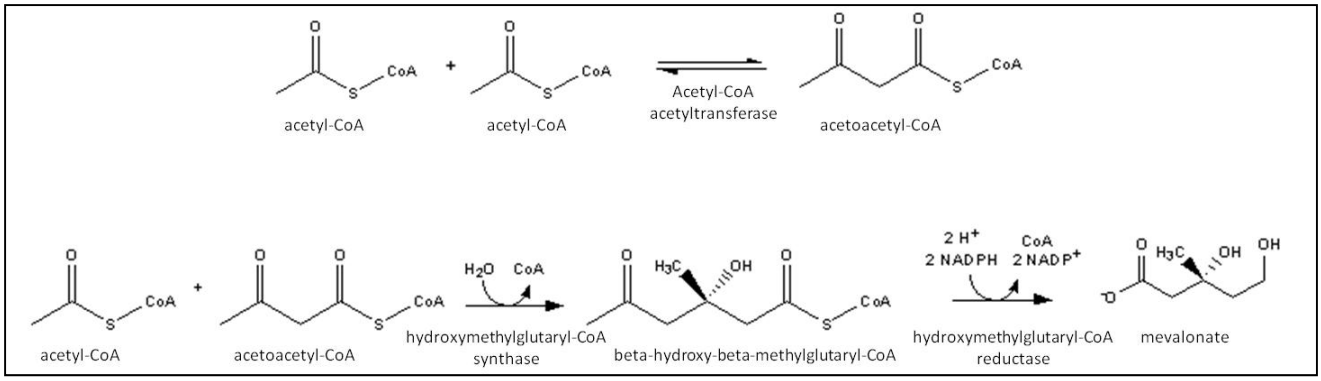

Figure 5: From Acetyl-Coa to mevalonate. The first step of cholesterol biosynthesis

intermediate beta-hydroxy-beta-methylglutaryl-CoA (HMG-CoA). HMG-CoA synthase is the principal regulatory enzyme for the isoprenoids biosynthesis.

HMG-CoA[4] is transported to the endoplasmatic reticulum membranes where is reduced to mevalonate (C6) by HMG-CoA reductase using 2 molecules of NADPH as reductant. Mevalonate is the immediate sterol synthesis precursor leading to the formation of the cholesterol ring structure, and HMG-CoA reductase is the key limiting enzyme of cholesterol production.

II. Activated isoprenoid generation

In this second reaction set, mevalonate is decarboxylated to form the C5 intermediate isoprene or isopentenyl pyrophosphate (figure 6). Isoprene is a key intermediate for different pathways including biosynthesis and degradation of glycoproteins and synthesis of coenzyme Q (ubiquinone), vitamin K and carotenoids.

Three reaction requiring ATP idrolisis occours in sequence: At first mevalonate (C6) generates 5-phospho-mevalonate (C6) through mevalonate kinase enzyme action. Then the enzyme phosphomevalonate chinase generates 5-pyrophospho-mevalonate (C6). The decarboxilase enzyme pyrophospho-mevalonate decarboxylase, using ATP and discarting CO_2 and P_i, generates isopentenylpyrophosphate (C5), that could be isomerizated in dimetihylallyl-pyrophosphate (C5). This two structure are in equilibrium one with the oter.

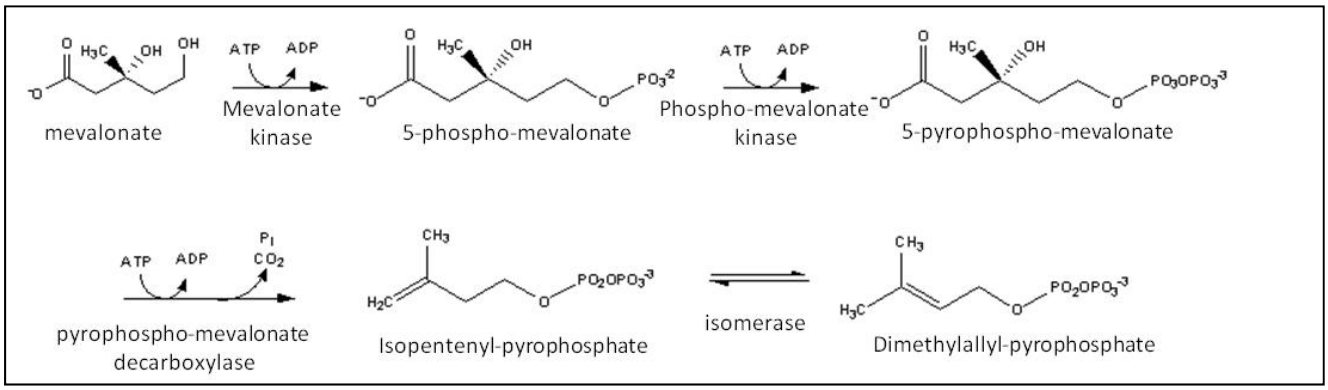

Figure 6: The generation of isoprene. From mevalonate to dimetihylallyl-pyrophosphate reaction set.

[4] HMG-CoA could be also employed in mitochondria as cheton precursor

III. Squalene generation

This third reaction set begins with C5 molecule that condensates to generate in sequences C10, C15 and C30 molecule (figure 7). The two C5 molecules: isopentenyl-pyrophosphate and its isomer dimetihylallyl-pyrophosphate condensate to generate geranyl-pyrophosphate (C10). A subsequent condensation occours between the geranyl-pyrophosphate and a second molecule of isopentenyl-pyrophosphate to produce farnesyl-pyrophosphate (C15). The riductive condensation of two farnesyl-pyrophosphate molecules generate Squalene (C30).

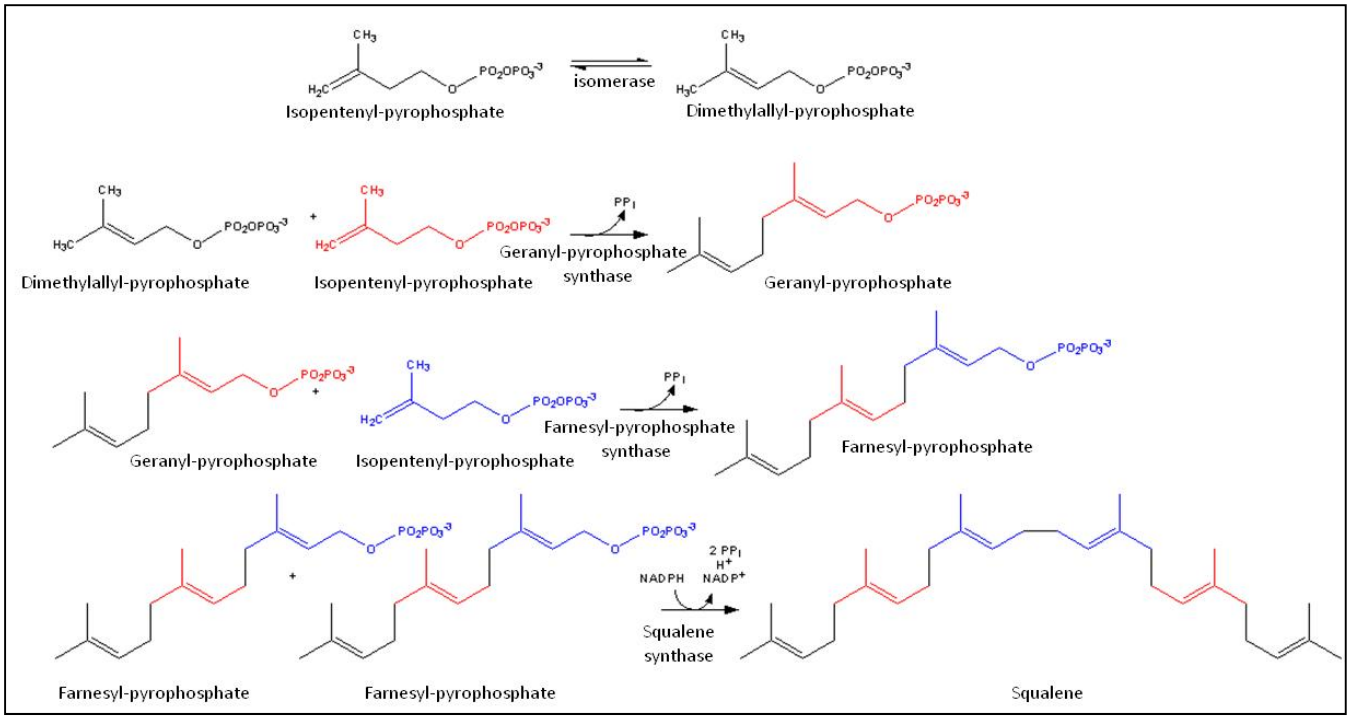

Figure 7: Condensation reactions that produce squalene from isopentenyl-pyrophosphate and dimetihylallyl-pyrophosphate.

IV. Squalene cyclization and cholesterol production

The last reaction set requires molecular oxygen (O_2) and takes place to cyclizate squalene, generating cholesterol. Only the main step of this complex pathway are schematically shown in figure 8.

Squalene is first oxidized by a squalene monooxygenase to squalene 2,3-epoxide, which undergoes cyclization catalysed by the enzyme squalene epoxide lanosterol-cyclase to form the first steroidal intermediate, lanosterol (C30). In this reaction there is a succession of concerted 1,2-methyl group and hydride shifts along the chain of the squalene molecule to bring about the formation of the four rings. This reaction takes place in the endoplasmic reticulum, but a cytosolic protein, sterol carrier protein 1, in the presence of the cofactors phosphatidylserine and flavin adenine dinucleotide (FAD), is required to bind squalene in an appropriate orientation. Finally, lanosterol is converted to cholesterol (C27) by multiple reactions that involve the removal of three

methyl groups, hydrogenation of the double bond in the side-chain, and a shift of the double bond from position 8,9 to 5,6 in ring B. A second cytosolic protein, sterol carrier protein 2, is required to bind 7-dehydrocholesterol, one of the intermediates of the process [8].

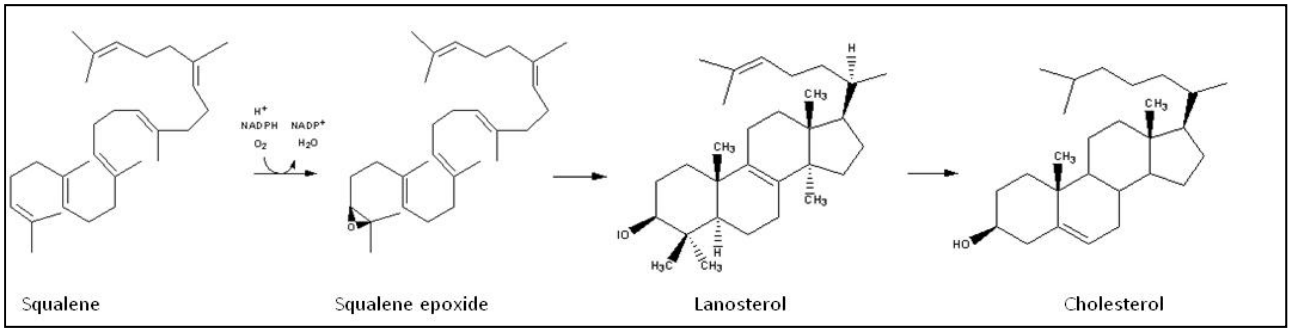

Figure 8: Main intermediates of squalene ciclyzation pathway that leads to cholesterol production.

2. Cholesterol transport

2.1 The cholesterol transporters.

Lipoproteins are aggregates of lipids and proteins, synthesized mainly in the liver and intestines, that allow lipids transport trough the aqueous environment of body fluids. Within the circulation, these aggregates are in a state of constant flux, changing in composition and physical structure as the peripheral tissues take up the various components before the remnants return to the liver. The most abundant lipid constituents are triacylglycerols, free cholesterol, cholesterol esters and phospholipids (phosphatidylcholine and sphingomyelin especially). Fat-soluble vitamins and anti-oxidants are also transported in this way.

Ideally lipoproteins should be classified via different apolipoproteins contents, as these determine the overall structures, metabolism and the interactions with receptor molecules in liver and peripheral tissues. However, the practical methods that have been used to segregate different lipoprotein classes, based on the relative densities of the aggregates on ultracentrifugation, have determined the nomenclature. Thus, the main groups are classified as chylomicrons (CM), very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). However, these classes can be further refined by improved separation procedures, and intermediate-density lipoproteins (IDL) and subdivisions of the HDL (e.g. HDL_1, HDL_2, HDL_3 and so forth) are often defined. Density is determined largely by the relative concentrations of triacylglycerols and proteins and by the diameters of the broadly spherical particles, which vary from about 6000Å in CM to 100Å or less in the smallest HDL.

I. Chylomicrons

Before having CM, prechylomicrons biosynthesis is needed. This process take place in the ER lumen and then lipoprotein molecules move along the secretory pathway to the Golgi. Prechylomicrons ER egress is defined as the rate-limiting step in their overall secretion from the enterocytes. Because of their very large size (average diameter 250 nm) how prechylomicrons traverse the ER and Golgi membranes is unclear. Prechylomicrons are processed into mature CM in the Golgi and then transported to the basolateral membrane via a separate vesicular system for exocytosis into the intestinal lamina propria. Fatty acids and monoacylglycerols entering the enterocyte via the basolateral membrane are also incorporated into triacylglycerol, but the basolaterally entering lipid is much more likely to enter the triacylglycerol storage pool than the lipid entering via the apical membrane (figure 9).

II. LDL/VLDL

Hepatic VLDL are lipid emulsion particles composed of a triglyceride-rich core and a surface monolayer of phospholipid. They transport endogenously synthesized lipids, particularly cholesteryl esters and triglyceride, from the liver to peripheral tissues. Their assemblage is a process consisting in two steps: first, during its translocation across the ER membrane, nascent apolipoprotein B100 (apoB) associates with lipids provided by microsomal triglyceride transfer protein. This results in the formation of incompletely lipidated "primordial" pre-VLDL particles. In the second step, additional lipid (primarily triglyceride) and other apolipoproteins (e.g. apoE) are added, resulting in the formation of mature, fully lipidated VLDL particles.

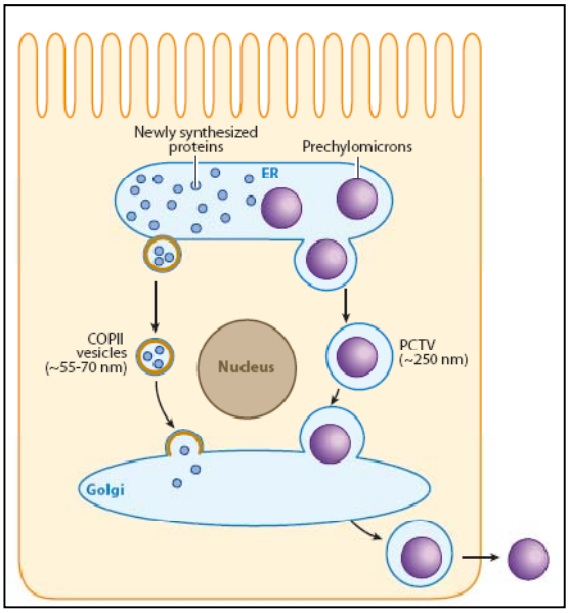

Figure 9: Intracellular prechylomicron transport. The assembly of prechylomicrons occurs in the lumen of ER. They are packaged into specialized vesicles known as prechylomicron transport vesicles (PCTVs) that bud off the ER membrane and move to and fuse with the cis-Golgi, delivering their prechylomicron cargo to the Golgi lumen. Nascent proteins are transported from the ER to the Golgi in coat protein complex II (COPII) vesicles. PCTVs bud from the ER membrane in the absence of COPII proteins, whereas the protein vesicles require the COPII machinery for their budding. After processing in the Golgi, mature chylomicrons are transported to the basolateral membrane via a separate vesicular system. Image from Mansbach et al. 2010

The circulating VLDL particles become progressively smaller as their core is removed by lipolysis and surface materials are transferred to HDL. In healthy individuals, most of the VLDL is converted to smaller LDL particles through an intermediary lipoprotein known as intermediate density lipoprotein (IDL). This has a density of 1006-1019 g/L, and possesses Apo E (which in the latter respect is similar to chylomicron remnants).

Low density lipoproteins (figure 10) are the main cholesterol carriers in the human circulation, and are thus key players in its transfer and metabolism. They have an average diameter of 22 nm, the core consisting of about 170 triglyceride and 1600 cholesteryl ester and the surface monolayer comprising about 700 phospholipid molecules and a single copy of apoB-100. In addition, the particles contain about 600 molecules of unesterified cholesterol (UC), of which about one-third is located in the core and two-thirds in the surface. It should also be noted that a few percent of the TG and CE molecules penetrate toward the surface. The main phospholipid components are phosphatidylcholine (PC) (about 450 molecules/LDL particle) and sphingomyelin (SM) (about 185 molecules/LDL particle). The LDL particles also contain lysophosphatidylcholine

(lyso-PC) (about 80 molecules/LDL particle), phosphatidylethanolamine (PE) (about 10 molecules/LDL particle), diacylglycerol (DAG) (about 7 molecules/LDL particle), ceramide (CER) (about 2 molecules/LDL particle), and some phosphatidylinositol (PI) . In addition to lipids, LDL particles also carry lipophilic vitamins and antioxidants, such as α-tocopherol (about 6 molecules/LDL particle) and minute amounts of γ-tocopherol, carotenoids, oxycarotenoids and ubiquinol-10. The particles are in a dynamic state, their structure and physical properties being dependent on their lipid composition as well as on the conformation of apoB-100.

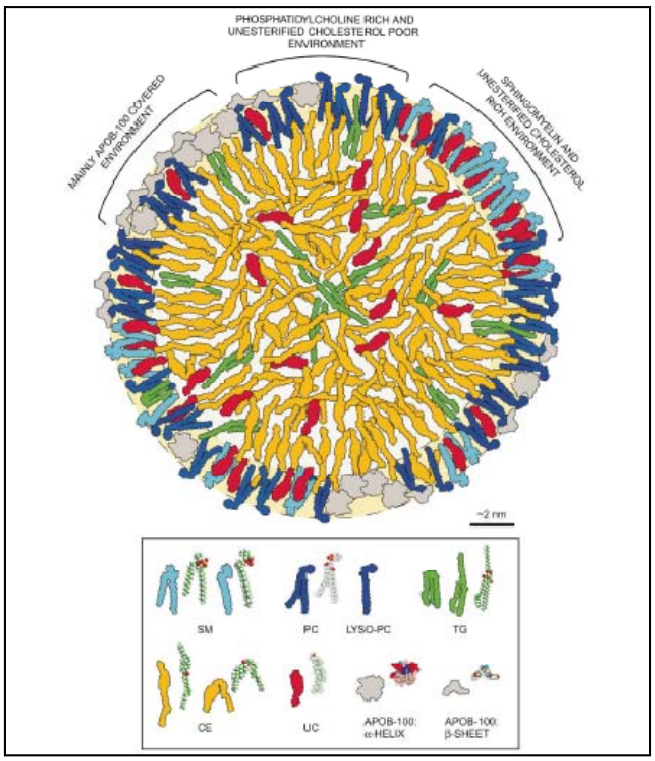

Figure 10: Schematic molecular model of an LDL particle. The depicted particle has a diameter of 20 nm, including a surface monolayer of 2 nm (yellowish background), and an average composition of 20% protein, 20% phospholipids, 40% CEs, 10% UC, and 5% TGs. The molecular components of the particle are drawn in both the correct percentages and size ratios. Note the different domains illustrated at the particle surface and the interpenetration of core and surface lipids. The individual molecules were built using Cerius^2 software (MSI Molecular Simulations Inc.). The chain compositions are illustrated as follows: SM (16:0); PC (16:0/18:2$^{\Delta 9,12}$); TG (16:0/18:2$^{\Delta 9,12}$/14:0); CE (18:2$^{\Delta 9,12}$). Image from Hevonoja et al. 2000.

The endocytic uptake of lipoproteins such as low-density lipoprotein represents the major source of cholesterol in not-steroidogenic cells (see 2.2)

A characteristic phenomenon of early atherogenesis is extracellular accumulation of LDL-derived lipids in the form of small lipid droplets and vesicles, which can lead to the development of atherosclerotic lesions in the arterial intima. Modifications in the structure of native LDL, that are capable of inducing aggregation and/or fusion of the particles, are currently recognized to be a prerequisite for the initiation of lipid accumulation.

ApoB-100 is the major protein component of very low, intermediate, and low density lipoproteins. These particles are linked in a delipidation cascade in which triglyceride-rich VLDL, released from the liver, is converted to cholesterol-rich LDL which is in turn catabolized by specific cell-membrane receptors throughout the body. Abnormalities in the metabolism of apoB-containing lipoproteins are responsible for the generation of hyperlipidemia and the associated increased risk of developing coronary heart disease.

III. HDL

High density lipoproteins are a heterogeneous group of particles whose principal physiological role is that of reverse cholesterol transport, but they also exert important anti-inflammatory and antithrombotic effects. They can be classified according to their chemical and physical characteristics, including hydrated density (1.063–1.210 kg/l), flotation rate (0–9 Svedbergs), diameter (7–12 nm), and electrophoretic mobility.

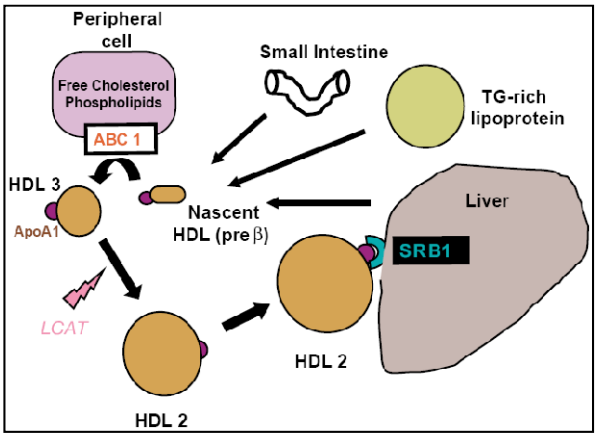

Figure 11: Schematic illustration of reverse cholesterol transport. Image from Fredenrich et al 2003

Small particles of discoid shape, named pre-HDL, are synthesized in liver and small intestine, or result from hydrolysis of triglyceride-rich particles. These pre-HDL uptake cholesterol from peripheral cells, and their shape change to spherical particles, named HDL3 then HDL2. The transformation of HDL3 into HDL2 is related to their enrichment in esterified cholesterol (via an esterifying enzyme, lecithin cholesterol-acyl-transferase (LCAT) associated with pre-HDL particles) and phospholipids. The final uptake of HDL2 by the liver involves a selective receptor, named scavenger receptor B1 (SR-B1) (figure 11)[9-17].

2.2 Cholesterol endocytosis

Endocytic uptake of lipoproteins such as low-density lipoprotein, and hydrolysis of their cholesterol esters in late endosomes and lysosomes (figure 12), represents a fundamental source of cell cholesterol.

LDL bind LDL receptors (LDLR) on cell surface and the complex is internalized via clathrin-coated pits[5]. These vesicles enter the clathrin-mediated endocytic pathway, drop their coats and fuse with early endosomes. The lower pH in early endosomes promotes dissociation of LDL from LDLR. The LDLR and other recycling proteins localize to early endosomal tubular extensions, which bud off of vesicles that fuse with the endocytic recycling compartment (ERC). They recycle back to the PM after approximately 10 minutes to be reutilized for many more rounds of LDL delivery. Eventually, the LDLRs enter the late endosomes en route to being degraded in the lysosomes. Some amount of early endosomal membrane free cholesterol, from both LDL and endocytosed PM, may also sort to the ERC via the same vesicles that carry recycling proteins.

[5] The coated pits are composed of clathrin and other accessory proteins. The complex is build to invaginate, pinch off from the PM, and form coated vesicles.

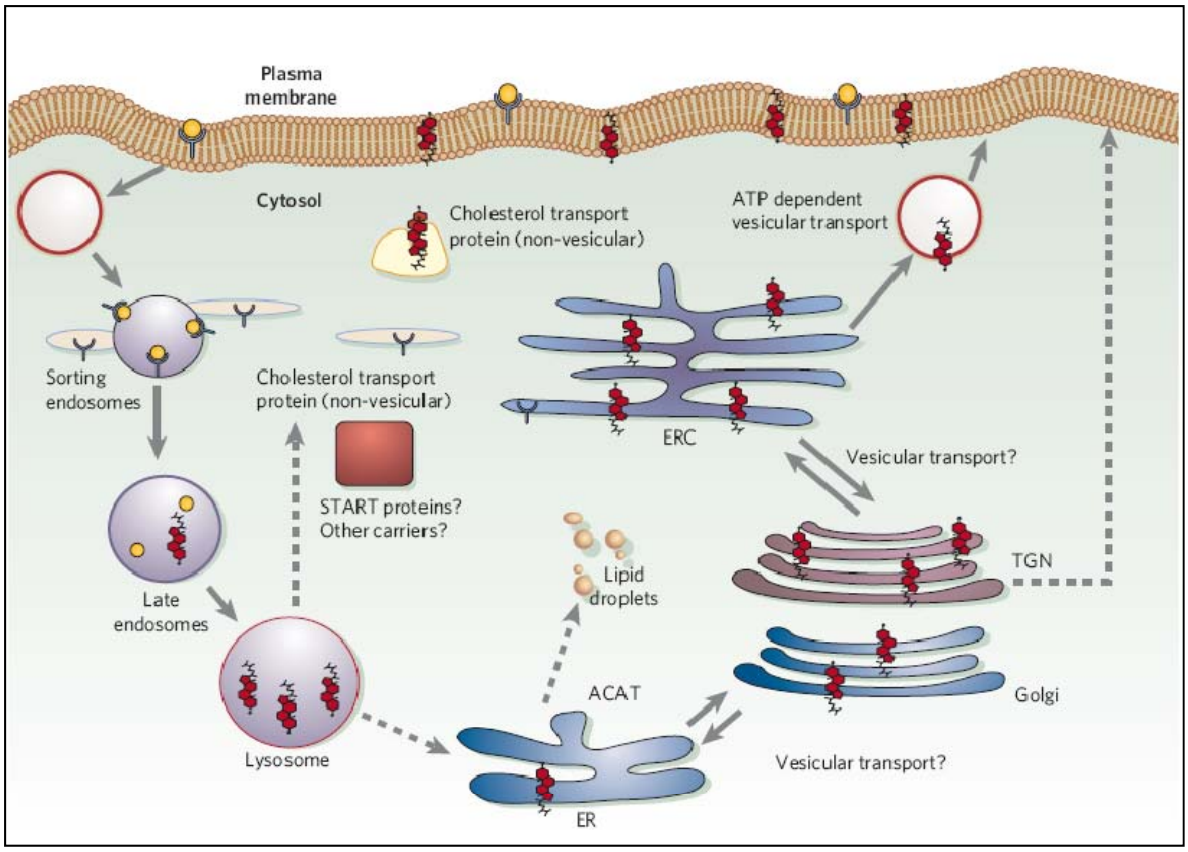

Figure 12: Intracellular cholesterol transport: LDL (yellow circles) carrying cholesterol and cholesterol esters bound to LDL receptors (light blue Y-shape) is internalized and transported to sorting endosomes and to late endosomes and lysosomes from which cholesterol can efflux to cellular compartments including the plasma membrane or the endoplasmic reticulum (ER). The LDL receptor recycles to the plasma membrane via the endocytic recycling compartment (ERC). Efflux from late endosomes and lysosomes is poorly characterized as indicated by the dashed lines. Cholesterol can move from the plasma membrane to the ERC by a non-vesicular, ATPindependent process. Recycling of cholesterol back to the plasma membrane occurs by nonvesicular transport and in membrane-recycling vesicles carrying other recycling membrane components. Newly synthesized cholesterol in the ER is mostly transported from the ER directly to the plasma membrane, bypassing the Golgi, but some follows the biosynthetic secretory pathway from the ER to the Golgi. Excess cholesterol in the ER becomes esterified by ACAT and stored in cytoplasmic lipid droplets. TGN, trans-Golgi network. Picture from Maxfield and Tabas 2005.

Cholesterol seems also traffics in the opposite direction from PM to ERC in non-vesicular, rapid and ATP-independent manner (figure 12 and 13).

Hydrolysis of LDL-chol esters to free cholesterol is widely thought to occur in late endosomes and lysosomes, but the acid lipase enzyme was recently localized to an earlier acidic compartment, so LDL cholesterol ester-derived free cholesterol may be generated soon after endocytosis..

The non-recycled contents of early endosomes proceed to late endosomes, by a process perhaps involving vesicular transport or the evolution of early to late endosomes. Late endosomes deliver the endocytosed materials to the trans-Golgi network (TGN) en route to the PMs. Late endosomes also receive various hydrolases and other protein components from the TGN, in this manner undergo successive maturation processes and become lysosomes. The materials not

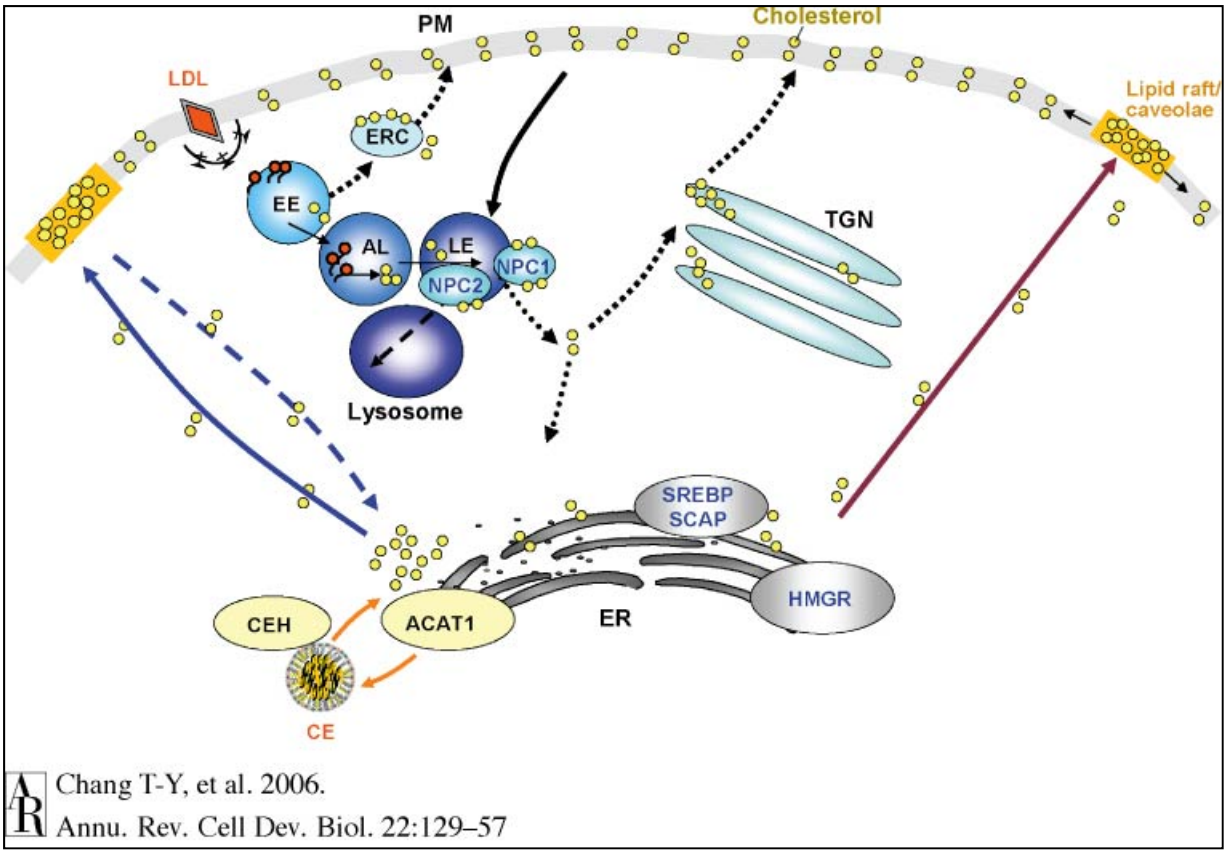

Figure 13: The plasma membranes (PMs) contain the highest concentration of cholesterol (yellow circles). The cholesterol-sensing membrane proteins are located in the ER:HMG-CoA reductase (HMGR), SREBP cleavage–activating protein (SCAP), and acyl-coenzyme A cholesterol acyltransferase 1 (ACAT1); or in the late endosomes Niemann-Pick type C1 (NPC1). The translocation of cholesterol between various compartments may involve both vesicular and non-vesicular mechanisms. The dotted lines represent cholesterol trafficking steps that are not well documented. Figure abbreviations : AL, acid lipase; CEH, cholesteryl ester hydrolase; EE, early endosome; ERC, endocytic recycling compartment; LE, late endosome; NPC2, Niemann-Pick type C2; SREBP, sterol-regulatory element–binding protein; TGN, trans-Golgi network. Image from Chang et al. 2006

retrieved/sorted in the endosomes end up in the lysosomes for degradation. It is unclear where cholesterol normally leaves the endosomal pathway and how it effluxes and redistributes to other sites. Studies of Niemann-Pick disease type C (NPC), an inherited lysosomal storage disorder that leads to accumulation of cholesterol and other lipids, have shown that a luminal protein (NPC2) and a transmembrane protein (NPC1) in late endosomes are required for efflux of cholesterol from these organelles, but the details of how these proteins work remain to be determined. In normal non-NPC cells, LDL-derived cholesterol leave late endosomes to reach other compartments like ER and PM. LDL-Chol also arrives at the endoplasmic reticulum and becomes available for esterification by the resident ER enzyme acyl-coenzyme A: cholesterol acyltransferase (ACAT).

Cholesterol that move to ER seem to use almost two pathways: the major one involves the PM as an intermediate and it has been divided into 2 steps:

I. From the proximal endosome to PM

II. From the distal PM to ER

Time-course studies in CHO cells show that it takes approximately 30 min for the LDL-CHOL to become available for efflux at the PM, but it takes approximately 60 min to 90 min before it becomes esterified at the ER (Cruz & Chang 2000, Sugii et al. 2003). Thus, LDL-CHOL may first arrive at the PM before it moves back to the ER. Alternatively, LDL-CHOL may move to the PM and ER by two separate transport mechanisms.

2.3 Intracellular cholesterol transport

Cholesterol that is synthesized in the ER need to be delivered to other compartments. The transport take place through a combination of vesicular and non-vesicular transport processes:

I. Vesicular transport

Cholesterol is present in the membranes of intracellular vesicles that shuttle among compartments. This kind of transfer typically requires an intact cytoskeleton, the tracks along which vesicles move, and ATP, providing energy for motor proteins.

II. Non-vesicular transport

This mechanisms is only partially understood but it seems to be the major way for cholesterol movement between organelles. Since cholesterol is extremely insoluble in water, it must be shuttled by diffusible carrier proteins, which have hydrophobic cavities to bind cholesterol and transport it across the aqueous cytosol. The best-documented example is the steroidogenic acute regulatory protein (StAR), which is the prototype for the StAR-related lipid transfer (START) family of transport proteins. This protein is essential for the delivery of cholesterol to mitochondria, where it is used in steroid hormone synthesis. Other members of this family can bind cholesterol or other lipids and facilitate their intracellular, non-vesicular transport.

Another form of non-vesicular transport may involve spontaneous desorption of cholesterol from one membrane and diffusion to another closely juxtaposed membrane, perhaps brought together at contact sites by specialized proteins.

The ER is the primary site of cholesterol synthesis but it is a cholesterol-poor organelle. 65% to 80% of total cellular cholesterol is in plasmatic membrane (PM), whereas only 0.1% to 2% is in ER. So, Chol leaving ER to rich PM moves against concentration gradient. Vesicular transport along the protein secretory pathway through the Golgi is one route from ER to PM but it is not the major pathway. Sites of close physical membrane apposition between ER and PM could facilitate this transport. Once biosynthesized, most of the endo-CHOL is rapidly (10–20 minutes) transported to

the cholesterol-rich, sphingolipid-rich domains (i.e., lipid rafts/caveolae) of the PM by an energy dependent non-vescicular trafficking process (figure 13). The molecular nature of this process is not well understood and some data support roles for SCP-2 and caveolin. Studies have identified two distinct cytosolic complexes of caveolin-1 and various chaperone proteins, one containing nascent cholesterol and another cholesterol esters. It has been proposed that these caveolin-cholesterol-chaperone complexes represent novel intracellular lipid particles analogous to plasma lipoproteins.

Excess cellular cholesterol from other compartments returns to the ER for esterification. PM cholesterol is thought to follow at least 2 paths to the ER (figure 13):

1) vesicular route via endosomes and/or Golgi;

2) non-vesicular alternative route.

However involved mechanism are not well understood

The principal steroidogenic organs in the body are the gonads, adrenal glands and the brain. *De novo* synthesis of steroid hormones from cholesterol is catalyzed by the cholesterol side-chain cleavage enzyme P450, which resides in the inner mitochondrial membrane. For this reason it is significant the mitochondrial capitation of cellular cholesterol in this tissue. In general mitochondria are considered cholesterol-poor organelles and outer mitochondrial membrane contains more cholesterol than the inner[6]. The steroidogenic acute regulatory element, a protein with a cholesterol-binding pocket, acts on the mitochondrial outer membrane to facilitate cholesterol transfer to the inner membrane (non vescicular transport). It is estimated that 400 molecules of cholesterol per minute are transferred into the mitochondria by each molecule of newly synthesized StAR. The transfer activity requires a conformational change in StAR. Spectroscopic and simulation studies suggest that the protein undergoes a molten globule transition in which the tertiary structure is altered and the secondary structure is preserved to open the sterol-binding pocket. However, according to other simulations, less distorting changes in protein conformation may be sufficient for cholesterol uptake and release.

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[6] Two important cholesterol-metabolizing P450 enzymes reside on the matrix side of the inner mitochondrial membrane:

I. The P450 side chain cleavage system (P450scc/Cyp11A1)
II. The sterol 27-hydroxylase (Cyp27)

both are expressed only in steroid hormone-producing cells. P450scc converts cholesterol to pregnenolone, which is modified by other enzymes to generate steroid hormones. The widely expressed Cyp27 converts cholesterol to 27-hydroxycholesterol, the most abundant oxysterol in plasma. This important oxysterol serves at least 4 functions

I. first intermediate in the alternative pathway of bile acid synthesis from cholesterol
II. a more soluble transport form of cholesterol in plasma
III. a potent repressor of SREBP processing
IV. a partial LXR agonist

Other proteins with StAR-related lipid-transfer domains that lack evident targeting sequences, such as STARD4, may be responsible for cytoplasmic delivery of cholesterol to the outer mitochondrial membrane. The peripheral benzodiazepine receptor (PBR) is an outer mitochondrial membrane protein that is also involved in the mitochondrial import of cholesterol for steroidogenesis. Increasing evidence points to a functional interaction between PBR and StAR. One possibility is that the cholesterol recognition domain of PBR contains a reservoir of labile cholesterol that StAR can mobilize for steroidogenesis. Recently, additional proteins that participate in the formation of a signalling complex were identified. In addition to PBR, this complex includes the PBR-associated protein PAP7, PKARIα (the PAP7-binding regulatory subunit of cyclic-AMP-dependent protein kinase) and StAR81. It was proposed that this complex forms a scaffold in the outer mitochondrial membrane and mediates the effects of hormones through cAMP on mitochondrial cholesterol transport [5, 18-22].

2.4 Cellular cholesterol efflux

Peripheral tissues cells can store but not degrade cholesterol, that need to be transported to the liver for reutilization and excretion.

High-density lipoproteins (§2.1) are the main acceptors for cholesterol released from the extra-hepatic cells. Mature HDLs transport CEs to the liver, the adrenals and other steroidogenic tissues. On surface of the liver cells and steroidogenic cells HDL are recognized by the HDL receptor: scavenger receptor type B class I. This receptor is also important for cholesterol efflux in the vessel wall, and it mediates uptake of CE by a selective process.

Once in the liver the HDL-derived cholesterol serves as an important precursor for bile acid synthesis. It can enter the bile duct to be excreted from the body. In steroidogenic tissues, the HDL-derived cholesterol is utilized for hormone synthesis.

For this retrograde transport of CHOL excess, HDL is recognized as an antiatherogenic lipoprotein. Raising plasma HDL is a potential therapeutic goal for treating atherosclerotic cardiovascular disease.

Cellular cholesterol effluxes occur by two distinct mechanisms:

I. Passive diffusion: driven by the cholesterol gradient between the cell surface and the HDLs in contact with the cell surface

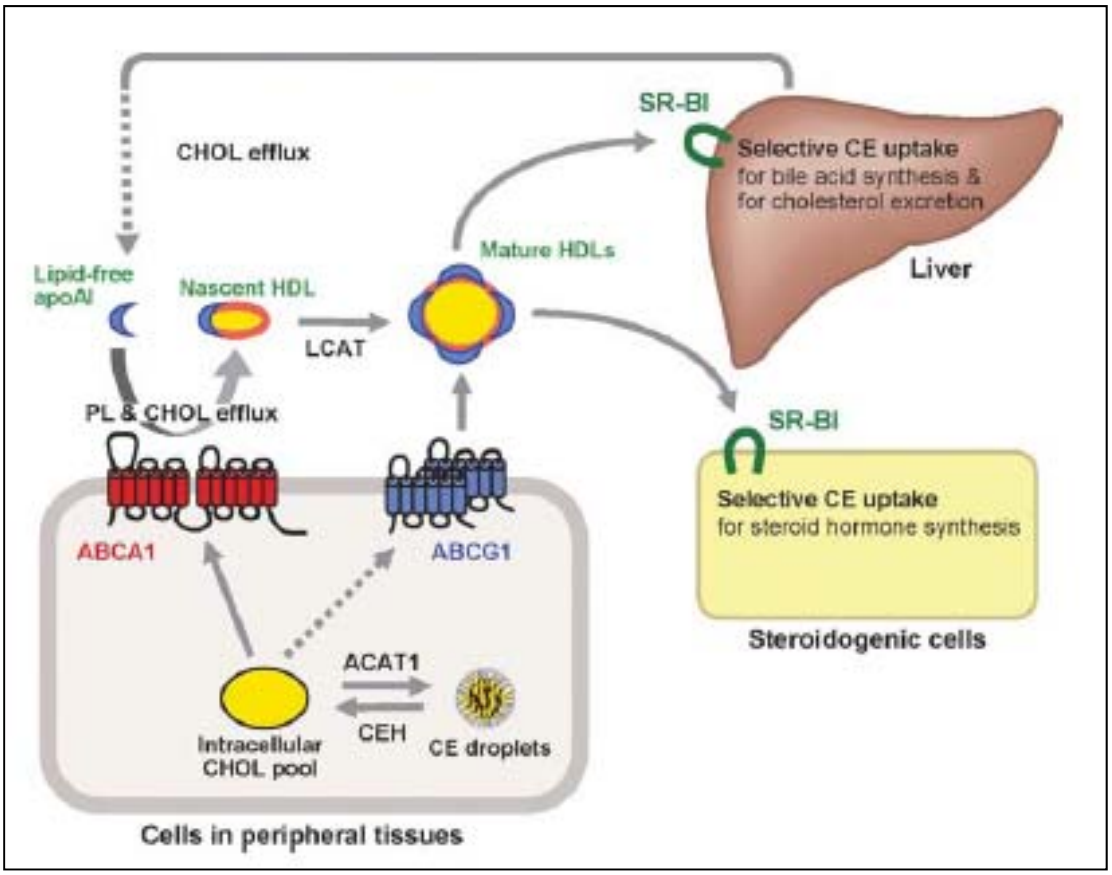

Figure 14: Summarizing scheme for cholesterol efflux. Cellular cholesterol efflux mediated by ABCA1 and ABCG1 and the dynamic relationship between the cholesterol pools for efflux and for esterification by acyl-coenzyme A:cholesterol acyltransferase 1 (ACAT1) within the cells of the peripheral tissues. ABCA1 facilitates apolipoprotein-mediated cholesterol and phospholipid efflux to generate nascent, disk-like high-density lipoprotein (HDL). Lecithin:cholesterol acyltransferase (LCAT), an enzyme present in the plasma, then uses phospholipids and cholesterol in the nascent HDL to esterify cholesterol and to produce mature, globular-shaped HDL. Mature HDL removes cholesterol from cells, facilitated by ABCG1. ABCA1 is widely distributed in various tissues. ABCG1 is highly expressed in macrophages. Other abbreviations used in figure: apoA-I, apolipoprotein A-I; CE, cholesteryl ester; CEH, cholesteryl ester hydrolase; CHOL, cholesterol; PL, phospholipid; SR-BI, scavenger receptor type B class I. Picture fromChang et al. 2006

II. Apolipoprotein-mediated pathway (figure 14). It consists in the production of the disk-like nascent HDL particles, using mainly the lipid-poor apoA-I (synthesized mainly in the liver), phospholipids and cholesterol (both donated from peripheral tissues cells) as substrates. The ABC protein ABCA1, located mainly in the PMs of various peripheral cells, plays an essential role in this process. Tangier Disease, a rare disease that lead to familial HDL deficiency, is caused by defective mutations within the coding regions of the Abca1 gene. How ABCA1 mediates cholesterol efflux is under intense investigation. Cellular ABCA1 gene expression is mainly controlled by transcription and by protein degradation. For transcriptional control, the liver X receptors (LXRs) bind to oxidized derivatives of cholesterol called oxysterols and forms heterodimers with the retinoid X receptors, which use 9-cis retinoic acid as natural ligand. The heterodimeric complex

then becomes active in stimulating expression of the Abca1 gene as well as of several other genes involved in cellular sterol efflux. Protein degradation regulation consist in ABCA1 binding with apolipoproteins that, in intact cells, stabilizes ABCA1 against degradation. The removal of cellular cholesterol by apoA-I mobilizes certain intracellular cholesterol pools and causes a decrease in ACAT1 activity. Conversely, blocking ACAT activity with ACAT inhibitors expands the cellular cholesterol pool available for apoA-I-mediated cholesterol efflux. In macrophages, in addition to ABCA1, another ABC transporter called ABCG1 mediates cellular cholesterol efflux to mature HDL by passive diffusion. This results in a huge reduction in cellular cholesterol. [20, 23]

3. Regulation of cholesterol homeostasis.

Later in 1950s and 1960s it was well established that dietary cholesterol inhibits its biosynthesis and in the 1970s it was shown that this regulation was primarily due to the inhibition of the rate limiting step in the pathway catalyzed by HMGCoA reductase. Various mechanisms by which this occurs have been reviewed.

Figure 15: a) Michael S. Brown . b) Joseph L. Goldstein the Nobel Prize in Physiology and Medicine

In the last quarter of the 20th century, Michael Brown and Joseph Goldstein (figure 15), working on hypercholesterolemia, elucidated the regulation of cholesterol biosynthesis and discovered the low density lipoprotein (LDL) receptor. For this work, in 1985 this two researcher were awarded the Nobel Prize in Physiology and Medicine. Once Brown and Goldstein had discovered the LDL receptor pathway, they addressed the mechanism by which cholesterol suppressed the expression of the genes encoding HMG-CoA reductase and the LDL receptor. Their work led to the discovery of the sterol regulatory element in the proximal promoter of these genes. Sterol regulatory element binding protein (SREBP) was subsequently found to bind to this element and promotes the expression of these and other genes involved in cholesterol and fatty acid biosynthesis.

Further research showed that cholesterol interacts with a protein on the endoplasmic reticulum and Golgi named SREBP cleavage activating protein (SCAP), which in turn governs the activity of a protease that cleaves a precursor of SREBP. When cholesterol levels are adequate, the protease is not activated and therefore SREBP is not released and does not migrate to the nucleus. As a result, expression of the genes for HMG-CoA reductase and LDL receptor is not induced. When cholesterol levels are insufficient, the SREBP precursor is cleaved, SREBP migrates to the nucleus and the expression of these genes is promoted. Thus, cholesterol levels in a cell are sensed by the SCAP-SREBP pathway to feedback regulate the biosynthesis of cholesterol (§3.2).
[2, 24, 25]

3.1 The SREBP family: structure and functions

SREBPs are basic-helix-loop-helix-leucine zipper (bHLHLZ) transcription factors synthetized as 1150 amino acid (AA) inactive precursors bound to the membranes of the endoplasmic reticulum. Both amino and carboxyl terminal project into the cytoplasm.

Each SREBP precursor is organized into three domains:

1. NH2-terminal domain ~ 480 AA that contains:
    a. the transactivation domain, a region rich in serine and proline
    b. the bHLH-LZ region for DNA binding and dimerization. This region contains the nuclear localization signal, which binds directly to importin (figure16), allowing the transport of nSREBPs into the nucleus.
2. two hydrophobic transmembrane spanning segments interrupted by a short loop of about 30 AA that projects into the lumen of the ER
3. COOH-terminal segment regulatory domain ~ 590 AA

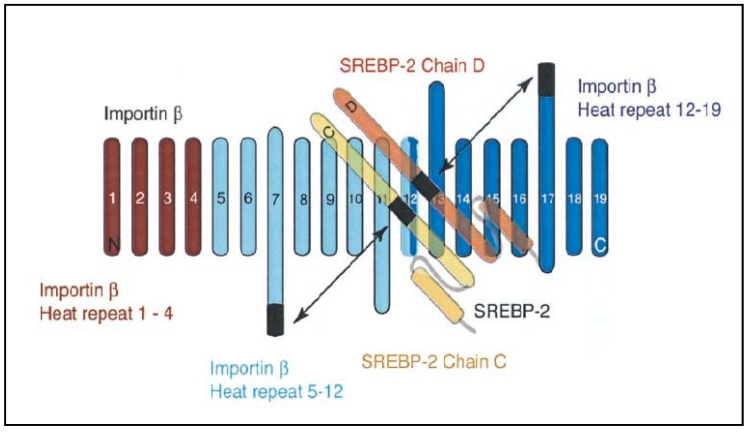

Figure 16: A schematic drawing of the 19 heat repeats of importin-b and the SREBP-2 HLHZ dimer. The importin-b molecule has a twofold symmetric arrangement of heat repeats 5 to 19, with heat repeat 12 in the center. In the importin-b molecule, the characteristic four long helices (heat repeats 7, 11, 13, and 17) are present. Major interactions occur between heat repeats 7 and 17 of importin-b and helix 2 of SREBP-2 (black). Importin-b uses the characteristic long helices 7 and 17 as a pair of chopsticks to pick up SREBP-2. Image modified from et Lee al 2003.

Three members of the SREBP family have been described in several mammalian species: SREBP-1a and 1c produced from a single gene (*srebf-1*) located on human chromosome 17p11.2 and SREBP-2 from a separate gene (*srebf-2*) located on human chromosome 22q13. SREBP1 and SREBP2 share ~47% sequence identity (figure 17).

SREBP-1a and 1c transcripts are produced through the use of alternative transcription start sites and differ in their first exon (exon 1a and exon 1c). The other exons are common to both isoforms. In humans, alternative splicing in the 3′ end has also been described (exon 18a and 19a or exon 18c and 19c). SREBP-1a is a more potent transcriptional activator than SREBP-1c due to its longer NH_2-terminal transactivation domain. However, SREBP-1c is the predominant isoform expressed in most of the tissues of mice and humans, with especially high levels in the liver, white adipose tissue (WAT), skeletal muscle, adrenal gland and brain. In contrast, SREBP-1a is highly expressed in cell lines and tissues with a high capacity for cell proliferation, such as spleen and intestine

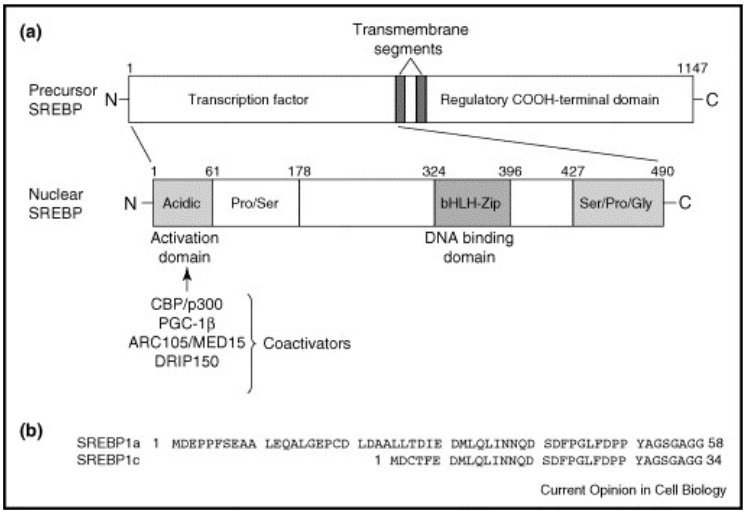

Figure 17: (a) Schematic illustrations of the membrane-associated (top) and nuclear (bottom) forms of SREBPs. The nuclear forms are generated by cleavage of the precursor forms in response to cholesterol depletion. There are three SREBP proteins: SREBP1a, SREBP1c and SREBP2. The amino acid numbers refer to human SREBP1a. Transcriptional co-activators that interact with the N-terminal trans-activation domains of nuclear SREBPs are indicated. (b) SREBP1a and SREBP1c originate from a single gene and only differ in the length of their N-terminal trans-activation domains, which makes SREBP1a a stronger activator.

Experimental data from mouse liver demonstrated that over-expression of:

- SREBP-1a markedly increases the expression of genes involved in cholesterol synthesis (HMG-CoA synthase and reductase, squalene synthase) and in FA synthesis (acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1).
- SREBP-1c isoform causes a selective induction of lipogenic genes, with no effect on genes of cholesterol synthesis.
- SREBP-2 isoform (figure 18) results in a preferential induction of genes involved in cholesterol biosynthesis, although a moderate induction of genes involved in FA synthesis is also observed.

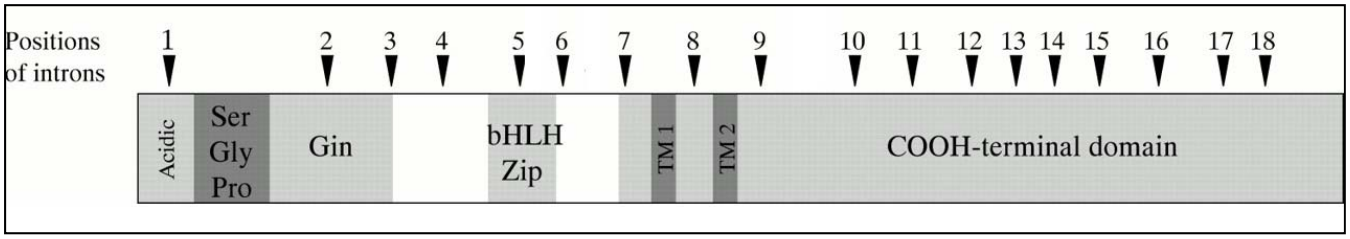

Figure 18: Domain structure of human SREBP-2. Ser Gly Pro, serine-glycine-proline rich domain; bHLH Zip, basic helix-loop-helix leucine zipper domain (transcriptionally active domain); TM 1, first transmembrane domain; TM 2, second transmembrane domain.Immage from Muller et al 2001

The observed selectivity of the SREBP isoforms for cholesterogenic and/or lipogenic genes is at least partly due to their different affinities for the various consensus sequences in the target gene (Amemiya-kudo et al).

The active form of the transcription factors is the amino terminal portion of the protein. SREBPs must be proteolytically cleaved to release the NH2-terminal segment that can enter the nucleus [26-33].

3.2 SREBP activation mechanism

Four proteins required for SREBP processing have been delineated (figure 19):

I. SREBP cleavage–activating protein (SCAP):

SCAP is a 1276 amino acid polytopic membrane protein that is both an escort for SREBPs and a sensor of sterols. It is required for movement of SREBPs from ER to Golgi. The NH2-terminal domain of SCAP consists of 730 amino acids organized into eight membranespanning segments. Within this segment, there are three N-linked oligosaccharide chains attached to the lumenal loops. The COOH-terminal cytoplasmic domain consists of 546 amino acids and includes five copies of a WD repeat (tryptophan–aspartate repeat), which represents a protein–protein interaction motif required for SCAP binding to the COOH-terminal domain of SREBP. SCAP/SREBP trafficking is regulated by cholesterol.

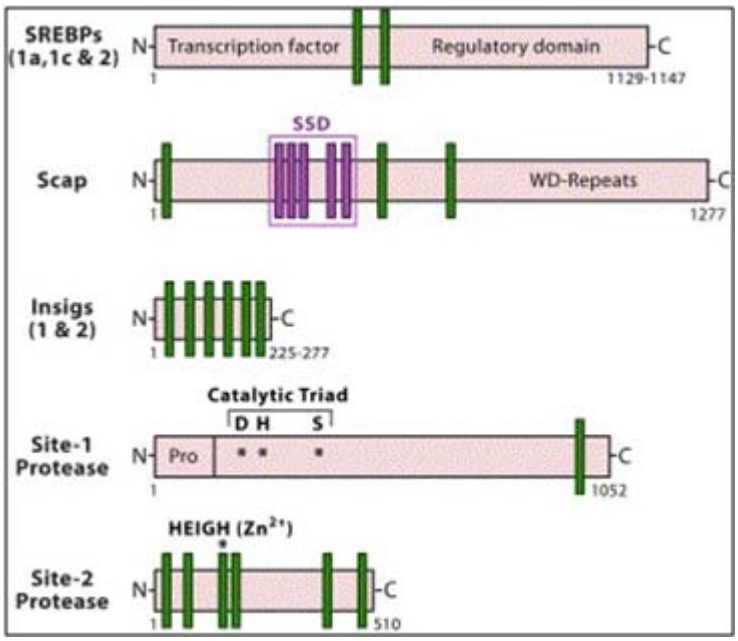

Figure 19: Domain Structures of Membrane Proteins that Regulate CholesterolAmino acid numbers refer to human proteins. Vertical bars denote transmembrane domains identified by hydropathy plots and verified by topology mapping (see text for references). SSD denotes sterol-sensing domain. Pro in Site-1 protease denotes propeptide sequence. The catalytic triad in Site-1 protease is the hallmark of a serine protease. The HExxH motif in Site-2 protease is the hallmark of a Zn^{2+} metalloprotease. Picture modified from Goldstein et al 2005

II. Insulin-induced gene (INSIG)

Two INSIG isoforms, designated INSIG1 and INSIG2, are known. Both the isoforms are expressed in most tissues, with especially high expression in the liver. Insigs are almost exclusively localized to the ER irrespective of sterol levels, suggesting that there might be an additional ER protein associated with them. Analysis of the Insig-1 sequence predicts a hydrophobic protein with multiple membrane-associated domains. Immunofluorescence data show that epitope-tagged Insig-1 is located in the ER membrane in both the presence and the absence of sterols. The two human INSIG proteins are 59% identical and both bind SREBP cleavage-activating protein in a sterol-dependent fashion. The major differences between these two proteins relate to the regulation of their expression. Thus, INSIG1 is itself an obligatory SREBP target gene, whereas INSIG2 is expressed

at a low but constitutive level, at least in cultured cells, and is not regulated by SREBPs. Yabe et al. [34] reported the existence of a liver-specific transcript of Insig2 in rodents, designated Insig2a, which differs from the ubiquitous transcript, called Insig2b, in the noncoding first exons that splice into a common second exon through the use of different promoters. Although both transcripts encode identical proteins, they differ in terms of regulation patterns.

III. Site-1 protease (S1P)

S1P is a 1,052-amino-acid serine protease, also known as subtilisin/kexin isozyme-1, SKI-1 (figure 20). It is anchored to the membrane by a single membrane-spanning helix, the bulk of the protease faces the lumen of the ER. The carboxy terminus is a short stretch of basic amino acids that face the cytoplasm, which makes S1P a type-1 membrane protein. S1P is synthesized as a proenzyme that undergoes autocatalytic cleavage of its prosegment to yield the active enzyme.

IV. Site-2 protease (S2P)

S2P, one of the most hydrophobic protease known (figure 20), contains an HEXXH zinc-binding motif, which is characteristic of the active site of many families of metalloproteinases. The unusual location of this motif within an otherwise hydrophobic region of the protein might be due to the nature of its substrates, as hydrophobic membrane-spanning helices are thought to lie within the plane of the lipid bilayer. S2P is sufficiently different from all other metalloproteinases to be included in its own family, M50, which consists of at least 68 proteins found in bacteria, archaea, plants and animals. S2P was identified using human genomic DNA to complement mutant cells that were unable to cleave SREBPs at site-2. Moreover a distal aspartate residue, Asp467, is believed to provide an additional coordinating ligand for the active-site metal atom. It is found in the sequence LDG that is highly conserved among homologues of S2P.

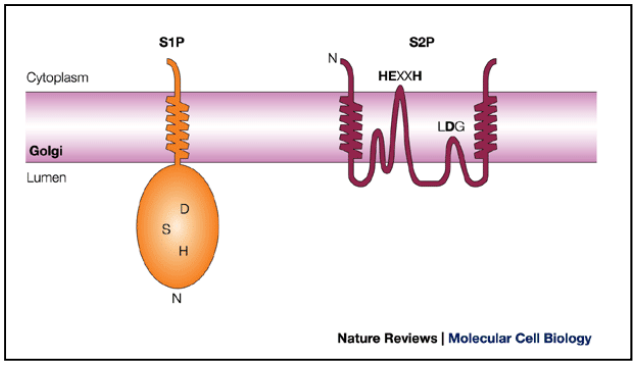

Figure 20: S1P is a subtilisin-like serine protease and the Asp-Ser-His (DSH) residues of the catalytic triad are necessary for S1P function32. S2P is an exceptionally hydrophobic metalloproteinase containing an HEXXH active-site motif in an otherwise hydrophobic portion of the protein (where H is histidine, E is glutamate and X can be any amino acid). Both histidine residues and the glutamate residue of the active site (indicated in bold) are necessary for S2P function43, as is a distal aspartate residue (D; bold), found within the LDG motif. This residue, which also lies within a hydrophobic portion of the protein, is believed to provide an additional ligand for the active-site metal atom47. N, amino-terminal. Picture modified from Rawson et al. 2003

Newly synthesized SREBP is inserted into the ER membranes, where its COOH-terminal regulatory domain binds the COOH-terminal domain of SCAP (figure 21). Integral-membrane proteins insulin induced Insig have been identified as ER proteins that bind SCAP and cause ER retention of the SCAP/SREBP complex when a certain amount of sterols is present. Cholesterol addition to the ER membranes causes a conformational change in the cytoplasmic loop between the sixth and seventh membrane-spanning segments of SCAP. The amount of cholesterol required to produce this conformational change is reduced in the presence of Insig proteins, thus facilitating the retention of SCAP/SREBP complexes in the ER. Sterol-insensitive SCAP mutants do not undergo this sterol-induced conformational change. As demonstrated by Goldstein in 2002 [35], SCAPs with

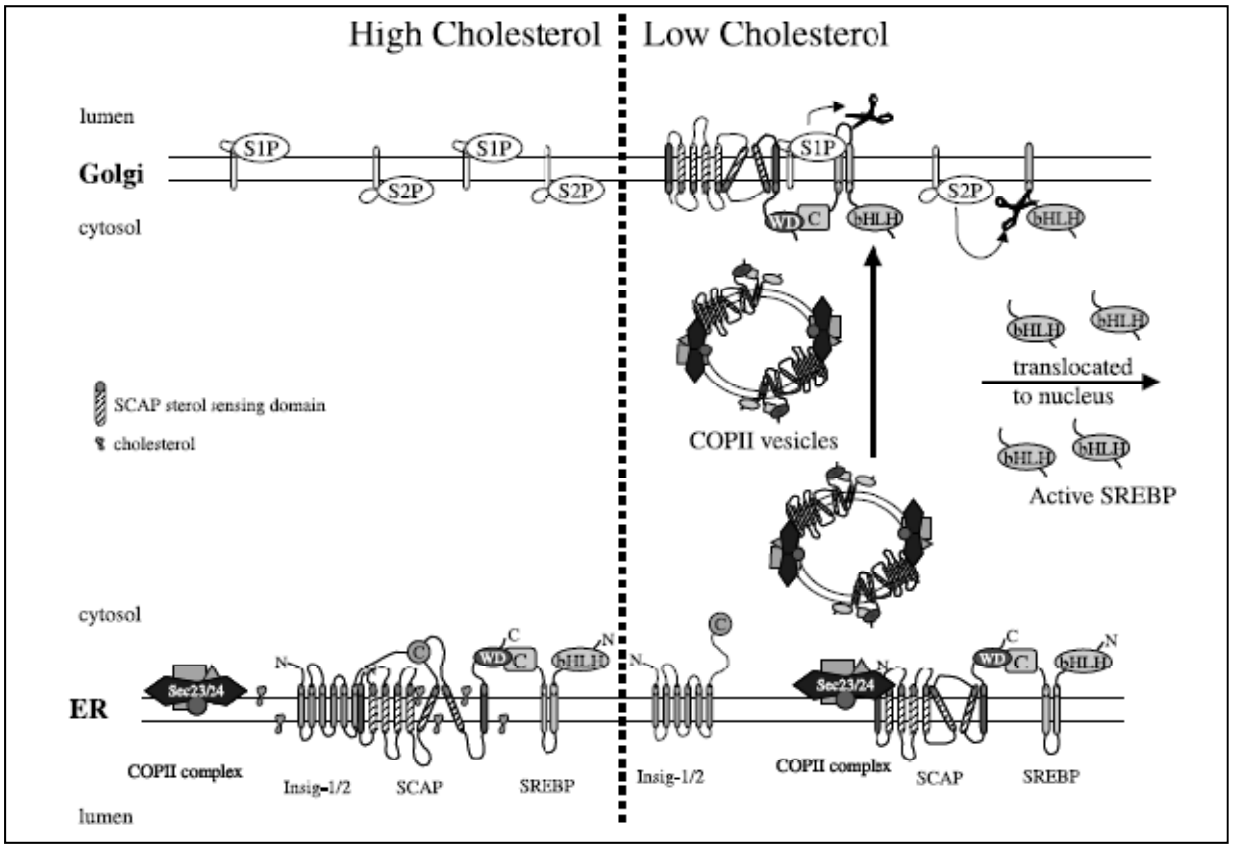

Figure 21: Model for the sterol regulation of SREBP trafficking and proteolysis. SCAP contains a ~170 amino acid putative sterol-sensing domain (helices 2–6). SCAP forms a tight complex with SREBP via the C-terminal domains of both proteins, consisting of five WD repeats in SCAP and the regulatory domain of SREBP. In the presence of high concentrations of cholesterol in the ER, SCAP undergoes a conformational change. This increases the affinity of SCAP for Insig-1 and -2. The Insig/SCAP/SREBP complex is thus retained in the ER. Excess cellular cholesterol also leads to the production of oxysterols, which increases SREBP-1a gene expression, yielding oleic acid for formation of cholesteryl esters. This provides a feed-forward mechanism to prevent the production or uptake of unesterified cholesterol. In the absence of cholesterol, the affinity of SCAP for Insig-1 is decreased. This allows the SCAP/SREBP complex to interact with the COPII proteins Sec23/24 and move into the transitional ER, eventually budding into COPII-coated vesicles for transport to the Golgi. In the Golgi, the SREBP undergoes two sequential proteolytic cleavages from the Golgi-resident S1P and S2P proteases. These cleavages are required for release of the transcriptionally active NH2 fragment, which can then enter the nucleus and regulate the transcription of a variety of sterol response genes. Insig-1 is itself regulated by SREBP and it can bind to SCAP under high protein concentrations independent of the cholesterol-dependent conformation of SCAP. Thus, under cholesterol-depleted conditions, there exists a feedback mechanism to prevent the overaccumulation of cholesterol. Picture from McPherson and Gauthier (2004)

point mutations at any positions within the cholesterol-sensing domain cannot bind Insig proteins. This fact facilitate SREBP processing also in the presence of elevated sterol levels.

The mechanism that Insigs play in the retention of SCAP/SREBP complexes is still not fully understood. It is possible that the binding of Insigs to SCAP in the presence of sterols, when SCAP conformation is changed, simply prevents the binding of the COPII proteins Sec23/24 and the formation of SCAP/SREBP-containing COPII vesicles. This implies that the binding of Sec23/24 is itself not regulated by the conformation of SCAP, and this is supported by evidence that over-expression of SCAP results in SREBP processing irrespective of sterol concentration. Additionally, Insig-1 has been shown to enhance the degradation of HMG-CoA reductase when sterol levels are high by binding to its sterol-sensing domain. This suggests a dual role for Insigs in the SCAP/SREBP pathway and in the regulation of cholesterol metabolism.

When cholesterol level drops down it is sensed by SCAP sterol sensor domain. The complex leaves the ER and moves to the Golgi apparatus membranes, where the two proteases S1P and S2P reside. Cleavage takes place in two steps: at first S1P cleaves the SREBP in the luminal loop between its two membrane-spanning segments, dividing the SREBP molecule in half. This cleavage allows a second protease S2P to clip the proteins at site 2 in the middle of the first membrane-spanning segment, and the NH2-terminal bHLHZip domain is released from the membrane. The NH2-terminal domain, designated nuclear SREBP (nSREBP), translocates to the nucleus, where it activates transcription by binding to non-palindromic sterol response elements (SREs) in the promoter/enhancer regions of multiple target genes [35-39].

3.3 Regulation of SREBP genes

SREBPs regulation occurs not only in posttranscriptional manner (S1P and S2P cleavage) but also transcriptionally. In this way SREBP-1c and SREBP-2 are subject to distinct forms of regulation, whereas SREBP-1a appears to be constitutively expressed at low levels in liver and most other tissues of adult animals.

One mechanism of regulation shared by SREBP-1c and SREBP-2 involves a feed-forward regulation mediated by SREs present in the enhancer/promoters of each gene. Through this feed-forward loop, nSREBPs activate the transcription of their own genes. In contrast, when nSREBPs decline (as in *Scap* or *S1p* knockout mice), there is a secondary decline in the mRNAs encoding SREBP-1c and SREBP-2.

Three factors selectively regulate the transcription of SREBP-1c:

I) liver X-activated receptors (LXRs)

II) insulin

III) glucagon

I. Liver X-activated receptors (LXRs)

LXRα and LXRβ are nuclear receptors that form heterodimers with retinoid X receptors. They are activated by a variety of sterols, including oxysterol intermediates that form during cholesterol biosynthesis. An LXR-binding site in the *SREBP-1c* promoter activates SREBP-1c transcription in the presence of LXR agonists. The functional significance of LXR-mediated SREBP-1c regulation has been confirmed in two animal models. Mice lacking both LXRα and LXRβ express reduced liver levels of SREBP-1c and its lipogenic target enzymes, and respond relatively weakly to treatment with a synthetic LXR agonist. Because a similar blunted response is found in mice that lack SREBP-1c, it appears that LXR increases fatty acid synthesis largely by inducing SREBP-1c. LXR-mediated activation of SREBP-1c transcription provides a mechanism for the cell to induce the synthesis of oleate when sterols are in excess. Oleate is the preferred fatty acid for the synthesis of cholesteryl esters, which are necessary for both the transport and the storage of cholesterol. LXR-mediated regulation of SREBP-1c appears also to be one mechanism by which unsaturated fatty acids suppress SREBP-1c transcription and thus fatty acid synthesis. Rodents fed diets enriched in polyunsaturated fatty acids manifest reduced SREBP-1c mRNA expression and low rates of lipogenesis in liver. In vitro, unsaturated fatty acids competitively block LXR activation by antagonizing with its endogenous ligands, and so decreasing LXR mediated SREBP-1c expression. In addition to LXR-mediated transcriptional inhibition, polyunsaturated fatty acids lower SREBP-1c levels by accelerating degradation of its mRNA. These combined effects may contribute to the long recognized ability of polyunsaturated fatty acids to lower plasma triglyceride levels.

II III. Insulin and glucagon

Transcription of some hepatic genes such as glucokinase (GK) are exclusively dependent on insulin. The mechanism is partially unknown but is clear that it involves SREBP-1c activation. It is established that insulin stimulates SREBP-1c gene transcription in the liver and in adipose tissue. Insulin-stimulated SREBP-1c gene transcription should logically result in an increased abundance of the precursor form of SREBP-1c in membranes of the hepatic endoplasmic reticulum. In hepatocytes insulin increases SREBP-1c mRNA levels concomitant with its ability to elevate mRNA levels for fatty acid biosynthetic genes. A dominant-negative form of SREBP-1c blocks the insulin effect on the fatty acid biosynthetic genes. The insulin effect on SREBP-1c mRNA is blocked when cAMP levels are elevated by glucagon, and this block is associated with a decline in

acetyl-CoA carboxylase and fatty acid synthetase, the two key enzymes of fatty acid biosynthesis. In the livers of living rodents, SREBP-1c mRNA levels have been shown to decline when insulin falls as a result of fasting or treatment with streptozotocin, and this fall is paralleled by decreases in lipogenic mRNAs. The opposite result has been observed when insulin is administered to streptozotocin-treated rats. In livers of lipodystrophic and ob/ob mice, SREBP-1c mRNA levels are elevated as a result of hyperinsulinemia secondary to leptin deficiency. Leptin treatment normalizes plasma insulin. As a result, SREBP-1c mRNA levels fall down, the mRNAs for lipogenic genes decline, and the fatty liver resolve.

SREBPs are also subject to regulation by rapid degradation via the ubiquitin-proteasome pathway (Hampton 2002), as it is shown in figure 22. In particular, nuclear SREBPs are targeted by various post-translational modifications, including phosphorylation, acetylation, sumoylation and ubiquitination. Nuclear SREBP1c was found to be phosphorylated and negatively regulated by GSK-3β[7]. Phosphorylation of these residues created a docking site for Fbw7, the substrate recognition component of a specific SCF ubiquitin ligase. Ubiquitinated SREBP is then degradated via proteasome. Knockdown of Fbw7 prevented the degradation of SREBP1 and enhanced SREBP-dependent transcription. Interestingly, the GSK-3-dependent phosphorylation of Thr426 and Ser430 in nuclear SREBP1 was enhanced in response to DNA binding. Accordingly, GSK-3β and Fbw7 were recruited to the promoters of SREBP target genes in vivo and DNA binding promoted Fbw7-dependent ubiquitination of SREBP1.

SREBPs are also modified by another protein, small ubiquitin-like modifier (SUMO). SUMO-1 is a 101 amino acid protein having 18% identity with ubiquitin, but with a remarkably similar secondary structure. Sumoylation of transcription factors, including SREBPs, is prone to result in attenuation of their transcriptional activities. The process requires a multistep reaction similar to that of ubiquitination, but the specific enzymes are distinct from those involved in ubiquitination. Ubc9 is a SUMO-conjugating enzyme (E2) that directly interacts with most sumoylated proteins, including SREBPs. In some cases, ubc9 itself plays, to a certain extent, a SUMO E3-like role in the absence of any E3 ligases. Unlike ubiquitination, which requires phosphorylation near the ubiquitination site, sumoylation competes with the phosphorylation close to the sumoylation site, which occurs in response to growth factor stimuli. This implies that growth factor stimuli interfere with sumoylation, thereby enhancing SREBP transcriptional activities, and lipid synthesis required for cell growth. Sumoylated SREBPs recruit a co-repressor complex containing histone deacetylase 3 to suppress their transcriptional activities [21]. Histone deacetylase

[7] Two of the residues targeted by GSK-3: Thr426 and Ser430; were mapped to the C terminus of nuclear SREBP1(Thr402 and Ser406 in SREBP1c)

3 is unable to directly interact with SREBPs, but a certain subunit in the co-repressor complex, which is not yet identified, is considered to be involved in the interaction

[36, 40-46].

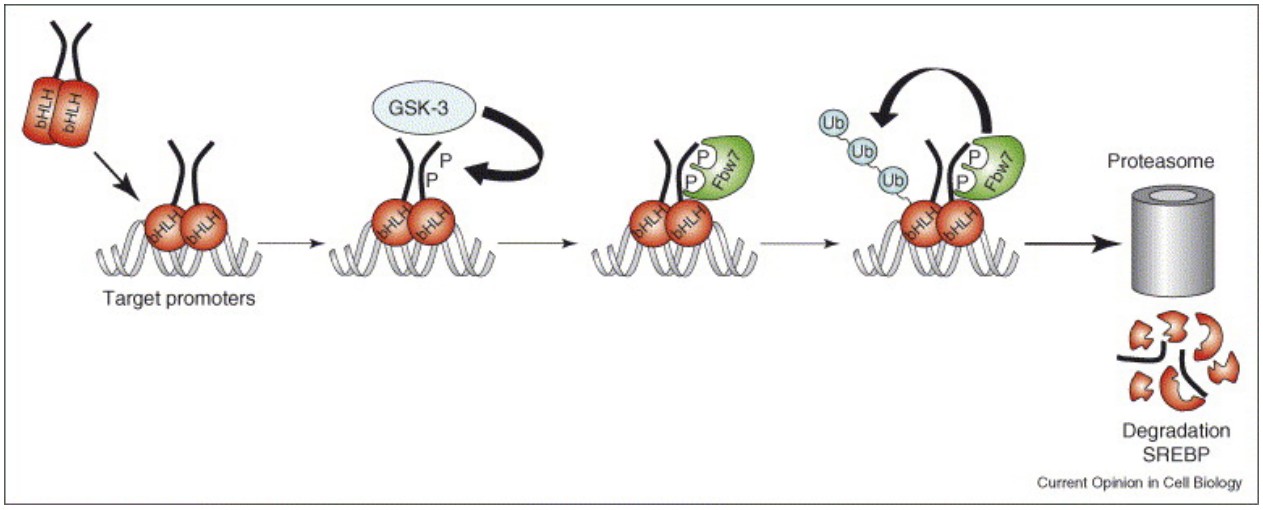

Figure 22: Phosphorylation-dependent degradation of active sterol regulatory element-binding protein 1 (SREBP1). Once in the nucleus, dimeric SREBP1 functions as a transcription factor by binding to specific DNA sequences in the promoters of its target genes. DNA binding enhances the interaction between SREBP1 and GSK-3β, resulting in the phosphorylation of Thr426 and Ser430 in the C terminus of nuclear SREBP1. As a result, the ubiquitin ligase SCF^{Fbw7} is recruited to SREBP target promoters and induces the ubiquitination and degradation of SREBP1. Most probably, both subunits of the SREBP dimer become phosphorylated and ubiquitinated in response to DNA binding. Only the substrate recognition component of the SCF complex is included in the figure. Figure from Bengoechea-Alonso and Ericsson 2007.

3.4 SREBPs are master regulator of gene expression

SREBPs are master regulator of a large number of genes (some genes are shown in table 1) involved in cholesterol biosynthesis, transport and esterification. The mechanism trough they act involves consensus sequence recognized on DNA and the interaction with co-activators factors.

SREBP-1a and -2 promote gene expression by interacting with two large ubiquitous transcriptional co-activators, CBP and P300. SREBP-1c, which has a much shorter activation domain, does not interact with CBP and P300.

SREBPs have recently been shown to be stabilized via acetylation by the intrinsic acetyltransferase activity of P300 and CBP. Independently of CBP and P300, the amino terminal of

SREBP-1a also interacts with the vitamin D receptor interacting protein (DRIP), also known as activator recruited cofactor (ARC).

Like all members of the bHLH-Zip family of transcription factors, SREBPs recognize the inverted E-box repeat 5′- CAXXTG-3′. Similar to Myc/Max and USF, the preferred SREBP E-box is 5′-CACGTC-3′. However, in contrast with other bHLH-Zip transcription factors, SREBPs contain a tyrosine residue in their basic domain in place of an arginine residue. This tyrosine residue confers flexibility, which allows SREBPs to bind also to the direct repeat sterol regulatory element (SRE) 5′- TCACNCCAC-3′.

All cholesterol-responsive promoters, including genes that regulate fatty acid as well as cholesterol metabolism, contain a direct repeat SRE site that is not accessible to other bHLH-Zip proteins, thus ensuring highly regulated cholesterol-dependent transcription. The functional importance of SREBPs in controlling transcription of sterol-regulated genes is well established. However, in all SREBP-regulated promoters studied to date, additional co-regulatory transcription factors are required.

SREBPs are inherently weak activators by themselves and function synergistically with ubiquitous factors such as nuclear factor-Y, CREB/ATF, and Sp1, that bind in proximity to achieve a high level of promoter activation. Sp1 has been shown to be a SREBP-1a coactivating factor for low-density lipoprotein receptor, and NF-Y has been shown to be a SREBP-1a coactivating factorfor steroidogenic acute regulatory protein (StAR), farnesyl diphosphate synthase, and HMG-CoA synthase. There is a requirement for both Sp1 and NF-Y in sterol regulation of the human fatty acid synthase promoter. Yin yang-1 (YY1) is a unique transcription factor that has the capability of acting as repressor, activator, or initiator of gene transcription via multiple mechanisms, including YY1 binding to an activator to occlude its recognition site. In binding to a promoter, YY1 may also bend the DNA and impair activator interaction. YY1 may also recruit histone deacetylases that confer transcriptional silencing. YY1 has been shown to repress in a dose-dependent manner the transcription of LDL receptor gene, by specifically targeting SREBP-1a interaction with Sp1. YYI has been shown to inhibit SREBP-1a/NF-Y enhancement of StAR activation by decreasing SREBP-1a binding to an SRE in the presence or absence of NF-Y.
[36, 47]

Gene	SRE promoter sequence
Cholesterol metabolism	
LDL receptor	ATCACCCCAC
HMG CoA synthase	CTCACCCCAC GCCACCCTAC
HMG CoA reductase	ACCGCACCAT CTCTCACCAC
Farnesyl diphosphate synthase	CTCACACGAG
Squalene synthase	ATCACGCCAG CTAGTGTGAG
SREBP-2	ATCACCCCAC
Lanosterol 14a-demethylase (Cyp51)	ATCACCTCAG
Fatty acid metabolism	
Acetyl CoA carboxylase	CCAT ----TCAC
Fatty acid synthase	GCCACGCCAC GTCAGCCCAT
Stearoyl CoA desaturase-1 and 2	AGCAGATTGTG
AcylCoA binding protein (Diazepam)	CTCGCCCGAG
SREBP1	CTCACCCGAG
ATP citrate lyase	TCAGGCTAG
Malic enzyme	TCACCCGTCGGTG
PPAR gamma	ATCACTTGAG
Acetyl CoA synthease	ATTCATGTGACAT ATCACTCCAC
Triglyceride synthesis	
Glycerol-3-phosphate acyltransferase	CTCAGCCTAG CTCACCCCAG
	GACACCCCAG
Plasma lipoprotein metabolism	
Lipoprotein lipase	CTCCCCCCAA
HDL receptor (SRBI)	GCCACCTGCA

Table 1: Gene recognized to have SRE consensus sequence. Table modified from Hitoshi and Shimano 2001.

4. Cholesterol and disease

Changes in the organization of membrane lipids can have profound effects on cellular functions such as signal transduction and membrane trafficking. These membrane effects can cause disease in humans as a result of genetic alterations or environmental effects (such as diet), or both. Cholesterol is one of the most important regulators of lipid organization, and mammals have developed sophisticated and complex mechanisms to maintain cellular cholesterol levels in membranes within a narrow range (as is shown in previous chapters). When these homeostatic mechanisms are overwhelmed, outcomes or progression of pathological conditions under cardiovascular system are facilitate.

4.1 Hypercholesterolemia

LDL excess is the major cause of injury to the vessels endothelium and underlying smooth muscle. When LDL particles become trapped in an artery, they can undergo progressive oxidation and be internalized by macrophages by means of the scavenger receptors on the surfaces of these cells. The internalization leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters resulting in the formation of foam cells. The degree to which LDLs are modified can vary greatly. Once modified and taken up by macrophages, LDLs activate the foam cells. Removal and sequestration of modified LDLs are important parts of the protective role of the macrophage in the inflammatory response, and minimize the effects of modified LDLs on endothelial and smooth-muscle cells. Antioxidants such as vitamin E can reduce free-radical formation by modified LDLs. Modified LDLs are chemotactic for other monocytes, and can up-regulate the expression of genes for macrophage colony-stimulating factor and monocyte chemotactic protein derived from endothelial cells. Thus, it may help expand the inflammatory response by stimulating the replication of monocyte-derived macrophages and the entry of new monocytes into lesions.

The inflammatory response itself can have a profound effect on lipoprotein movement within the artery. Specifically, mediators of inflammation such as tumor necrosis factor α, interleukin-1, and macrophage colony-stimulating factor, increase binding of LDLs to endothelium and smooth muscle and increase the transcription of the LDL-receptor gene. After binding to scavenger receptors in vitro, modified LDL initiates a series of intracellular events that include the induction of urokinase and inflammatory cytokines, such as interleukin-1. Thus, a vicious circle of inflammation, modification of lipoproteins, and further inflammation can be maintained in the artery by the presence of these lipids.

Oxidized LDLs are present in atherosclerotic lesions in humans. In animals with hypercholesterolemia, antioxidants can reduce the size of lesions, and they reduce fatty streaks in non human primates [18, 48].

4.2 Atherosclerosis

Atherosclerosis is the major human disease associated with cholesterol and lipid metabolism. The earliest event in atherogenesis (Figure 23 and 24) is the atheromas formation, that consists in the accumulation of plasma lipoproteins in the sub-endothelium, or intima, of focal areas of the arterial tree. The lipoproteins are retained owing to a combination of proteoglycan binding and lipoprotein aggregation. This leads to the increasing in particle size, that blocks egress from the arterial wall. These retained lipoproteins, particularly those that are modified by oxidation, aggregation and other means, stimulate atherogenesis trough the promotion of inflammation. Monocytes and T cells, but not neutrophils, infiltrate the arterial wall. More specifically, certain types of oxidized phospholipid derived from modified lipoproteins can activate the overlying endothelium to secrete chemokines, and express adhesion molecules for monocytes and T cells. These leukocytes migrate through an otherwise intact endothelial layer, and the monocytes eventually differentiate into macrophages in the intima.

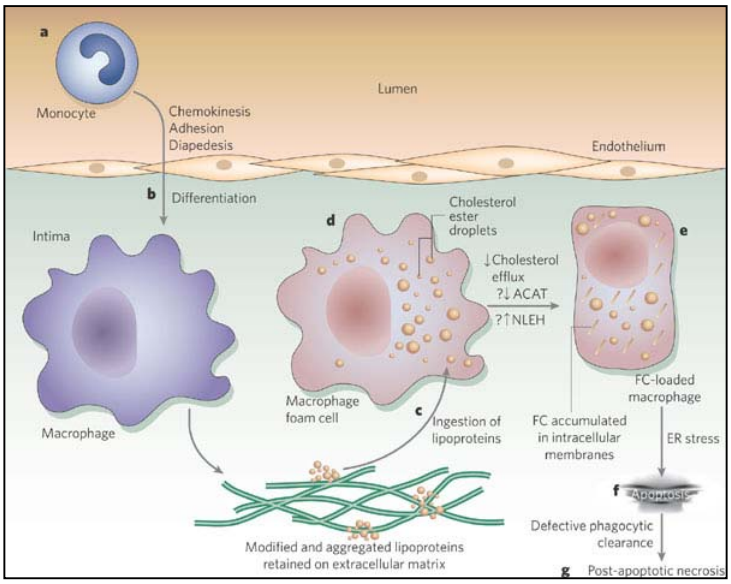

Figure 23:**a)** Monocytes are attracted to focal areas of the arterial wall in which atherogenic lipoproteins have been retained on the extracellular matrix. These retained lipoproteins signal to the endothelium to express chemokines and adhesion molecules. **b)** The monocytes migrate through the endothelial layer and differentiate into macrophages. **c)** The macrophages ingest the retained lipoproteins by endocytic and phagocytic mechanisms and thus acquire a large load of lipoprotein-derived cholesterol. **d)** In early lesions, the cholesterol is stored as ACAT-derived cholesteryl esters and thus acquire a foamy appearance. **e)** In advanced lesions, unesterified or 'free' cholesterol (FC) accumulates **f)** leading to macrophage apoptosis **g)** and necrosis. Image from Maxfieeld and Tabas (2005)

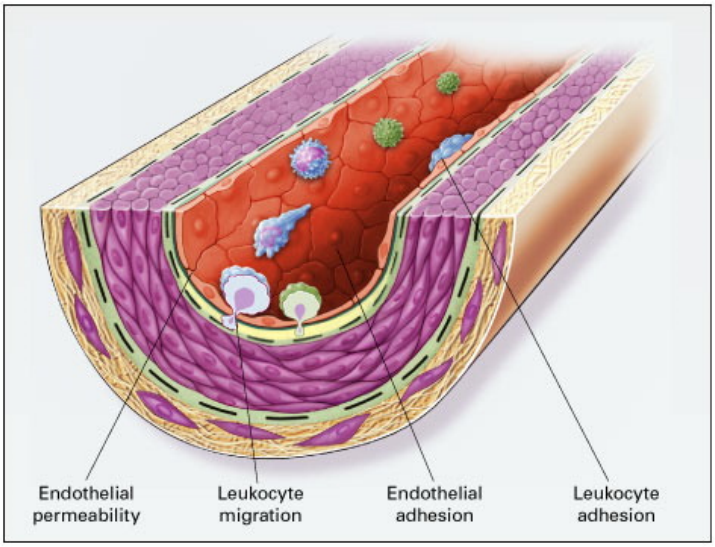

Figure 24: Endothelial Dysfunction in Atherosclerosis. Image from Ross 1999

Once embedded in the intima, the macrophages, through a process that is only partly understood, ingest the lipoprotein particles[8] bound to the matrix. Interestingly, just as cholesterol depletion inhibits signal-dependent actin assembly. In some cells, loading of macrophages with cholesterol through uptake of modified lipoproteins or through a cyclodextrin carrier (that is, without a lipoprotein) can lead to increased actin assembly and protrusion of membrane processes in macrophages. It seems likely that these cholesterol-dependent effects are mediated by changes in lipid organization, which can affect activation of the small GTPase Rac17. In the blood vessel wall, the initial contact with lipoproteins could lead to cholesterol transfer to the macrophages, leading to actin-dependent protrusions. This would enhance the further uptake of cholesterol into the cells.

Most of the cholesterol in lipoproteins is in the form CE. These esters are hydrolyzed to cholesterol and fatty acids in acidic degradative organelles such as late endosomes, and then free cholesterol is transported to other sites in the macrophage:

- transport to the plasma membrane is important for cholesterol efflux;

- transport to the ER is necessary for intracellular cholesterol homeostasis (through SREBP) and for re-esterification by ACAT;

- transport to the mitochondria leads to the formation of oxysterols, which, in turn, may have roles in LXR activation and sterol efflux.

ACAT-mediated re-esterification is a major fate of lipoprotein-derived cholesterol in intimal macrophages. The resulting cholesteryl ester molecules coalesce into membrane-bound neutral lipid droplets in the cytoplasm, a feature that has given rise to the term 'foam cell' (figure 25).

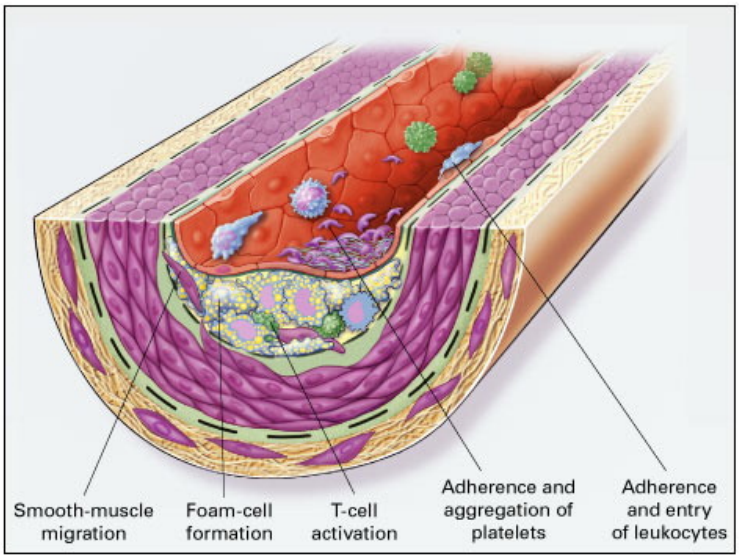

Figure 25. Atherosclerosis progression: Plaque formation. Image from Ross 1999

Receptor-mediated uptake by means of the LDL receptor is usually limited because of its homeostatic down-regulation by cholesterol. However, aggregated LDL can deliver enormous amounts of cholesterol to macrophages and cause foam-cell formation. The likely explanation is that one or more receptors other than LDL receptors are involved and/or that

[8] In a cell-culture model of the initial interaction of macrophages with retained and aggregated lipoproteins, significant rearrangement of the actin cytoskeleton and protrusion of membrane processes is seen, and this is required for the continued uptake of cholesterol into the cells

LDL receptor down-regulation is not complete in these macrophages. Foam cells seems to be formed mostly, if not exclusively, by the uptake of oxidized LDL and may also participate in other early atherogenic processes, such as smooth-muscle-cell migration and T-cell-mediated inflammatory and immune responses. Moreover, remnant lipoproteins are avidly internalized by cultured macrophages and are potent inducers of ACAT activation and foam-cell formation. They are abundant in the intima of atherosclerotic lesions, and their levels in plasma are strongly associated with the presence of foam cells and the incidence of atherosclerotic vascular disease in animal models and humans.

Foam-cell secretion of inflammatory cytokines and matrix metalloproteinases contributes to atherosclerosis. Macrophage foam cells may also participate in other early atherogenic processes, such as smooth-muscle-cell migration and T-cell-mediated inflammatory and immune responses.

Early atherosclerotic lesions are not symptomatic because arterial lumen occlusion is not great enough to compromise blood flow. This lack of occlusion is aided by outward remodelling of the affected region of the arterial wall. After years of gradual lesion development, foam cells, smooth muscle cells, extracellular matrix material and smooth-muscle-cell-derived scar tissue can lead to slowly progressive lumen occlusion. Symptoms are usually absent because organ blood flow is restored by compensatory, hypoxia-driven neo-vascularization. If this compensatory process becomes compromised, the patient may experience stable, exercise-induced compromise of blood flow (for example, exercise-induced angina), but not acute cardiovascular events. Importantly, the smooth-muscle-cell-derived scar tissue forms a fibrous cap that covers and essentially 'protects' the underlying lesion, and these lesions tend to be relatively stable.

Some lesions progress leading to acute vascular events, including sudden death, acute myocardial infarction, unstable angina or ischemic stroke (figure 26). These events are caused by acute, occlusive luminal thrombosis, which leads to organ damage because of the suddenness of lumen occlusion. This process occurs over minutes, so there is not enough time for compensatory responses. Pathological observations of affected arteries in patients suffering from acute events led to the plaque-disruption theory of acute

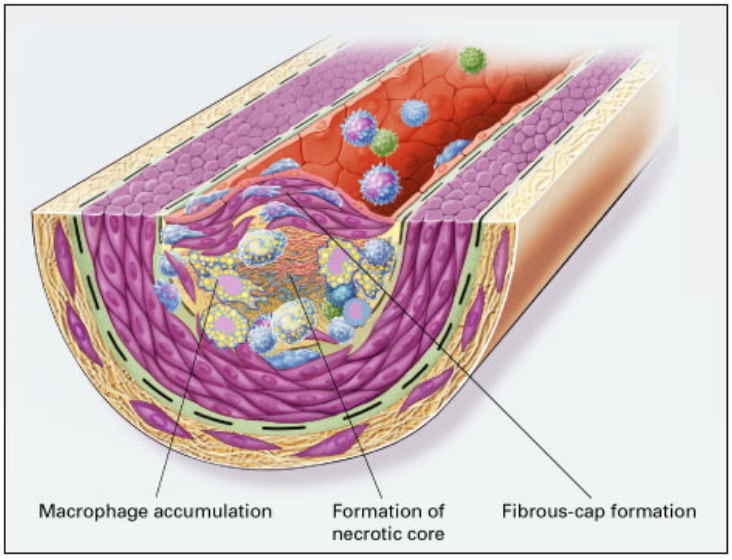

Figure 26: Formation of advanced lesion of Atherosclerosis. Image from Ross 1999

athero-thrombosis. According to this theory, a minority of plaques become necrotic and highly inflammatory, which eventually leads to breakdown of the protective fibrous cap or to erosion of the endothelial cell layer. These events, in turn, expose the luminal blood to underlying plaque material, which promotes coagulation and thrombosis. Of interest, these rare events do not necessarily occur in the largest plaques, but rather those that have large areas of necrosis.
[7, 18, 48, 49]

4.3 Alzheimer's disease.

The role of cholesterol and lipids in Alzheimer's disease has been actively studied for over a decade, on the basis of the observation that there is a genetic linkage between age of onset of Alzheimer's disease and the presence of the ε4 allele of ApoE. Polymorphisms in other proteins involved in cholesterol metabolism may also have a genetic linkage with this disease.

ApoE is one of the main carriers of cholesterol in the brain, and it seems possible that alterations in cholesterol distribution or levels have a role in formation of amyloid deposits. The amyloid in Alzheimer's disease is formed by aggregation of a 39–42-residue peptide, the Aβ peptide, which is formed by two proteolytic cleavages of a transmembrane protein, the amyloid precursor protein (APP). These cleavages take place in intracellular organelles. In tissue culture studies, severe lowering of cellular cholesterol (more than 35% reduction) partly inhibited the formation of the Aβ peptide, but moderate reduction in cellular cholesterol increased the formation of Aβ peptide. Furthermore, rodents treated with statins can have increased amyloid production, and recent studies indicate that treatment with statins does not reduce the amyloid burden in humans. Nevertheless, statins may be neuroprotective, perhaps because of their pleiotropic effects on endothelial cell function and as suppressors of inflammation. There is still not a good mechanistic explanation for the association of the ε4 allele of ApoE with age of onset of Alzheimer's disease.

Studies using animal models of AD[9] show a strong connection between plasma cholesterol levels and Aβ generation. In a study done by Refolo, L.M. *et al* et al, is shown that high fat/high cholesterol diet raises cholesterol levels in plasma and CNS of transgenic mice expressing the FAD mutants $APP_{K670N,M671L}$ and $PS1_{M146V}$. Because both β-APP C-terminal fragment (CTF) and Aβ levels are increased in the brain of these animals, it may be possible that cholesterol levels can regulate APP processing and Aβ generation *in vivo.* Neuropathological analysis showed that a high cholesterol diet also increases the deposition of amyloid plaques. Additionally, cholesterol-lowering agents reverses the effect of high fat/high cholesterol diet on Aβ accumulation and cholesterol

[9] Rabbits, transgenic mice and guinea pigs

levels in the plasma and CNS. In a study on mice harboring a different APP FAD mutation (Swedish mutant) (Howland, D.S. *et al*, anno) a high-cholesterol diet elevated cholesterol levels in plasma and CNS, but had an opposite effect on Aβ generation. In this model mice, the levels of both $A\beta_{40}$ and $A\beta_{42}$ were reduced in the brain. The reason for the apparent discrepancy may reside in either the genetic background of the mice or, more likely, in the different transgene introduced. Species differences in mice may affect cholesterol generation, and therefore the choice of APP transgenic mouse model used becomes extremely important for the outcome of studies in which cholesterol levels are manipulated. A recent study in guinea pigs has strengthened the role of lipid-lowering drugs in the regulation of Aβ generation, as simvastatin reduced cholesterol and both $A\beta_{40}$ and $A\beta_{42}$ levels in guinea pig plasma.

All this evidence underline that there are multiple strands of research pointing at an important role for cholesterol in the pathogenesis of AD, but the process is however far from a clear explanation. AD is a complex multifactorial disease, and cholesterol metabolism is subject to a complex regulation at many different levels and in different compartments, so that increase or decrease in cholesterol levels bring about re-adjustments of the system in way that may not be immediately evident. Certainly more research is needed in this rapidly evolving and promising field.

[18, 50-53]

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NUTRITIONAL REGULATION OF CHOLESTEROL HOMEOSTASIS

Cardiovascular diseases (CVDs) are the major cause of death in industrialized country and has been strongly linked to an excessive dietary intake of saturated fatty acids and cholesterol, over production of endogenous cholesterol, and chronic inflammation due to oxidative stress. The combination of these conditions causes accumulation and oxidation of plasma low-density lipoprotein–cholesterol (LDL-c), vascular inflammation, and endothelial dysfunction.

Dietary and pharmacologic reduction in total and LDL cholesterol decreases the risk of coronary events, and dietary intervention is the first-line approach. Many nutritional compounds can act on cholesterol absorption, metabolism, and biosynthesis.

There is a large amount of evidence suggesting that a high consumption of fruits, vegetables and fish can help reduce the risk of CVD. Certain phytochemicals, including flavonoids and phytosterols are the food components to which the protective effect is ascribed. A considerable amount of epidemiological data suggests that phytochemicals contained in plant derived foods are able to reduce the risk of age-related chronic illness such as CVD. The initial ideas about how these compounds provide protection following ingestion, centred upon their direct antioxidant activity. However, recent research propose that many of these compounds do not act as direct antioxidants but through more subtle effects on a variety of cell signalling pathways, and by modulating gene expression. Now it appears clear that phytochemicals act not only as direct anti-oxidants but also through more subtle routes such as interaction with cell signalling pathways, or modulation of gene expression.

Other important nutrients that possess a recognized protective effect in CVD are n-3 polyunsaturated fatty acids (PUFAs). Even in this case, the mechanism of action appears multiple, also involving cell signalling and molecular events.

n-3 PUFAs and some phytochemicals such as Epigallocatechingallate from green tea and phytosterols from soybeans, share the characteristic of regulating both inflammation and cholesterol biosynthesis, so influencing two of the main risk factors for CVD.

5. Poly Unsaturated Fatty Acid (PUFA)

5.1 Structure and metabolism.

Fatty acids are composed by a hydrocarbon chain with a carboxyl group at one end and a methyl group at the other. The most abundant fatty acids have straight chains of an even number of carbon atoms and the length of this chain varies from 2 to 30 or more, and may contain double bonds. If double bonds are present in the acyl chain the fatty acid is called unsaturated (UFA). A fatty acid containing two or more double bonds is called a poly unsaturated fatty acid (PUFA).

Unsaturated fatty acids are named by identifying the number of double bonds and the position of the first double bond counted from the methyl terminus (with the methyl, or ω, carbon as number 1) of the acyl chain. The 18-carbon fatty acid with two double bonds in the acyl chain with the first double bond on carbon number six from the methyl terminus is notated as 18:2ω–6, or 18:2*n*–6. It is commonly named linoleic acid and it is the first member of the *n*–6 family. α-linolenic acid (18:3*n*–3) is the simplest member of the ω –3 or *n*–3 family. Mammals, but not plants, lack the desaturase enzymes necessary to synthesize linoleic and α-linolenic acids, which are essential fatty acids (EFAs) and must be introduced with food. Since EFAs are widely distributed in foods their deficiency is rare in humans.

Once introduced by diet, linoleic acid can be converted in longer and more unsaturated ω-3 PUFAs, while α-linolenic acid is the precursor of ω-3 PUFAs (figure 27). Thus linoleic acid can be converted into γ-linolenic acid (18:3*n*–6), and dihomo-γ-linolenic acid (20:3*n*–6) into arachidonic acid (20:4*n*–6). Using the same series of enzymes, α-linolenic acid is converted into EPA (eicosapentaenoic acid; 20:5*n*–3). Further conversion of EPA into DHA (docosahexaenoic acid; 22:6*n*–3) involves the addition of two carbons to form docosapentaenoic acid (22:5*n*–3), the addition of two more carbons to produce 24:5*n*–3, desaturation to form 24:6*n*–3 and removal of two carbons by limited β-oxidation to yield DHA. Arachidonic acid can also be metabolized by the same series of enzymes. In mammals, the pathway of desaturation and elongation occurs mainly in the liver.

It is important to note that a competition exist between the metabolism of *n*–6 and *n*–3 fatty acids. Δ6-desaturase preferred substrate is α-linolenic acid, but linoleic acid is much more prevalent in the human diet, so the metabolism of *n*–6 fatty acids is quantitatively more important.

Plant seed oils are rich in PUFAs. Corn, sunflower, safflower and soya-bean oils are rich in linoleic acid, which may comprise as much as 75% of the fatty acids present. Thus these oils and foods made from them are important dietary sources of linoleic acid. Some plant oils (e.g. soya-

bean oil) also contain α-linolenic acid in smaller amounts; green plant tissues are also a source of this fatty acid. Data from U.K. indicate that the typical intakes of linoleic and α-linolenic acid are in the range of 10–15 and 0.75–1.5 g/day respectively. As to longer chain PUFAs, they are consumed in smaller amounts than linoleic and α-linolenic acids. Estimates of the intake of arachidonic acid in Western populations vary between 50 and 300 mg/day for adults. Fish, especially oily fish (salmon, herring, tuna and mackerel) and fish oils are a rich source of EPA and DHA. In the absence of oily fish or fish oil consumption, α-linolenic acid is by far the principal dietary *n*–3 PUFA. Average intake of the long-chain *n*–3 PUFAs in the U.K. is estimated at <250 mg/day. [1, 2]

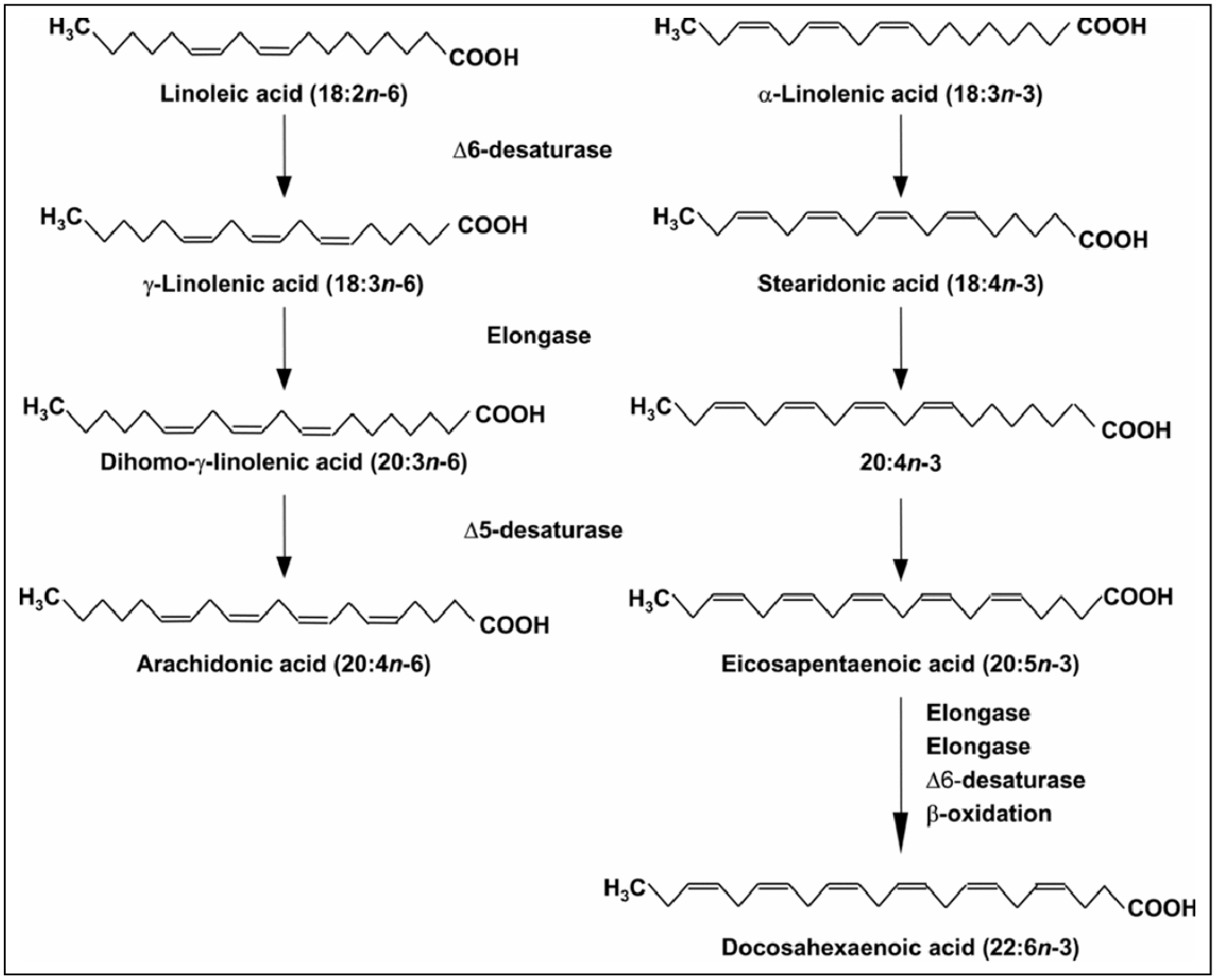

Figure 27: Structure and metabolism of n-6 and n-3 PUFAs. Image from Calder 2005.

5.2 Occurrence in food

Omega-3 fatty acids have ever been a part of the human diet and it has been estimated that the ratio n-6 /n-3 in the diet of early humans was 1:1. Nowadays the ratio calculated in the United

States has risen to ~10:1 because of the combination of reduced omega-3 fatty acid intake and the widespread use of vegetable oils rich in linoleic acid. Because of the competition between the linolenic acid and the α-linoleic acid for metabolic conversion to longer-chain, physiologically active metabolites, reducing the former while increasing the latter (or simply increasing the latter) is a strategy for increasing tissue levels of n-3 fatty acids. Another obvious strategy is to simply consume more EPA and DHA, an approach that minimizes the significance of the ratio.

The major food sources of α-linolenic acid are vegetable oils, principally canola and soybean oils (Table 2). Although some α-linolenic acid is converted to the longer-chain omega-3 fatty acids, the extent of this conversion is modest and controversial (0,2-15%) and the conversion to DHA is much less than that to EPA. This is the reason why it is better to favour the assumption of food in which EPA and DHA are already present. The primary source of very long chain *n*−3 PUFA in the diet is derived from the consumption of cold-water fatty fish such as salmon and tuna (table 3).

	Linolenic Acid Content g/tbsp
Olive oil	0.01
Walnuts, English	0.07
Soybean oil	0.09
Canola oil	1.03
Walnut oil	1.04
Flaxseeds	2.02
Flaxseed (linseed) oil	8.05
Adapted from USDA Nutrient Data Laboratory	

Table 2: α-Linolenic Acid Content of Selected Vegetable Oils, Nuts, and Seeds. Table modified from Kris-Etherton et al 2003.

A number of countries (Canada, Sweden, United Kingdom, Australia, Japan) as well as the World Health Organization and North Atlantic Treaty Organisation have made formal population-based dietary recommendations for omega-3 fatty acids. Typical recommendations are 0.3 to 0.5 g/die of EPA DHA and 0.8 to 1.1 g/die of α-linolenic acid. Recently, the Food and Nutrition Board, Institute of Medicine, and The National Academies, in collaboration with Health Canada, released the Dietary Reference Intakes for Energy and Macronutrients. The Acceptable Macronutrient Distribution Range (AMDR) for α-linolenic acid is estimated to be 0.6% to 1.2% of energy, or 1.3 to 2.7 g/die on the basis of a 2000-calorie diet. This is ~10 times the current intake of EPA and DHA. The lower boundary of the range is based on an Adequate Intake set for α-linolenic acid, which represents median intake levels that prevent an essential fatty acid deficiency. The upper boundary corresponds to the highest α-linolenic acid intakes from foods consumed by individuals in the United States and Canada. Thus, the intent of the AMDR range for n-3 fatty acids is to provide guidance for healthy people, not to prevent chronic disease. These recommendations can easily be

Fish	EPA+DHA Content g/3-oz Serving Fish (Edible Portion) or g/g oil	Amount Required to Provide 1 g of EPA+DHA per Day oz (Fish) or g (Oil)
Tuna		
Light, canned in water, drained	0.26	12
White, canned in water, drained	0,051	4
Fresh	0.24–1.28	2.5–12
Sardines	0.98–1.70	2–3
Salmon		
Chum	0,047	4.05
Sockeye	0,047	4.05
Pink	1.09	2.05
Chinook	1.48	2
Atlantic, farmed	1.09–1.83	1.5–2.5
Atlantic, wild	0.9–1.56	2–3.5
Mackerel	0.34–1.57	2–8.5
Herring		
Pacific	0,098	1.05
Atlantic	0,091	2
Trout, rainbow		
Farmed	0,068	3
Wild	0,058	3.05
Halibut	0.4–1.0	3–7.5
Cod		
Pacific	0.13	23
Atlantic	0.24	12.05
Haddock	0.02	15
Catfish		
Farmed	0.15	20
Wild	0.02	15
Flounder/Sole	0.42	7
Oyster		
Pacific	1.17	2.05
Eastern	0.47	6.05
Farmed	0.37	8
Lobster	0.07–0.41	7.5–42.5
Crab, Alaskan King	0.35	8.05
Shrimp, mixed species	0.27	11
Clam	0.24	12.05
Scallop	0.17	17.05
Capsules		
Cod liver oil*	0.19	5
Standard fish body oil	0.30	3
Omega-3 fatty acid concentrate	0.50	2
Omacor (Pronova Biocare)	0,059	1
Data from the USDA Nutrient Data Laboratory.104The intakes of fish given above are very rough stimates because oil content can vary markedly (>300%) with species, season, diet, and packaging e and cooking methods.		
*This intake of cod liver oil would provide approximately the Recommended Dietary Allowance of vitamins A and D.		

Table 3: Fish rich in PUFA. Amounts of EPA_DHA in Fish and Fish Oils and the Amount of Fish Consumption Required to Provide ~1 g of EPA+DHA per Day. Table modified from Kris-Etherton et al 2003.

met by following the AHA Dietary Guidelines to consume two fish meals per week, with an emphasis on fatty fish (for exempla: salmon, herring, and mackerel), and by using liquid vegetable oils containing α-linolenic acid. Commercially prepared fried fish (such as fast food establishments, as well as many frozen fried fish products) should be avoided because they are low in omega-3 and high in trans-fatty acids. Patients with CHD should be encouraged to increase their consumption of EPA and DHA to ~1 g/die, which is the dose used in the GISSI-Prevention Study. Table 3 presents n-3 fatty acid content of various fish and supplements as well as the amount required each day to provide ~1 g/die of EPA-DHA. Although this level of EPA and DHA intake potentially can be attained through fish consumption, the requisite amount of fish intake may be difficult to achieve and sustain over the long term. For those individuals who do not eat fish, have limited access to a variety of fish, or cannot afford to purchase fish, a fish oil supplement may be considered. Depending on the preparation, up to three 1-g fish oil capsules per day will be necessary to provide ~1 g/d of omega-3 fatty acids[1].

	Age	n-6		n-3	
	(years)	% energy	g/die	% energy	g/die
Infants	0,5 - 1	4,5	4	0,2-0,5	0,5
Children	1 to 3	3	4	0,5	0,7
	4 to 6	2	4	0,5	1
	7 to 10	2	4	0,5	1
Male	11 to 14	2	5	0,5	1
	15 to 17	2	6	0,5	1,5
	18	2	6	0,5	1,5
Female	11 to 14	2	4	0,5	1
	15 to 17	2	5	0,5	1
	18	2	4,5	0,5	1
Pregnant		2	5	0,5	1
Premium		2	5,5	0,5	1

Table 4: PUFA recommended intake in Italy . Table modified from SINU raccomandation.

In Italy, recommendations on PUFA intake are given by the Italian Society of Human Nutrition (SINU- Società Italiana Nutrizione Umana)[3]. They are summarised in table 4.
[3-7]

5.3 Safety of PUFA intake

The FDA (Food and Drugs Administration) has ruled that intakes of up to 3 g/die of marine omega-3 fatty acids are GRAS (Generally Recognized As Safe) for inclusion in the diet. This ruling included specific consideration of the reported effects of omega-3 fatty acids on glycemic control in patients with diabetes, on bleeding tendencies, and on LDL cholesterol. [4]

[1] The most common fish oil capsules in the United States today provide 180 mg of EPA and 120 mg DHA per capsule.

5.4 PUFA biological effects and mechanisms of action

Dietary *n*-3 PUFA are known to exert positive effects on several physiological processes involved in the development of chronic diseases, such as the regulation of plasma lipid levels, cardiovascular system, immune function, insulin action, neuronal development and visual function (table 5).

Ingestion of *n*-3 PUFAs will lead to their distribution in cell with effects on membrane composition and function, eicosanoid synthesis, and signalling, as well as the regulation of gene expression. Cell-specific lipid metabolism, as well as the expression of fatty acid-regulated transcription factors, probable plays an important role in determining how cells respond to changes in PUFA composition.

Target System	Effect	Likely Mechanism
CNS	Improves cognitive functions	Membrane composition; RXRα
Retina	Enhances visual acuity	Membrane composition
Immune	Immunosuppressive	Membrane composition/Rafts
	Anti-inflammatory	Eicosanoids synthesis/Action; NFkB
Cardiovascular	Anti-arrhythmia	Membrane composition/Rafts
	Anti-thrombotic	Eicosanoids synthesis/Action; NFkB
Serum Lipids	Lowers triglycerides	PPARα & γ;SREBP-1c;LXRα & β
Liver	Suppresses lipogenesis	SREBP-1c;LXRα & b
	Increases fatty acid oxidation	PPARα
	Suppresses VLDL synthesis	PPARα/HNF4a
Skeletal Muscle	Improves insulin sensitivity	Membrane composition 9,10 PPARα & γ; NFkB

Table 5: PUFA effects on humans and mechanism involved. Table modified from Donald B. Jump.

I) Eicosanoids production: relation with inflammation

The key link between fatty acids and inflammation relates to the fact that the family of inflammatory mediators termed eicosanoids is generated from 20-carbon PUFAs liberated from cell-membrane phospholipids. Inflammatory cells typically contain a high proportion of the *n*–6 PUFA arachidonic acid and low proportions of *n*–3 PUFAs, especially EPA. Thus arachidonic acid is typically the dominant substrate for eicosanoids synthesis.

Eicosanoids include PGs (prostaglandins), TXs (thromboxanes), LTs (leukotrienes), HETEs (hydroxyeicosatetraenoic acids). Arachidonic acid in cell membranes can be mobilized by various phospholipase enzymes, e.g. phospholipase A_2. The free acid can subsequently act as a substrate for the enzymes that synthesize eicosanoids. Metabolism by COX (cyclo-oxygenase) enzymes gives rise to the 2-series PG and TX (figure 28). There are two isoforms of COX: COX-1 is a constitutive enzyme, COX-2 is induced in inflammatory cells as a result of stimulation. COX-2 is responsible for the markedly increased production of PG that occurs on cellular activation. PGs are formed in a cell-specific manner.

Increased consumption of fish oil, rich in EPA and DHA, results in increased proportions of these fatty acids in inflammatory cell phospholipids, partly at the expense of arachidonic acid. Consequently, in view of the fact that there is less substrate available for the synthesis of eicosanoids from arachidonic acid, fish oil supplementation has been shown to decrease production of PGE_2, TXB_2 , LTB_4 and 5-HETE and LTE_4 by inflammatory cells.

EPA also acts as a substrate for COX and LOX enzymes, giving rise to a different family of eicosanoids: the 3-series PGs and TXs, the 5-series LTs and the hydroxy-EPAs. Fish oil supplementation results in the increased production of LTB_5, LTE_5 and 5-hydroxy-EPA by inflammatory cells, that are believed to be less potent compared with those formed from arachidonic acid (figure 28).

Additionally, a novel group of mediators, termed E-series resolvins, has been identified. They are formed from EPA by COX-2, and exert anti-inflammatory actions. DHA-derived mediators (D-series resolvins, docosatrienes and neuroprotectins) are also produced by COX-2 under some conditions, and to be anti-inflammatory.

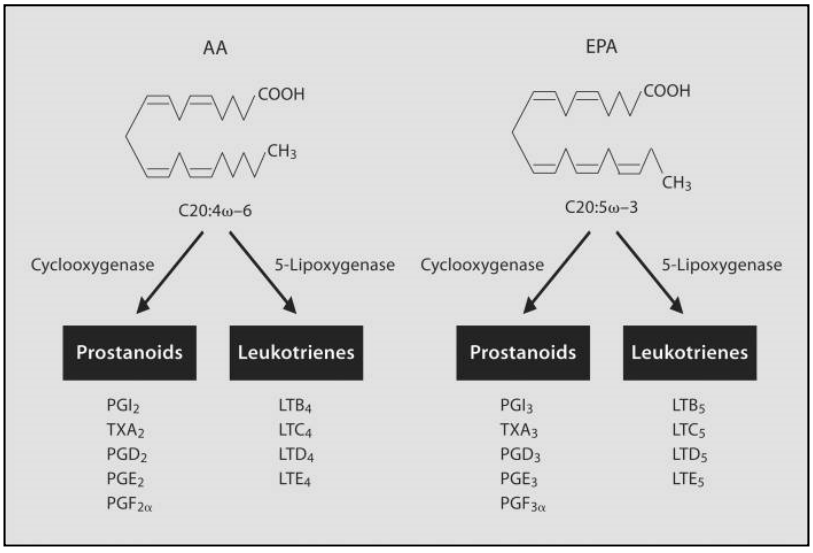

Figure 28: Generation of eicosanoids from AA and EPA. Metabolism of AA and EPA by the COX and 5-LO pathways leads to the 2,4- or 3,5-series prostanoids and LTs, respectively. The 2,4-series eicosanoids produced from AA promote inflammatory and prothrombotic effects, whereas the corresponding 3,5-series eicosanoids produced from EPA have either the opposite effects or possess much lower levels of biological activity. Image from Hooman et al.(2009)

Many of the anti-inflammatory effects of *n*–3 PUFAs appear to be exerted at the level of altered gene expression. However, these effects have been demonstrated only a limited number of times and often in *in vitro* settings, and thus the extent of these effects *in vivo* is not yet clear.

Nevertheless, cell-culture and animal feeding studies indicate potentially very potent effects of *n*–3 PUFA on the expression of a range of inflammatory genes.

The *n*–3 PUFAs effect on inflammatory gene expression seems to happen through direct actions on the intracellular signalling pathways that lead to activation of one or more transcription factors such as NF-κB (nuclear factor κB). Previous studies have shown that *n*–3 PUFAs can down-regulate the activity of the nuclear transcription factor NF-κB. EPA prevented NF-κB activation by TNFα in cultured pancreatic cells, an effect that involved decreased degradation of the inhibitory subunit of NF-κB (IκB), perhaps through decreased phosphorylation. Similarly, EPA or fish oil decrease endotoxin-induced activation of NF-κB in human monocytes. This is associated with decreased IκB phosphorylation, perhaps due to decreased activation of mitogen-activated protein kinases. These observations suggest direct effects of long-chain *n*–3 fatty acids on inflammatory gene expression through the inhibition of activation of NF-κB.

Recently Bordoni et al. (2007) demonstrated in neonatal rat cardiomyocytes that n-3 PUFA down-regulate genes related to cardiac remodeling, apoptosis or inflammation.

II) Membranes composition and gene expression

To understand how PUFAs affect membrane composition and gene expression is important to introduce lipid micro domain called rafts (figure 29 panel a). Rafts are well-studied micro-domain characterized by a "liquid-ordered" phase structure in which sphingomyelin and cholesterol co-localize resulting tightly packed. For this reason they do not integrate well into the fluid phospholipid bilayer, causing them to form micro-domains. This unique environment is suitable for signalling protein localization. Caveolae (figure 29 panel b) are specialized rafts enriched with the structural protein caveolin-1. This specific kind of raft has several functions including cholesterol transport and signal transduction. Several studies showed that hydrolysis of plasma membrane SM by bacterial sphingomyelinase (SMase) C (which degrades it to ceramide) results in a rapid translocation of plasma membrane cholesterol to endoplasmic reticulum (ER).

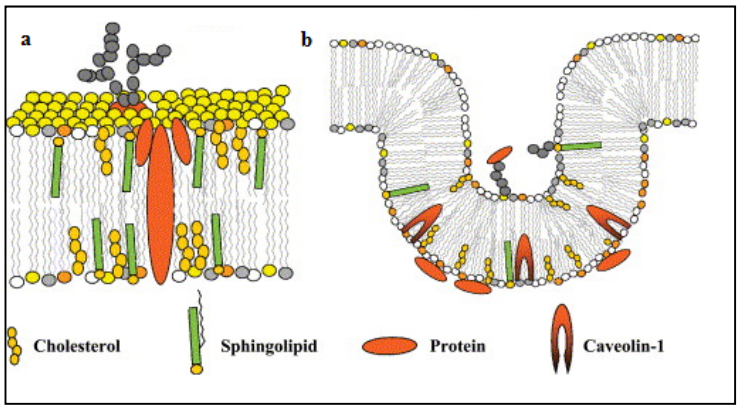

Figure 29: a) Lipid raft enriched with cholesterol and sphingolipids. b) Caveola enriched with caveolin-1, cholesterol and sphingolipid. Image modified from Ma et al.(2004).

N−3 polyunsaturated fatty acids (PUFA) are able to modulate plasma membrane composition and cellular signalling in relation to lipid micro-domains.

In *in vivo* model, Ma et al.(2004) have shown that dietary *n*−3 PUFA markedly alter the lipid composition of colonic caveolae/lipid rafts in mice fed with fish oil. The *n*−3 fatty acids, EPA and DHA, from fish oil were incorporated into the fatty acyl groups of caveolae phospholipids and decreased the caveolar content of cholesterol and caveolin-1, the major structural component of caveolae by ~50%. The effect of diet on caveolar cholesterol content is particularly notable since *n*−3 PUFA, unlike cholesterol-sequestering reagents that have been used to perturb micro-domain integrity in vitro, selectively reduced caveolar cholesterol content without affecting total cellular cholesterol.

The intracellular membrane cholesterol displacement mechanism may involve the ability of fatty acids to cause sphingomyelinase induced sphingomyelin hydrolysis. Sphingomyelin hydrolysis results in the generation of ceramide, which can decrease levels of transcriptionally active mSREBP and SRE-mediated gene transcription. Cholesterol binds to sphingomyelin with higher affinity than to other membrane phospholipids. In the presence of cholesterol acceptors, sphingomyelin hydrolysis forces cholesterol translocation extracellularly. In the absence of acceptors, cholesterol is displaced to intracellular compartments (figure 30).

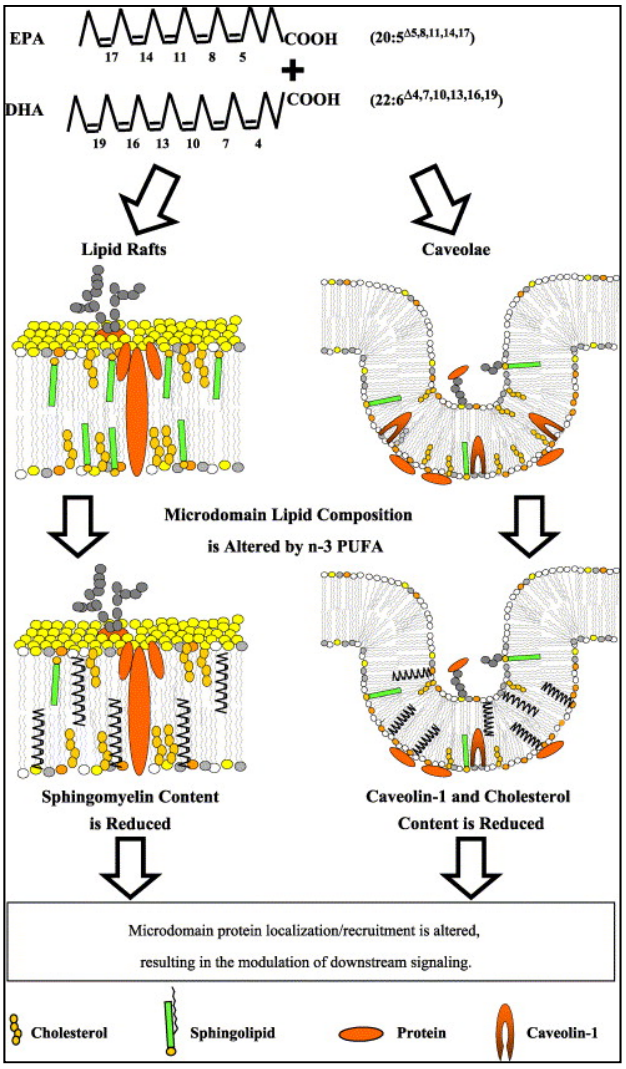

Figura 30: Dietary *n*−3 PUFA readily incorporate into lipid rafts and caveolae. EPA and DHA are acylated to phospholipids within both rafts and caveolae. The presence of highly flexible very long chain unsaturated fatty acids is not compatible with the ordered phase properties of rafts and caveolae. Consequently, the localization of microdomain-specific proteins is perturbed, resulting in the displacement of cholesterol and ceramide that affect the modulation of downstream cellular signaling events. Image from Ma et al.(2004).

It is also well established that ceramide has several signalling effects in cells, including activation of ceramide activated protein kinase, protein kinase C, and protein phosphatases. Moreover, intracellular cholesterol transport has been shown to be regulated by protein phosphorylation, and the activity of the rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase, is regulated by phosphorylation and dephosphorylation. In a recent study, Papasani et al. demonstrated that both exogenous and endogenous ceramide modulate the HMGCR activity

independent of the membrane SM levels. Furthermore, part of the effects of ceramide on the enzyme appear unrelated to the increase in ER cholesterol, indicating an independent effect on sterol homeostasis. Moreover the depletion of SM alone may not be sufficient to account for the substantial translocation of PM cholesterol to ER that occurs following SMase C treatment. Ceramide and SMase effects are additive suggesting that the depletion of SM and the generation of ceramide have independent effects on cholesterol trafficking, probably affecting different aspects of the process. Authors also hypothesize that ceramide increase the phosphorylation of HMGCR, inactivating it.

Ceramide selectively displaces cholesterol from the membrane rafts, possibly by competing for association with other raft lipids. This could result in increased availability of cholesterol for transport to ER, as well as to exogenous acceptors such as apo AI and phospholipids. The sterol regulatory element (SRE)-mediated gene transcription that controls the biosynthesis, cellular uptake and catabolism of cholesterol is inhibited by ceramide, independent of cellular cholesterol levels. In addition, Gallardo et al. reported that ceramide inhibits the expression of HMGCR in human U-937 and HL-60 cells, whereas it increases the expression and activity of the enzyme in HepG2 cells, apparently by inducing maturation of SRE binding protein. The ceramide degradation product sphingosine has also been shown to be an inhibitor of cholesterol esterification by ACAT (by Harmala et al., 1993).

An alternative mechanism to explain PUFA cholesterol lowering effect hypothesizes that cholesterol displaced from rafts could be sensed from SCAP as a high intercellular cholesterol condition, resulting in the retention of SREBP-SCAP complex into PM.

In many models PUFAs suppress SREBP-1 but not SREBP-2 expression. The decline in SREBP-1 accounts for the PUFA-mediated suppression of de novo lipogenesis. The mechanism for the suppression of nSREBP-1 has been attributed to the inhibition of SREBP-1c gene transcription, enhanced $mRNA_{SREBP\text{-}1}$ degradation, and inhibition of SREBP-1 proteolytic processing. Recently Botolino et al. puropsed that PUFA accelerates the nSREBP-1 degradation by a 26S proteasome-dependent pathway while having little impact on microsomal SREBP-1 or nSREBP-2 [1, 2, 8-20].

5.5 PUFA and CVD

PUFAs reduce inflammation acting on eicosanoids production, and they modulate gene expression and membrane composition (§5.4). Other effect ascribed to PUFAs against CVD are the reduction of serum triglyceride levels, anti-arrhythmic effects, decreased platelet aggregation, plaque stabilization, and/or reduction of blood pressure.

I. Reduction of serum triglyceride levels

Inflammatory response seems to be enhanced by the exposure of endothelium to triglyceride-rich lipoproteins. One of the most consistent and best recognized anti-atherogenic properties of n-3 is in the reduction of fasting and postprandial serum triglycerides and free FA (FFA). Such effect occurs by a reduction in the synthesis of triglycerides, and hence the secretion rates of hepatic very low density lipoproteins (VLDL), through the interference with most of the transcription factors that control the expression of enzymes responsible for both triglyceride assembly and FA oxidation. n-3 PUFAs reduce the expression of the sterol regulatory element-binding proteins (SREBP), transcription factors that regulate cholesterol-, FA-, and triglyceride-synthesizing enzymes (§5.4). This effect seems to be due to EPA- and DHA-induced inhibition of liver X receptor alpha/retinoid X receptor alpha (LXRα/RXRα) heterodimer binding to the promoter of the SREBP-1c gene. The increase by n-3 PUFAs of the mitochondrial and peroxisomal FA β-oxidation rates is well documented. This effect is mediated by n-3 FA activation of the peroxisome proliferator-activated receptor (PPAR)α, a ligand-activated transcription factor that up-regulates the expression of acyl-coenzyme A oxidase, the rate-limiting enzyme in the β-oxidation pathway. Finally, a possible effect by n-3 FA on the farnesoid X receptor (FXR) activity was also postulated. FXR is a nuclear receptor for bile acids that plays a central role in lipid homeostasis.

II. Anti-arrhythmic effects:

There are several mechanisms able to explain how n-3 PUFAs may prevent arrhythmias. It is long known that n-3 PUFAs inhibit voltage-gated sodium channels, resulting in a longer relative refractory period and in an increased voltage required for membrane depolarization, which reduces heart rate. Furthermore, n-3 PUSAs maintain the integrity of L-type calcium channels, thus preventing cytosolic calcium overload during periods of ischemic stress. n-3 PUFA may also indirectly contribute to the decrease of heart rate by improving left ventricular efficiency and reducing blood pressure.

III. Decrease of platelet aggregation

PUFA n-6 are precursors for the 2-series eicosanoids (§5.4), which have a wide range of potential effects on metabolic pathways relevant to thrombosis. In fact, although the AA derivative PGI_2 is a potent vasodilator, TXA_2 stimulates platelet aggregation and produces vasoconstriction. 5-LO metabolites, LT, have been linked to inflammation and atherogenesis. EPA and DHA consumption lower tissue levels of AA by inhibiting its synthesis and by taking its place in

membrane phospholipids. EPA-derived 3-series eicosanoids are typically less vaso-constrictive and produce less platelet aggregation than those made from AA. Fish oil could, therefore, be considered as "positive conditioners" of the thromboxane–prostaglandin balance. In general, however, the reduction in platelet function by EPA and DHA, when measurable, is limited in extent.

IV. Plaque stabilization

A recent study by Thies et al. has highlighted a potential role of n-3 FA in decreasing the risk of atherosclerotic plaques rupture in patients awaiting carotid endarterectomy. In this study, plaques from patients taking fish oil featured a net incorporation of n-3 PUFA into plaque lipids, and this correlated with a reduced macrophage infiltration and thicker fibrous caps compared with plaques from patients assuming sunflower oil-enriched control capsules.

In recent years, supportive experimental evidence has indicated that plaque neovascularization and exacerbated release of metalloproteinases (MMP) by the activated endothelium and macrophages play a pathogenetic role in plaque progression and instabilization. Particularly, in the orchestration of angiogenesis, COX-2 activity seems to have a central role. Cell treatment with NS-398, an inhibitor of COX-2 activity, abolished such vascular organization. It is therefore plausible that DHA exerts an anti-angiogenic effect at least in part by reducing stimulated COX-2 expression. Moreover cell treatment with DHA for 48 h before stimulation resulted in a significant inhibition of MMP-9 release, thus potentially having a plaque-stabilizing effect.

V. Reduction of blood pressure

N-3 PUFA seems also to exert antihypertensive effect acting on blood pressure. By a combined analysis of 36 randomized older trials, fish-oil intake (median dose 3.7 g/day EPA plus DHA) has been found to reduce systolic blood pressure by 2.1 mmHg and diastolic blood pressure by 1.6 mmHg. These findings have been corroborated by those obtained in a large international cross-sectional epidemiologic study conducted on 4680 middle age men and women, in which n-3 FA intake was found to be inversely related to blood pressure, both in hypertensive and in non-hypertensive subjects. At least two mechanisms may account for this effect. Firstly, incorporation of EPA and DHA into membrane phospholipids was shown to increase systemic arterial compliance. Secondly, EPA and DHA seem to improve endothelial function by enhancing the release of NO. This effect seems to be due to the lipid and structural modification of caveolae, plasma membrane microdomains that act as regulators of endothelial nitric oxide synthase (eNOS) activity [20-23]

6. Catechins from tea.

6.1 Structure, biosynthesis and occurrence in tea plants.

Catechins belong to the group of compounds generically known as flavonoids, which have a C6-C3-C6 carbon structure and are composed of two aromatic rings, A and B (figure 31).

Tea plant is known to contain seven kinds of major catechins and traces of various other catechin derivatives. Catechins could be divided into two classes:

I) Free catechins:
- (-)-catechin,
- (-)-gallocatechin,
- (-)-epicatechin,
- (-)-epigallocatechin;

II) Esterified or galloyl catechins:
- (-)-epicatechin gallate,
- (-)-epigallocatechin gallate,
- (-)-gallocatechin gallate.

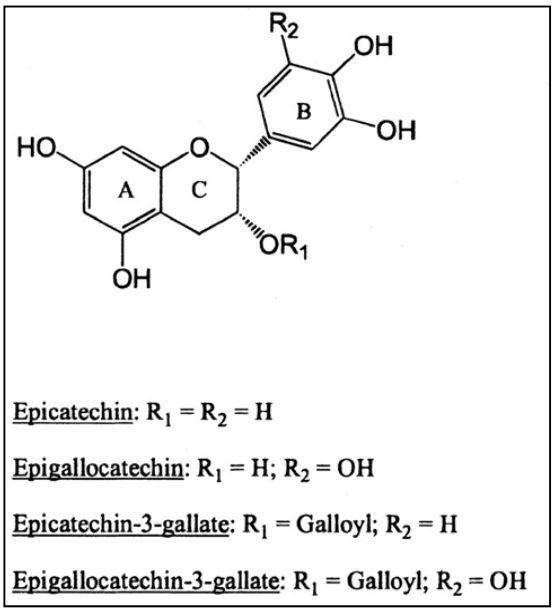

Figure 31: Catechins structure. image from Lee et al. (2004)

These catechins are present in all parts of the tea plant; 15–30% in the tea shoots, and there is also a high content in the second and third leaves[2].

The C6 (A) catechin ring is produced by the acetic–malonic acid pathway and C3-C6 (B) is sensitized by the shikimic–cinnamic acid pathway starting from the glucose pool. When 14C-acetic acid is supplied, metabolism occurs only at the A-ring of quercetin, and when 14C-shikimic acid or 14C-*t*-cinnamic acid is supplied, 14C occurs at the C3-C6 structure. In this way the C6-C3-C6 structure of flavanoid is synthesized with the decarboxylation of three molecules of acetic-malonyl-CoA and cinnamic-coumaroyl-CoA. The first stable C6-C3-C6 compound is confirmed to be chalcone from which catechin is synthesized by way of flavanone. (_) Epigallocatechin is produced by hydroxylation of (_)- epicatechin. Then (_)-epicatechin gallate and (_)-epigallocatechin gallate are synthesized by esterification of catechins with gallic acid (2). It was confirmed that carbons in the *N*-ethyl group of theanine are incorporated into catechin, although this pathway of catechin synthesis seems to be a minor one.

[2] In August, when the sun's rays are the strongest, catechin content is the highest.

The production of catechins in the tea plant increases on exposure to light and decreases in the shade. These phenomena are related to the activity of phenylalanine-ammonialyase, which is a key enzyme in the biosynthesis of catechin B ring. When the tea plant is covered (blocking out light), this enzyme activity decreases rapidly. The biosynthesis of catechin is also increased by a rise in temperature. Once catechin is synthesized, it is stored in the vacuole of the cell and hardly undergoes any metabolism or decomposition.

According to the variety of tea plant, the catechin content varies. Green tea is rich in polyphenols, about 70% of which are catechins, and is the main dietary source of catechin gallatesand gallocatechins. In comparison, black tea contains about 30% of the catechins found in green tea and is mainly composed of complex condensation products such as thearubigins and theaflavins. Other dietary sources of catechins include red wine, grapes, apples and chocolate.

EGCG is one of the most abundant green tea catechins, with a cup of green tea containing 20–200 mg EGCG. It has also been suggested to be one of the most active compound in green tea, mediating most of the biological effects and accounting for 32% of the antioxidant potential. The remaining catechins include: (2)-epicatechin ((2)-EC), (2)-epigallocatechin (EGC) and epicatechin-3-gallate (ECG), which provide 5–7, 9–12 and 9–12% of the antioxidant potential of green tea, respectively. Catechins are thought to be primarily responsible for the beneficial effects of green tea. [24, 25]

6.2 Bioavailability and adsorption

After oral administration green tea catechins are absorbed intestinally, predominately in the jejunum and ileum, with peak absorption occurring at 1.5–2.5 h after consumption. EGCG and ECG are thought to be mainly transported across the intestinal epithelial cell layer via paracellular diffusion. However, bioavailability is low due to instability under digestive conditions, poor absorption, and rapid metabolism and excretion, leading to only about 5% of consumed catechins appearing in the plasma. EGCG is the only known polyphenol to be mainly in the free form (77–90%) in the plasma, whereas EGC and EC are mostly in the conjugated form (31 and 21% in the free form, respectively). The extensive metabolism in the intestine and liver leads to the formation of glucoronides, sulfides and methylated metabolites. This biotransformation alters the chemico-physical properties of the catechins, such as the mass, charge and polarity, aiding the deactivation and rapid excretion of the biologically active compounds.

Green tea catechins are methylated by the enzyme catechol-O-methyltransferase, glucuronidated by UDP-glucuronosyltransferases (UGT) and sulfadated by sulfotransferases (SULT).

Glucuronidation and sulfation can occur to previously methylated EGCG and vice versa, forming mixed metabolites.

Green tea catechins have also been shown to undergo microbial degradation in the colon, forming compounds such as valerolactones, and phenolic and benzoic acids which may be absorbed or passed out in the feces. In comparison with the methylated, glucuronidated and sulfadated metabolites mentioned previously, studies have shown the valerolactones to appear in the blood and urine considerably later.

The major products of EGCG metabolism are 4^{II}-O-methyl-(-)-EGCG and 4^{I},4^{II}-O-dimethyl-(-)-EGCG (at high or low concentrations of EGCG, respectively), (-)-5-(3^{I},4^{I},5^{I} -trihydroxyphenyl)-γ-valerolactone and (-)-5-(3^{I},4^{I} -dihydroxyphenyl)-γ-valerolactone and the respective sulfates and glucuronides. Also EGC and EC are metabolized in several products.

In most cases, the biological activity of green tea catechin metabolites is limited compared with the parent form; however, a small selection of metabolites have been shown to have similar or in some cases higher activity than the parent compound. Lu et al. (2003) found EGCG-3^{I}-glucuronide and EGCG-3^{II}-glucuronide to have similar radical-scavenging ability compared with free EGCG. Exposure of human aortic endothelial cells to catechin metabolites extracted from the plasma of (+)-catechin-administered rats was found to inhibit monocytes adhesion when cells were pretreated with the cytokine IL-1b. The unconjugated form, (+)-catechin, had no such effect. [25-27]

6.3 Mechanism of action

Epidemiological evidence suggests that tea consumption lowers the risk of CVD. This protective effect is probably due to multiple mechanisms of action which are ascribed mainly to epigallocatechin gallate (EGCG), the main catechin in tea.

EGCG is a potent inhibitor of lipid absorption, due to the formation of complexes with lipids and lipolytic enzymes which interfere with the luminal process of emulsification, hydrolysis, micellar solubilization, and subsequent uptake of lipids. EGCG intake is associated with lower plasma cholesterol level. Bursill et al (2006) demonstrated that this catechin regulates cholesterol metabolism in HepG2 cells via modulation of the conversion of sterol regulatory element binding protein-1 (SREBP-1) to its active form, and the increase of LDL receptor binding activity. In a study performed on animal models, green tea feeding reduced cholesterol and triglyceride plasma levels by 28 and 48%, respectively, and increased HDL cholesterol by 88%.

EGCG and other catechins are also potent antioxidants. Pearson et al. (1998) demonstrated with human LDL and human aortic endothelial cells (ex vivo study) a 3.9% inhibition of LDL oxidation after 12 h incubation with 0.08 μg/ml green tea extract, and a 98% inhibition after incubation with

5 μg/ml. These concentrations are physiologically relevant and are achievable in plasma by normal dietary consumption of tea.

EGCG is also reported to be a powerful anti-inflammatory agent. It directly inhibits neutrophil adhesion and suppresses chemokine production in the inflammatory site. Moreover EGCG inhibits the activity of the transcription factor NF-kB, so reducing the expression of target genes indispensable to the inflammatory process [28-33].

6.4 Green tea and cardiovascular diseases

Cardiovascular diseases (CVDs) are caused by many endogenous or exogenous factors such as cigarette smoking, diabetes mellitus, hypertension and hyperlipidemia. These factors can trigger an inflammatory response in vessels, the establishment of this inflammatory status, mediated in part by ROS, results in damage to vascular endothelial and smooth muscle cells. Endothelial dysfunction, characterized by pathological changes in endothelial cell anticoagulant, anti-inflammatory and vasorelaxation properties, promotes recruitment of monocytes, macrophages, growth factors and cellular hypertrophy, which contribute to the formation of atherosclerotic plaques. ROS activity helps to drive many of these processes, but also altering transcription and signal transduction. Some important effects observed are: up-regulation of leukocyte adhesion molecules, increased apoptosis of endothelial cells, activation of matrix metalloproteinases, and altered vasomotor activity. These events mediate recruitment of inflammatory cells. Additionally, ROS level influences the accumulation and hypertrophy of vascular smooth muscle cells (VSMC) observed in atherosclerotic, restenotic lesions and hypertensive vascular diseases. ROS can also suppress growth and/or induce apoptosis.

The correlation between tea consumption and cardiovascular health has been extensively investigated. Experimental and clinical data underline the evidence that green tea consumption lowers the risk of cardiovascular diseases and mortality, and also cohort studies generally indicate the beneficial effects of green tea on CVDs mortality. These protective effects are probably due to multiple mechanisms of action of tea catechins.

EGCG is a potent inhibitor of lipid absorption. This catechin forms complexes with lipids and lipolytic enzymes, thereby interfering with the luminal process of emulsification, hydrolysis, micellar solubilization, and subsequent uptake of lipids. In a study performed on animals, green tea feeding reduced cholesterol and triglyceride plasma levels by 28 and 48%, respectively, and increased HDL cholesterol by 88%. These effects on lipid profile lower cardiovascular risk.

LDL oxidation is inhibited by 3.9% after 12 h incubation with 0.08 ppm green tea extract, and by 98% after incubation with 5 ppm green tea extract. Interestingly, these concentrations,

particularly the first one, represent doses potentially achievable by natural intake. Two clinical trials substantiate these findings.

Another important aspect that characterize CVDs is atherogenesis, process dramatically influenced by chronic inflammation of the vessel wall, activation of the vascular endothelium, increased adhesion of mononuclear cells to the injured endothelial layer and their subsequent extravasation into the vessel wall. EGCG directly inhibits neutrophil adhesion and also suppresses chemokine production in the inflammatory site. Furthermore, some evidences indicate that EGCG can modulate adhesion-molecule expression. In addition, EGCG and ECG strongly induce apoptosis of monocytes, a cell type largely involved in atherogenesis, by dose-dependent activation of caspase 8 and 9, and further downstream caspase 3. EGCG also inhibits the activity of the transcription factor NF-kB, reducing the expression of target genes indispensable to the inflammatory process.

In *in vitro* studies, catechins induced endothelium-dependent vasorelaxation via nitric oxide release from the endothelium. In other works emerged that green tea catechins directly enhances endothelial nitric oxide synthase (eNOS) activity by rapid phosphorylation and activation through phosphatidylinositol 3-kinase-dependent, cyclic adenosine monophosphate (cAMP)-dependent protein kinase, and Akt-dependent pathway. Nitric oxide (NO) is a reactive specie but is also the endothelial-derived relaxing factor which modulates vasodilatation essential for CV health. It has been demonstrated that under patho-physiological conditions such as hypoxia with subsequent reperfusion, when the circumstances allow the formation of substantial amounts of reactive oxygen species and NO, these molecules react avidly and RNS are generated, causing damage to cellular components. A recent study showed that the supplementation of moderate concentration of a green tea extract to the medium of cultured cardiomyocytes just before the induction of hypoxia and subsequent reoxygenation is able to prevent the increase of inducible NO synthase expression. This could reduce NO overproduction, allowing to hypothesize that GT components act not only on reactive oxygen species via an antioxidant mechanism, but also regulating NO synthesis.

It must be considered that the bioavailability of potential antioxidants from plant foods is generally too low to have any substantial direct effect on reactive oxygen species. It has been postulated that the mechanism of action of polyphenols, even in very low concentrations, might entail their interaction with cell signaling and might influence gene expression, with the consequent modulation of several cell activities. The study by Danesi et al (2008) evidenced that a moderate GT concentration supplemented to cultured rat cardiomyocytes selectively activates the PPAR-β isoform.

Another issue involving catechins is their antithrombotic properties. Moreno et al (2004) in a humans study showed that the density of microvessels was increased in ruptured plaques and in

lesions with macrophage infiltration. The plaque base microvessel density appeared to be an independent marker for plaque rupture. Endothelial cells produce essential factors for angiogenesis. Tea compounds have been demonstrated to effectively impair growth factor signalling pathways and inhibit receptor tyrosine kinase activities, thereby affecting angiogenic processes in endothelial cells.

[28, 29, 31-37]

7. Phytosterols

7.1 Structure

Phytosterols are plant-derived sterols that are structurally similar and functionally analogous to cholesterol in vertebrate animals. The chemical structure of common phytosterols is shown in figure 32. In many foods sitosterol is the most abundant form.

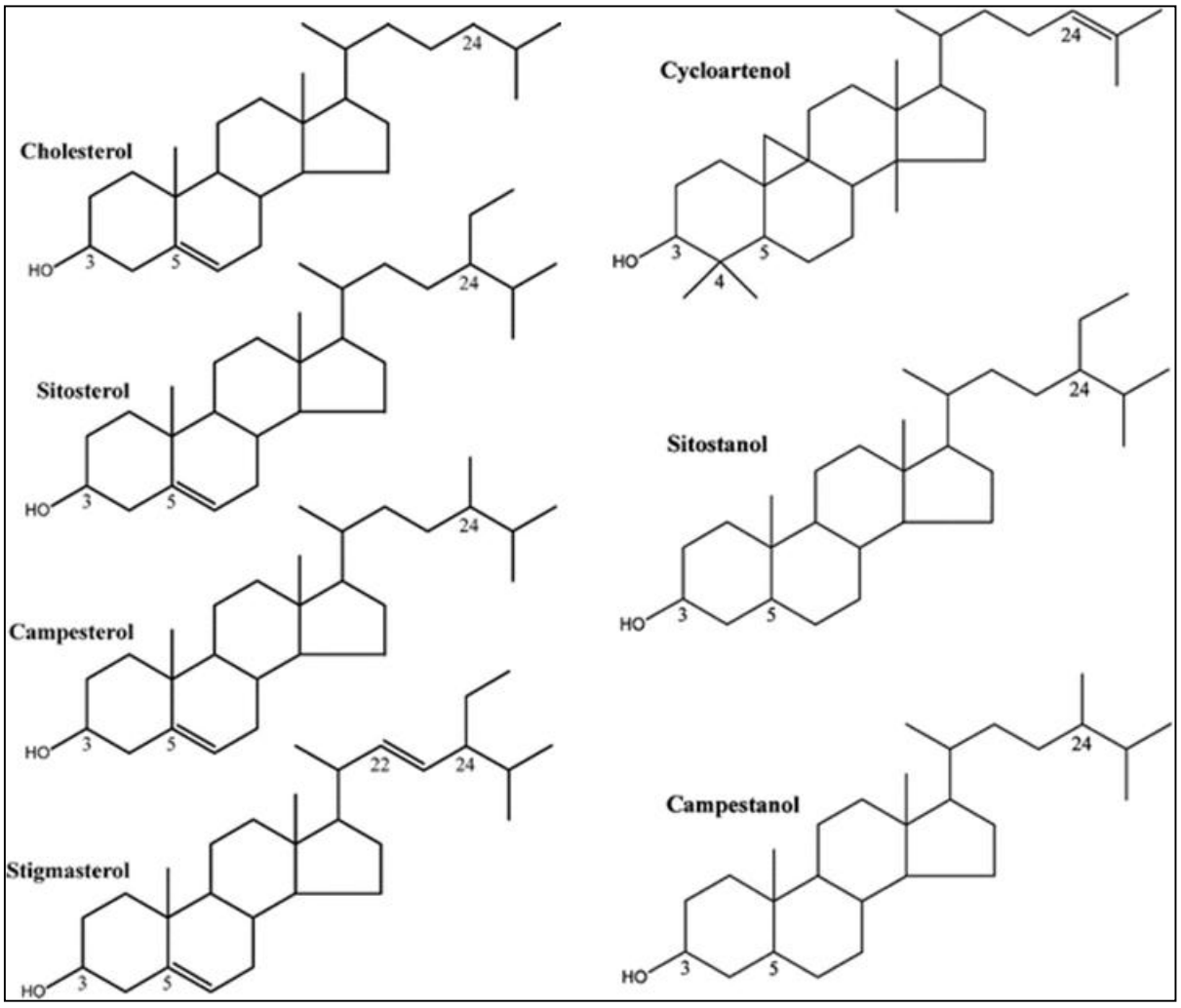

Figure 32: Main phytosterols structure. Image from Ostlund et al (2002).

Phytosterols are divided into two categories:

I) Sterols or Δ^5-sterols, possessing a double bond at position 5

II) Stanols, with a 5α-reduction of that double bond

These classes are reflected in the common names of the compounds. For example, sitosterol is structurally identical to sitostanol except for the double bond at position 5, and campesterol bears the same relationship to campestanol. Reduction of the double bond to give a β-hydrogen at

position 5 is a common intestinal bacterial degradation product of cholesterol and plant sterols but is not generally found in foods. [38]

7.2 Biosynthesis and metabolism

Plants and animals sterol biosynthesis and metabolism are quite different. Cholesterol is synthesized from acetate through the straight-chain compound squalene and then cyclized into many intermediate precursor sterols (§ 1.2.2), but the final product is most often nearly pure cholesterol, and only trace amounts of related precursors are found in humans. In plant phytosterols are also synthesized from acetate subunits through squalene, but the first cyclized product after squalene is the cycloarterol (compound unique of plant). Some foods, such as rice, contain cycloartenol or related compounds in significant amounts. Plants then produce a variety of final sterol products, and each species has a characteristic distribution of sterols[3]. Moreover there are modifications to the sterol nucleus at the level of the side chain.

In animals most of the total body cholesterol is free, with a relatively small aggregate amount present as long-chain fatty acyl esters in plasma lipoproteins and specialized cells. Plants have a more diverse variety of derivatives at the 3-position.

I) Long-chain fatty acyl esters[4]

II) Ferrulate esters.

III) Glycosylate derivatives.

The complexity of phytosterols is increased further by steric asymmetry. Addition of alkyl groups in the side chain results in the substituted carbon becoming asymmetric. For example, soybeans contain two 24-methylcholesterol isomers, campesterol (24-α-configuration) and dihydrobrassicasterol (24-β-configuration). Generally the α-configuration is more common in higher plants, whereas the β-configuration predominates in algae. Stereochemistry has not generally been considered in human and animal studies of phytosterols.

In foods three compounds account for most of the phytosterol mass:

I) sitosterol (24-ethylcholesterol)

II) campesterol (24-methylcholesterol)

III) stigmasterol (Δ^{22}-24-ethylcholesterol).

[3] This characteristic distribution of sterols is sometimes used as a chemical fingerprint for the identification of food product sources

[4] Present in most plants and constitute over 50% of the total sterol in foods such as corn oil.

The structural similarity to cholesterol is striking, and it is difficult to separate cholesterol from phytosterols or different phytosterols from each other by physical methods unless powerful techniques such as high pressure liquid chromatography [38].

7.3 Occurrence in food

All vegetable foods contain appreciable quantities of phytosterols. The most important natural sources of plant sterols in the human diet are oils and margarines, although they are also found in a range of seeds, legumes, vegetables and unrefined vegetable. Cereal products are a significant source of plant sterols, their contents, expressed on a fresh weight basis, being higher than in vegetables.

Current food databases do not have comprehensive estimation of phytosterols content. Thus, they cannot be routinely calculated in test diets.

Since the most concentrated source of phytosterols is vegetable oil, a person consuming 30 g/die of corn oil would receive 286 mg of phytosterols, an amount that has been shown to be active in reducing cholesterol absorption. An exception is palm oil, which is deficient in phytosterols after refining for western markets. Smaller unit amounts are found in nuts, breads, and whole vegetables, but these items also have larger portion sizes. Except for highly refined carbohydrates and animal products, nearly all foods contribute appreciably to phytosterols intake. The daily phytosterol consumption range is estimated to be in the range 167-437 mg/die, approximately the same as cholesterol intake.

Stanols are present in un-hydrogenated vegetable oils and cereals. An estimate of stanol consumption can be obtained from fecal analyses because bacterial conversion of Δ^5-sterols in the large bowel results in 5β-reduced rather than 5α-reduced metabolites. In subjects not receiving stanol treatment, stanol excretion has been reported to be ~24–29 mg/die. This suggests that stanols may comprise about 10% of dietary phytosterol intake. [38, 39]

7.4 Adsorption

Cholesterol absorption in normal subjects is in the range of 56.2±12.1%. Phytosterols traditionally have been considered to be non absorbable. In humans consuming solid food diets more than 90% of sitosterol is recovered in the stool, and sitosterol has been used as a non absorbable recovery standard in measurements of cholesterol balance and absorption. Despite this a small but definite absorption occurs. Several studies using radioactive sitosterol have reported that absorption is 0.6–7.5% of the administered amount. Percentage absorption varies inversely with the

dose, suggesting that a limitation in absorption exists at high intake levels. Campesterol absorption has been measured at 9.6% by losses during intestinal intubation and 16% from stool losses determined by mass spectrometry. Campestanol has been reported to be 12.5% absorbed by losses during intubation, and 5.5% absorbed by radioactive assay of plasma. The most desirable conditions for measuring phytosterol absorption involve the use of serum samples to establish unequivocal absorption into the systemic circulation. Recent works using these methods report the following generally lower values for percent absorption: sitosterol, 0.51%; campesterol, 1.9%; sitostanol, 0.04; and campestanol, 0.16%. Taken together, these studies show that phytosterol absorption is quite low. Particularly, 5α-stanols are absorbed with about 10% of the efficiency of the corresponding Δ5-sterols.

In humans serum, levels of sitosterol and campesterol are only 0.1–0.14% of the cholesterol concentration. When dietary Δ5-sterols are supplemented at 2–3 g/day, serum sitosterol and campesterol levels increase by 34–73%, with most values remaining in the normal range. The same dose of stanols reduces serum Δ5-sterol levels by 17–36%, presumably by inhibiting absorption. Baseline serum stanols were difficult to measure, with levels estimated at 0.003–0.004% of cholesterol.

The same as cholesterol, phytosterols must be solubilized in micellar form for absorption. The sterol-laden micelle interacts with the intestinal brush border thereby facilitating the uptake of sterols by enterocytes. The precise molecular mechanisms for sterol absorption are not well defined but cholesterol and phytosterols absorption both require the Niemann-Pick C1 Like 1 Protein (NPC1L1). NPC1L1-deficient transgenic mice have virtually undetectable plasma phytosterols concentrations suggesting that NPC1L1 is the primary pathway for their absorption. Within the enterocyte, cholesterol is esterified by acyl cholesterol acyl transferase (ACAT2), packaged into chylomicrons at the basolateral membrane, and secreted into the lymphatic system for portage to the left subclavian vein. The intestine can secrete cholesterol back into the lumen of the intestine: unesterified cholesterol and phytosterols are transported back into the intestinal lumen by a specific pump called the “sterolin pump” containing the ATP Binding Cassette (ABC) proteins, ABCG5 and ABCG8. Thus, ABCG5 and ABCG8 may selectively ‘pump’ sterols out of the intestinal cells as a first-line defense to dietary input, but in the fasting state, the liver can continually pump sterols (cholesterol and non cholesterol sterols) into bile and thus maintain a low non cholesterol sterol level in the body. In the fasting state, the intestine may also be able to reduce whole body sterol pools by continuing to pump sterols (presumably delivered to the intestinal enterocytes via the high-density lipoprotein pathway) .

Defective ABCG5 or ABCG8 genes generate the pathological condition of phytosterolemia[5]. Since ABCG5 and G8 not only regulate sterol absorption, but are also responsible for biliary excretion of phytosterols; consequently, the total body phytosterol pool in phytosterolemia is increased because of both increased absorption and decreased excretion [38, 40-42].

7.5 Safety of phytosterols supplementation

A high intake of phytosterol could reduce not only cholesterol, but also fat-soluble vitamins and other vegetable-derived compounds adsorption. since these lipophilic materials are carried in plasma lipoproteins, most studies have normalized their plasma levels to those of lipids or cholesterol. In this way, in some studies serum α- and β-carotene level has been reported to be reduced by 11–22%, while other studies found only a small reduction without statistical significance. However, a correlation has been noted between estimated phytosterol-induced reduction in cholesterol absorption and decrease of circulating carotenes and lycopene. The most affected compounds are the non polar hydrocarbons α-carotene, β-carotene, and lycopene. Serum concentrations of 25-hydroxyvitamin D, retinol, and vitamin K are not reduced.

One potential safety concern is the rare inherited disorder phytosterolemia. Phytosterolaemia or sitosterolaemia is a rare autosomal recessive condition caused by mutations on the ABCG5 and ABCG8 gut transporter proteins. This leads to accumulation of phytosterols in blood and tissues. Although increased absorption of phytosterols occurs in this illness and is an important clinical marker, it is possible that abnormal handling of cholesterol contributes most strongly to the resulting atherosclerosis. It is not clear what effect phytosterol feeding might have on sitosterolemic subjects [38, 43].

[5] Phytosterolemia is an autosomal recessive disease that occurs 1 in 5 million people.

8. Probiotics

8.1 Colonic microbiota

The human large intestine can be described as a complex microbial ecosystem where the community is in the range of 10^3 organisms, and in its diversity count over 400 different species. The behavior of colonies bacteria are affected by the physiology and architecture of the hindgut. The bacteria present have fluctuating activities in response to substrate availability, redox potential, pH, O_2 tension and colon distribution.

During birth, the gastrointestinal tract of the newborn is inoculated by the mother's vaginal and fecal flora. Initially a predominance of facultative anaerobic strains such as Escherichia coli or enterococci exists. These bacteria may therefore create an highly reduced environment that then allows the growth of strict anaerobes. After weaning a pattern that looks like the adult flora becomes to be established. Most human large intestinal microorganisms have a strictly anaerobic metabolism, and numbers of facultative anaerobes are many orders of magnitude lower than those of the obligate anaerobes. Because the native gut microflora are so well adapted to their environment, it is difficult for other organisms (including pathogens) to colonize in the lumen. This creates colonization resistance or non-specific disease resistance in the gut which acts as an important line of defense against pathogens. However disturbances in the normal intestinal microbial community structure can result in the proliferation of pathogens.

In this huge amount of bacteria, the numerically predominant anaerobes were Gram-negative rods of the genus Bacteroides. These microorganisms can constitute to 30% of the total fecal flora and as such can have an important impact on colony microbiological processes. Other numerically predominant groups are bifidobacteria (Gram-positive rods), eubacteria (Gram-positive rods), clostridia (Gram-positive rods), lactobacilli (Gram-positive rods) and Gram-positive. Other groups exist in lower proportions, including enterococci, coliforms, methanogens and dissimilatory sulfate-reducing bacteria.

Considering this wide range of bacterial species residing in the large gut, as well as the different growth substrates available, the colony microbial ecosystem harbors a multiplicity of nutritional patterns. A variety of different metabolic niches, bacterial habitats and interrelationships have arisen. The gut microflora exerts a considerable influence on host biochemistry including:

- enzymatic activity of intestinal contents
- short chain fatty acid (SCFA) production in the lumen
- oxidation-reduction potential of luminal contents

- host physiology
- host immunology
- Modification of host-synthesized molecules

In general, intestinal bacteria may be divided into species that exert either harmful or beneficial effects on the host (figure 33). Pathogenic effects include diarrhea, infections, liver

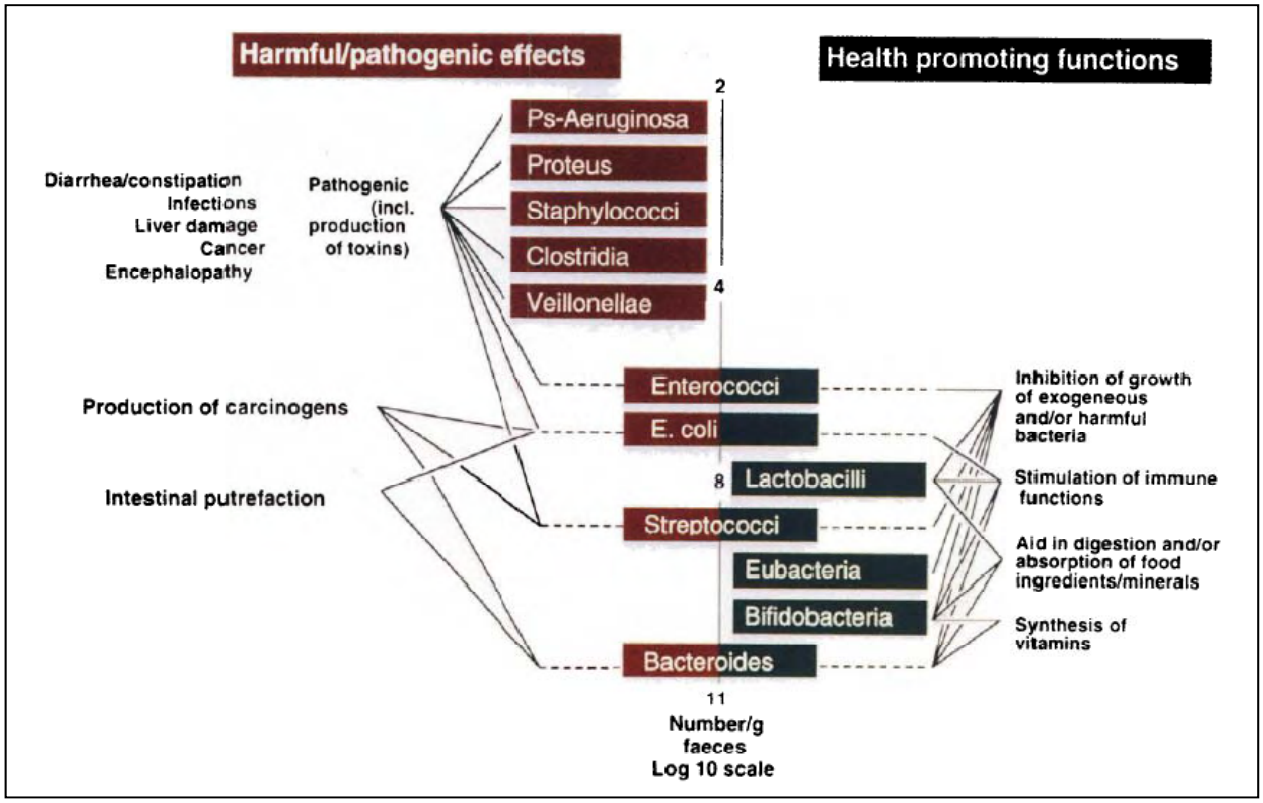

Figure 33: Scheme of the composition and health effects of predominant human fecal bacteria.

The bacteria are split into those groups that have harmful or pathogenic influences on human health, and health promoting function are mentioned. Image from Gibson et al. 1994

damage, carcinogenesis and intestinal putrefaction; health-promoting effects may be caused by the inhibition of growth of harmful bacteria, stimulation of immune functions, lowering of gas distention problems, improved digestion and absorption of essential nutrients, and synthesis of vitamins. [44, 45]

8.2 Probiotics

R. Fuller in 1989 [46] gave the definition that well represent probiotics: “probiotics are live microbial food supplements, which benefit the health of consumers by maintaining, or improving their intestinal microbial balance”. In parallel the development of such compounds that are able to improve probiotic life, prebiotics, is fundamental. Gibson and Roberfroid in 1995 [44] defined prebiotic as “non-digestible food ingredient that beneficially affects the host by selectively

stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" This non-viable food component moves to the colon and has a selective fermentation. Food ingredients classified as prebiotics must not be hydrolyzed or absorbed in the upper gastro intestinal tract, need to be a selective substrate for one or a limited number of colonic bacteria, must alter the microbiota in the colon to a healthier composition and should induce luminal or systematic effects that are beneficial to host health.

A number of beneficial roles for probiotic strains have been reported or theorized. These benefits include:

- Re-establishment of balanced intestinal microflora
- Improving colonization resistance and/or prevention of diarrhea
- Systemic reduction of serum cholesterol
- Reduction of fecal enzymes, potential mutagens which may induce tumors
- Metabolism of lactose and reduction of lactose intolerance
- Enhancement of immune system response
- Improved calcium absorption
- Synthesis of vitamins and pre-digestion of proteins

Due to their perceived health benefits probiotic bacteria have been increasingly included in food such as yoghurts and fermented milks during the past two decades. Most commonly they have been lactobacilli such as Lactobacillus acidophilus, and bifid bacteria.

Before a probiotic can benefit human health it must accomplish several criteria:

- It must have good technological properties to be manufactured and incorporated into food products without loose viability and functionality or creating unpleasant flavors or textures.
- It must survive passage through the upper gastrointestinal tract and arrive alive at its site of action.
- It must be able to function in the gut environment.
- It must establish the effect of the probiotic strain on the intestinal microbiota and on the host. This includes not only positive health benefits, but also demonstration that probiotic strains do not have any deleterious effects.

[44-49]

8.3 Cholesterol lowering effect of probiotics in human

There is a large number of study reporting probiotic effects on serum cholesterol level in humans. It has been observed that whole-fat milk and skim milk consumption reduce serum cholesterol, while the same amount of milk fat, as butter intake, increase it. Yoghurt has also been shown to decrease serum cholesterol levels in humans, but several studies designed to evaluate the potential reduction of its levels by the consumption of certain cultured dairy products have given variable data and no solid conclusions. In most of these studies a decrease in serum cholesterol was only observed during the consumption of very high doses of fermented dairy products. Other investigators using more 'normal' doses of the fermented milk product failed to confirm such findings. It was suggested that the contradictory results obtained could be, at least in part, related to experimental design. Some of the factors addressed were lack of statistical power, use of inadequate sample sizes, failure to control nutrient intake and energy expenditure during the experiments and variations in the baseline levels of blood lipids. The intra-individual variation in blood cholesterol over a few months ranged from 5 to 15%. Moreover differences in experimental design (type and quantity of the fermented milk product; age and sex distribution, and starting plasma cholesterol levels of the subjects studied; and length of study period) make direct comparisons difficult. Furthermore, more recent studies underline different effects that are ascribable to different probiotic used in fermented products.

Agerbaek et al. in 1995 [50] reported that a fermented milk product (Gaio®), that is produced through the action of a bacterial culture containing a strain of *Enterococcus faecium* and two strains of *Streptococcus thermophilus* (CAUSIDO® culture), was effective in reducing plasma cholesterol at relatively modest levels of intake. The bacterial strains contained in this product were isolated from the intestinal flora of inhabitants of Abkhasia (Caucasus), a region reputed for the longevity of its people and where fermented milk is a major part of the diet. Richelsen et al. [51] , using the same product in a six month trial, initially observed similar resultsIn the follow up, , concentrations of *Enterococcus faecium* in the test product dropped, and LDL-cholesterol level of treated subjects was similar to placebo. For this reason it was suggested that *Enterococcus faecium* plays a significant role in the potential beneficial effect of this fermented milk product.

Larsen et al in 2000 [52] evaluated the effects of the Gaio® product and two alternative products on the serum cholesterol levels of overweight and obese subjects. Five groups were randomly formed. One of the groups consumed the test Gaio®; two groups consumed two new yoghurts with different bacterial cultures (one was fermented with two strains of *S. thermophilus* and two strains of *L. acidophilus*, and the other with two strains of *S. thermophilus* and one strain of *L. rhamnosus*); the fourth group consumed placebo yoghurt. The last group was given two placebo tablets daily. When comparing all five treatment groups, a significant decrease in LDL-cholesterol was reported in the Gaio® product group only (8.4%,$P<0.05$). The reason for the observed hypocholesterolemic effect was once again

proposed to be related to the CAUSIDO® bacterial culture, particularly *E. faecium.* Although the specific *in vivo* action of the strain remains to be investigated.

Rossi et al. [53] tested the ability of *E. faecium*, *L. acidophilus*, *L. jugurti*, *S. thermophilus,* and *L. delbrueckii* to decrease cholesterol *in vitro* and to grow in the presence of bile salts. The study showed that among the strains tested, *E. faecium* and the mixture of *E. faecium* plus *L. acidophilus* produced the greater cholesterol reduction in the medium after 24 h anaerobic incubation (53 and 65%, respectively).

De Roos et al. [54], in a randomized, placebo-controlled parallel trial, attempted to evaluate whether the intake of *Lactobacillus acidophilus* strain L-1 lowered serum cholesterol levels in healthy men and women with normal to borderline high-cholesterol levels. The 78 subjects in this study consumed 500 ml of control yoghurt daily for 2 weeks. They were then randomly allocated to receive 500 ml per day of either control yoghurt or yoghurt enriched with *Lactobacillus acidophilus* L-1 for another 6 weeks. No significant reduction of serum cholesterol levels in the subjects consuming the yoghurt enriched with *L. acidophilus* L-1 was observed after the trial.

Schaarmann et al., 2001[55] aimed studying the interrelationship between the intake of probiotic yoghurt and the concentration of cholesterol fractions. In this study, 29 healthy women consumed 300 g per day of a probiotic yoghurt (*Lactobacillus acidophilus* and *Bifidobacterium* longum) after a period of eating standard yoghurt (*Streptococcus thermophilus* and *Lactobacillus lactis*). The volunteers were divided in a normocholesterolemic group (total cholesterol <250 mg/dL) and a hypercholesterolemic group (total cholesterol >250 mg/ dL). The experiment consisted of three periods (placebo, standard yoghurt, and probiotic yoghurt), each lasting for 51 days. A decrease in the concentration of LDL-cholesterol and triacylglycerides after consumption of standard and probiotic yoghurts was reported. A larger decrease was observed after intake of the probiotic yoghurt when compared with the standard yoghurt in the hypercholesterolemic group, but this, however, was not significant. Inversely, a increase in the HDL cholesterol was observed with the probiotic yoghurt that resulted in a smaller atherogenic ratio (LDL/ HDL-cholesterol). No significant differences were observed when comparing the normocholesterolemic and the hypercholesterolemic groups.[50-56]

8.4 Mechanism of action

Existing evidence from human and animal studies suggests a moderate cholesterol lowering action of some fermented dairy products. However, the potential mechanisms for this claimed effect remain to be clarified.

Several *in vitro* experiments with lactobacilli and bifidobacteria evidenced the ability of some strains to assimilate cholesterol in the presence of bile acids. Gilliland et al [57] in an *in vitro* study found that certain *Lactobacillus acidophilus* strains could remove cholesterol from a growth

medium only in the presence of bile and under anaerobic conditions. Because these conditions are expected to occur in the intestine, the authors concluded that this should enable the organisms to assimilate at least part of the cholesterol ingested in the diet, thus making it unavailable for absorption into the blood.

To corroborate *in vivo* this *in vitro* findings, the same authors tested *L. acidophilus* RP32 using young pigs[6]. Feeding of pigs with *L. acidophilus* RP32, selected for its ability to grow in the presence of bile and to remove cholesterol from a laboratory medium, significantly inhibited the increase in serum cholesterol when fed a high-lipid diet ($P<0.05$). The mechanism underlying this effect was referred to as cholesterol assimilation by the *L. acidophilus* strain. Other studies reported that *L. acidophilus* possesses a significantly greater cholesterol uptake ability than *Streptococcus thermophilus*. However, variations in the ability of cultures of yoghurt bacteria and bifidobacteria to assimilate cholesterol were observed. It has been suggested that the diverse results obtained in different human studies can be, at least in part, be due to the different bacterial strains used in fermentation. Viability of the ingested bacterial strains in the human gut, and the ability to colonize the small intestine (where most of the cholesterol absorption would take place), could ultimately be expected to be of key importance for this effect.

Subsequent work done by Klaver and van der Meer (1993) [58] considered the mechanism of the proposed assimilation of cholesterol by *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Authors concluded that removal of cholesterol from the culture medium by *L. acidophilus* RP32 and other species was not due to bacterial uptake of cholesterol, but rather could relate to co-precipitation with de-conjugated bile salts in an acidic environment. De-conjugated bile acids are less soluble and less likely to be absorbed from the intestinal lumen than conjugated bile salts. Thus, de-conjugation of bile acids in the small intestine could result in a greater excretion of bile acids from the intestinal tract, especially as free bile acids are excreted more rapidly than their conjugated forms. Increased excretion of bile acids should result in lowered serum concentrations, which in turn would decrease the amount of bile acids reaching the liver for secretion back into the intestine through enterohepatic circulation. To replace the excreted bile acids, more would have to be synthesized from cholesterol in the liver. Thus, it has been suggested that in a steady state situation, de-conjugation of bile acids could lead to the reduction of serum cholesterol by increasing the formation of new bile acids or by reducing the absorption of cholesterol throughout the intestinal lumen. However, it remains unclear whether microbial bile de-conjugation properties are related to the hypocholesterolemic effects observed *in vivo,* because pH in the intestinal tract of humans is usually neutral to alkaline.

[6] Pigs were used because their digestive system, distribution of coronary arteries, and atherosclerotic tendencies resemble those of humans.

Tahri et al. [59, 60] studied the hypothesis of the proposed assimilation or co-precipitation of cholesterol by *Bifidobacterium* species. They observed the existence of an intense binding between cell surface and cholesterol, which was considered as an uptake of cholesterol into the cells. This assimilation was dependent on cell growth and the presence of bile salts. They concluded that the observed removal of cholesterol from the broth could be attributed not only to the co-precipitation, but also to a conjugation. It still remains necessary to determine whether the requirement for bile salts is related to their detergent effect and if an inhibition of bile salt de-conjugation influences the assimilation of cholesterol. The same authors, in *in vitro* study in the absence of bacterial cells, observed that cholesterol was partially removed from the medium at pH values lower than 5.5 when de-conjugated bile salts were added. It was a transient phenomenon, and the precipitated cholesterol was re-dissolved when solutions were adjusted to pH 7. They concluded that resting cells of bifidobacteria do not interact with cholesterol, while growing cells of bifidobacteria assimilate cholesterol in their cell membrane. No significant relationship was, however, noticed between cholesterol assimilation and the degree of bile salt de-conjugation by bifidobacteria, as previously reported for lactobacilli (Gilliland et al., 1985). It has been suggested that bile salts hydrolase activity might have a role in the mechanism of assimilation, as non de-conjugating bifidobacteria were not able to assimilate appreciable amounts of cholesterol even if conjugated or de-conjugated forms of bile salts were present in the growth medium. The authors concluded that this cholesterol assimilation model could explain the *in vivo* observed hypocholesterolemic effect. Further investigations are still needed to determine the mechanism of cholesterol assimilation and localization of the assimilated cholesterol in the cells.

In 1997 Noh et al.[61] reported that cholesterol assimilated by *L. acidophilus* ATCC43121 was not metabolically degraded, as most of it was recovered with the cells. They observed that cells grown in the presence of cholesterol micelles and bile salts were more resistant to lysis by sonication. This could suggest a possible alteration of the cell wall or membrane by cholesterol.

Meei-YN Lin et al. [62] investigated the *in vitro* cholesterol reducing abilities of six *L. acidophilus* strains. A maximum cholesterol uptake of 57% was reported when *L. acidophilus* ATCC4356 was grown anaerobically for 24 h in a medium supplemented with bile acids . The authors concluded that the *in vivo* hypocholesterolemic effect of *L. acidophilus* cells was due to the direct assimilation by the cells and/or attachment of cholesterol to their surface. As previously mentioned, coprecipitation of cholesterol with decon-jugated bile acids, which occurs *in vitro*, is not likely to take place *in vivo* because pH of the small intestine is higher than 6.0.

Lactic acid bacteria with active BSH, or cultured products containing them, were suggested . to lower serum cholesterol levels through an interaction with the host bile salt metabolism [63]. The proposed mechanism is comparable to cholestyramine treatment which, like other bile salt sequestrants, binds bile salts and prevents them from being reabsorbed. Thus, less bile salts would return to the liver, resulting in a loss of feedback inhibition of bile salt synthesis, and an increased conversion

of cholesterol to bile salts. This led the authors to suggest that the ingestion of lactic acid bacteria containing active BSH might be regarded as a 'biological' alternative to common medical or surgical interventions to treat hypercholesterolemia.

Usman and Hosono [64, 65] focused on the binding abilities of the intact cells toward cholesterol. The authors found that all 28 strains of *L. acidophilus* were able to bind cholesterol, but the binding abilities varied widely. It was suggested that differences were related to chemical and structural properties of the bacterial cell wall peptidoglycans. In another study the same authors investigated the effect of a non-fermented milk supplemented with *L. gasseri* (SBT0270 and SBT0274), which have previously been shown to exhibit high cholesterol- binding and taurocholate-deconjugating activities *in vitro.* The total and LDL-cholesterol levels were reported to be 42 and 64% lower, respectively, in the group given *L. gasseri* SBT0270 supplemented milk than in the milk-only group. Triglyceride levels decreased when rats were given milk and non-fermented milk supplemented with the bacterial strains in relation to the control group (water), while milk alone had no observed effect on total and LDL-cholesterol. The authors attributed the observed hypocholesterolemic effect of *L. gasseri* SBT0270 to its ability to suppress the re-absorption of bile acids into the enterohepatic circulation (by de-conjugation), and to enhance the excretion of acidic steroids in feces.

Probiotic bacteria, once resident in the human gut, ferment food-derived indigestible carbohydrates. This results in an increased production of short-chain fatty acids (SCFA). This has been suggested to cause a decrease in the systemic levels of blood lipids either by inhibiting hepatic cholesterol synthesis, or by redistributing cholesterol from plasma to the liver . The SCFA production in the large intestine has been reported to be 100 to 450 mmol/day, with relative proportions of acetate, propionate, and butyrate being about 60:20:15, depending on the substrate. Acetate in the serum seems to increase total cholesterol, while propionate increases blood glucose and tends to lower the hypocholesterolemic response caused by acetate by reducing its utilization by the liver for fatty acid and cholesterol synthesis. Indeed, a study by Wolever and colleagues [66] showed that serum acetate and propionate concentrations were related to serum lipid concentrations in both males and females, serum propionate being strongly negatively related to both total and LDL cholesterol ($P < 0.001$). However, sufficient propionate must be produced to offset the effects of acetate generation as a precursor for lipid synthesis. Therefore, depending on the proportion of fatty acids produced during bacterial fermentation, plasma cholesterol concentrations may be altered. Additionally, some bacteria may interfere with cholesterol absorption from the gut either by de-conjugating bile saltsor by directly assimilating cholesterol. A number of bacteria have been reported to hydrolyze conjugated bile acids, such as *Bacteroides* spp., bifidobacteria, fusobacteria, clostridia, lactobacilli, and streptococci. There is evidence that the gut flora not only hydrogenates, dehydrogenates, and oxidizes bile acids, but also cleaves side chains to yield steroids. *In vivo*, removal of cholesterol would occur because de-conjugated bile acids are not well absorbed by the gut mucosa and are excreted through the feces and urine. The excretion of

bile acids results in decreased enterohepatic recirculation and therefore more cholesterol, which is the precursor of bile acids, needs to be utilized for *de novo* bile acid synthesis. Moreover *in vitro* studies using different strains of *L. acidophilus* grown on media containing bile have shown that certain strains can modify cholesterol metabolism. Because the amount of bile in the medium did not exceed concentrations normally found in the intestine, it can be expected that this cholesterol assimilation would also occur *in vivo*. The uptake of cholesterol by bacteria would make it unavailable for absorption into the circulation. However, some work still needs to be undertaken in this field leading toward more conclusive clinical evaluations to understanding of the *in vivo* mechanisms of probiotic effect on blood lipids and to improvement of strain stability characteristics. Also, the *in vitro* ability to reduce cholesterol observed with some strains needs to be confirmed in mixed culture and mixed substrate environments [56-66]

8.5 Food supplementation and safety

In Europe, the European Food Safety Authority (EFSA), established and funded by the European Community as an independent agency in 2002, standardize food safety and the ability of regulatory authorities to fully protect consumers. In 2004 (13-14 December, Brussels, Belgium) EFSA defined the QPS: Qualified Presumption of Safety of Micro-organisms in Food and Feed [67].

The purpose of this document was to explore the possibility of introducing a system, similar in concept and purpose to the GRAS (Generally Recognised As Safe) [68] definition used in the USA, which could be applied to micro-organisms and eventually their products and to invite comments on its practicality. This claims do not look for reproducing the GRAS system but take account of the different social and regulatory climate present in Europe. This is necessary since issues of importance to Europe would not necessary influence a GRAS listing. An example of this would be the presence of acquired antibiotic resistance factors, considered highly undesirable in Europe but currently of lesser issue in the USA.

QPS are defined as “an assumption based on reasonable evidence” and qualified to allow certain restrictions to apply. The scheme aims to have consistent generic safety assessment of micro-organisms through the food chain without compromising safety standards. Individual evaluations would be limited to aspects particular to the organism, such as acquired antibiotic resistance determinants in lactic acid bacteria. QPS status would not apply to a micro-organism that commonly causes pathogenicity. A micro-organism would not necessarily be considered a potential

pathogen where there are infrequent reports of clinical isolates from severely ill people. Broadly the characteristics to be evaluated for QPS approval are:

- Unambiguous identification at the claimed taxonomic level.
- Relationship of taxonomic identity to existing or historic nomenclature.
- Degree of familiarity with organism, based on weight of evidence.
- Potential for pathogenicity to humans and animals.
- The end use of the micro-organism. This will influence any qualifications imposed, depending on whether the organism is to be directly consumed; is a component of a food product not intended to enter the food chain, but which may adventitiously; or is used as a production strain in a product intended to be free of live organisms.

Summarizing the concept, it is only when a probiotic strain has been unequivocally identified; characterized, screened and its mechanisms of action elucidated with scientific rigor; labeled accurately and truthfully; tested for safe and efficacious human use in randomized, blinded placebo-controlled clinical trials, ideally with independent verification; and undergone a risk-benefit comparison with existing treatments that there will be evidence of sufficient quality to support the unjustified beneficial claims made to date for many proposed probiotics. [67-69]

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EXPERIMENTAL STUDIES

High serum cholesterol level is one of the major risk factor for cardiovascular diseases. Although different cholesterol lowering drugs exist, the possibility of nutritional interventions in the prevention and counteraction of hypercholesterolemia remains a very important issue. Beside an equilibrated and correct diet, different bioactive compounds could represent an additional strategy for reducing serum cholesterol, and therefore the incidence of cardiovascular diseases.

During my PhD course I focused my attention on some of these bioactives, performing studies in cultured cells and experimental animals. Some of these studies were preliminary to the use of these compounds for lowering cholesterol.

In the following chapter an overview of the performed experimental studies sorted by argument is reported.

I. High within-sample variation in the expression of cholesterol metabolism and trafficking genes in human abdominal aortic aneurysm tissue precludes its use as a tissue culture model

Note. The following study was carried out during my six month period abroad. It was performed at the Institute of Food Research IFR (Norwich, UK), and Norfolk and Norwich University Hospital NNUH (Norwich, UK) under the supervision of Prof. Paul A. Kroon.

The aim of the investigation was to evaluate the potential of using cultured human abdominal aortic aneurysm tissue obtained during elective open surgery as a model to assess the vascular effects of bioactives.

There is considerable interest in dietary bioactives such as n-3 polyunsaturated fatty acids and epigallocatechin-3-gallate that possess anti-hypercholesterolemic and anti-inflammatory activities. We investigated the potential of using cultured human abdominal aortic aneurysm (AAA) tissue obtained during elective open surgery as a model for assessing the vascular effects of bioactives. We quantified the mRNA levels for 5 genes involved in cholesterol metabolism and

trafficking. Inter-individual variation in gene expression of untreated tissue was extremely high (18, 5-, 185-, 198-, and 317-fold for *Srebp1*, *Srebp2*, *Ldlr*, *Acat2* and *Hmgcr*, respectively; n=4 patients). Within-patient variation in *Srebp2* expression was also high (3-fold). The shortcomings of the tissue model precluded us from observing possible eicosapentaenoic acid, docosapentaenoic acid or epigallocatechin-3-gallate induced changes in gene expression. Human AAA tissue exhibits highly variable gene expression and is therefore not suitable as an in vitro model for evaluating the effects of xenobiotics on transcription.

II. Effect of n-3 LC-PUFA supplementation on the lipid environment: a HR-MAS NMR and GC study in cultured rat cardiomyocytes

Note. N-3 polyunsaturated fatty acids are known to be involved in the prevention of cardiovascular diseases. However less is known on PUFA metabolic destiny and effects once inside the cell. This study aimed to elucidate the modifications occurring in the cell lipid environment after EPA and DHA supplementation. Investigation were carried out on cultured neonatal rat cardiomyocytes using high resolution magic angle spinning nuclear magnetic resonance technique in combination with gas chromatography. This research was carried out under the supervision of Prof. Vitaliano Tugnoli and Prof. Alessandra Bordoni, and was the result of an intense collaboration with The Department of Chemistry of the University of Modena and Reggio Emilia under the supervision of Prof. Luisa Schenetti and Prof. Adele Mucci.

The manuscript has been accepted for publication in Lipids.

It is well recognized that a high dietary intake of long chain polyunsaturated fatty acids (LC-PUFA) has profound benefits on health and prevention of chronic diseases. Particularly, in recent years there has been a dramatic surge in interest on the health effects of n-3 LC-PUFA derived from fish, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. Notwithstanding, the metabolic destiny and the effects of these fatty acids once inside the cell has been seldom investigated in a global way. Using cultured neonatal rat cardiomyocytes as model system, we have investigated for the first time the modification occurring in the cell lipid environment after EPA and DHA supplementation by means of high resolution magic angle spinning nuclear magnetic resonance technique in combination with gas chromatography.

III. N-3 PUFA regulation of genes related to cholesterol metabolism is related to genotype

Note. This *in vivo* investigation aimed to elucidate the effects of EPA and DHA supplementation in a rat model of metabolic syndrome. We focused on the modifications of hepatic gene expression after supplementation. The investigation was carried out using real time PCR as main technique. Experiments were done under the supervision of Prof. Alessandra Bordoni and Prof. Vitaliano Tugnoli. Some NMR analysis were also performed, in collaboration with the Department of Chemistry of the University of Modena and Reggio Emilia, under the supervision of Prof. Luisa Schenetti and Prof. Adele Mucci.

Aim: To evaluate in liver the effect of EPA and DHA supplementation on the expression of genes related to cholesterol metabolism in two different strains of rat, SH and WK.
Methods: 14 male WK rats and 14 male SH rats were used. SH rats are the most commonly used animal models for the metabolic syndrome, since they develop insulin resistance, hyper-triglyceridemia, abdominal obesity, hypertension, and hypercholesterolemia 7 rats from each group received a control diet and 7 a diet enriched in n-3PUFA for 90 days. Plasma lipid profile, total lipid fatty acid composition in plasma and liver, and hepatic tissue cholesterol concentration were evaluated. The expression of *Srebf-1, Srebf-2, Ldlr, Acat2,* and *Hmgcr* was analysed by two step q-PCR.
Results: Dietary n-3 PUFA were absorbed, and incorporated in liver. In SH rats, the supplementation recovered the imbalanced blood parameters, lowering the atherogenic index. Gene expression analysis underlined that in control condition some genes were differently transcribed in the two strains, and PUFA molecular effects were different, according to the different genotype.
Conclusions: genotype seems to influence the effects of n-3 PUFA at molecular level. Although further studies are needed, this observation could be useful while considering n-3 PUFA supplementation in humans with familial forms of hypercholesterolemia

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IV. Phytosterols supplementation reduces metabolic activity and slows cell growth in cultured rat cardiomyocytes

Note. Plant sterols are known to decrease cholesterol level, but they also inhibit the growth and development of tumours. Since these two effects are exerted at similar phytosterol

concentrations, this work aimed to evidence possible adverse effects of phytosterols in non-neoplastic cells. The study was carried out under the supervision of Prof. Alessandra Bordoni, in tight collaboration with Prof. Maria Fiorenza Caboni (Department of Food Sciences of the University of Bologna) and Prof. Andrea Pession (Department of Pediatrics Hematology and Oncology Unit "Lalla Seragnoli", St. Orsola-Malpighi Hospital).

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Phytosterols or plant sterols (PSs) are essential phytochemicals which have been shown to decrease serum total and LDL-cholesterol levels, and to inhibit the growth and development of tumours. PSs are poorly absorbed, but despite the low plasma concentration they can be incorporated into cell membranes, and their anti-neoplastic activity is accounted to the resulting interference of membrane composition and functionality. The similarity between the cholesterol-lowering and anti-neoplastic PS effective doses deserves attention on the possible adverse effects even in non-neoplastic cells, but to date few studies have been addressed to the clarification of this important issue. In this study we supplemented primary, non-neoplastic neonatal rat cardiomyocytes with two different PS concentrations, both within the range of plasma concentration considered as effective for cholesterol-lowering. Cardiac cells were chosen as experimental model since the heart has been reported as the target organ for PS sub-chronic toxicity. Following supplementation a dose-dependent incorporation of PSs, and a decrease in cholesterol content were clearly evidenced. The replacement of cholesterol by PSs could have been the cause of the observed slowing down of cardiomyocyte growth and reduction in metabolic activity. The herein reported study is the first report on the effect of PSs in cardiac cells, and although it is difficult to translate the obtained results to the health of heart tissue, it raises the suspect that the long-term exposure to physiologically relevant PS concentrations is a potential hazard.

V. Hypocholesterolemic effect of a new bifidobacteria mix on rat induced for hypercholesterolemia.

Note. Probiotics are a large heterogeneous group of active non pharmacological compounds. Some strains seem to be able to decrease cholesterol absorption. Since different strains exert diverse outcomes on cholesterol metabolism, the aim of the following study was to evaluate the hypocholesterolemic effect of a probiotic mix on rat induced for hypercholesterolemia. This study was carried out under the supervisions of Prof. Alessandra Bordoni. The probiotic mix was a kind

gift of Prof. Diego Matteuzzi (University of Bologna) and Prof. Maddalena Rossi (University of Modena).

Aim. To evaluate the hypocholesterolemic effect of a probiotic mix (*B. breve* WC 0463, *B. breve* WC 0420, and *B. bifidum* WC 0417) on 30 days old male Wistar Kyoto rat nutritionally induced for hypercholesterolemia.

Methods. After 15 day on 3% p/p cholesterol diet, total cholesterol, LDL-cholesterol, HDL-cholesterol, atherogenic index, and triglyceride were evaluated in rats, evidencing a shift toward an atherogenic condition. Then rats were randomly divided into three groups, one fed a 1% hypercholesterolemic diet, one receiving the same diet plus the probiotics mix, and the third fed a standard diet. After further 30 days, total cholesterol, LDL-cholesterol, HDL-cholesterol, atherogenic index and triglyceride were evaluated.

Results. The negative effects of a hypercholesterolemic diet were already evident after 15 days of treatment, and were reversed coming back to a standard diet. Treatment with the probiotic mix partially mitigated the effects of 1% hypercholesterolemic diet. Actually, administration of the probiotic mix reduced by 30% LDL-cholesterol, without affecting HDL-cholesterol.

Conclusion. Our findings strength the hypothesized ability of *bifidobacteria* in reducing serum cholesterol level. In particular, the probiotic mix counteracted the effect of a high-cholesterol diet reducing LDL-cholesterol fraction by 30%. The addition of suitable prebiotics could further enhance the observed effect.

I. High within-sample variation in the expression of cholesterol metabolism and trafficking genes in human abdominal aortic aneurysm tissue precludes its use as a tissue culture model

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Introduction

Atherosclerotic cardiovascular disease (ACVD) is the leading global health problem as assessed by mortality [1]. Considerable research effort has focused on understanding the processes that lead to atherosclerotic plaque formation and growth, and also on interventions that can arrest, slow or reverse atherosclerosis. ACVD is understood to be a disorder of lipid accumulation and is also associated with inflammation [2]. Evidence from epidemiological and clinical trials, and from in vitro studies, indicates that certain diets and dietary components have the potential to inhibit atherosclerosis and reduce cardiovascular disease risk [3-5]. Some food bioactives, including flavonoids and n-3 polyunsaturated fatty acids (PUFAs), are recognized as both anti-hypercholesterolemic and anti-inflammatory agents [3, 6-9]. The anti-inflammatory properties of epigallocatechingallate (EGCG) are well documented [10]. In addition, EGCG has been reported to

Abbreviations: AAA: abdominal aortic aneurysm; *Acat2:* acyl-coenzyme A cholesterol transferase 2; ACVD: atherosclerotic cardiovascular disease; DHA: docosahexaenoic acid; EGCG: epigallocatechingallate; EPA: eicosapentaenoic; *Gapdh*: glyceraldehyde phosphate dehydrogenase; HDL: high density lipoprotein; *Hmgcr:* hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase; LDH: lactate dehydrogenase; *Ldlr:* low-density lipoprotein receptor; PUFAs: poly unsaturated fatty acids; *Srebp:* sterol regulatory element binding proteins.

prevent lipid accumulation and alter lipoprotein levels in rats [11]. Eicosapentaenoic (EPA; C20 : 5 n-3) and docosahexaenoic acid (DHA; C22 : 6 n-3), the main long chain n-3 poly unsaturated fatty acids (PUFAs), have been shown to lower serum triglycerides and total cholesterol levels, whilst increasing the levels of high density lipoprotein HDL-cholesterol ('good cholesterol') [12-14]. Furthermore, they are precursors of anti-inflammatory eicosanoids [15].

Due to the high incidence of ACVD globally, and the associated mortality, the identification of nutritional preventative strategies is crucial if the rate of the disease is to be controlled or reduced. Cultured cells or tissues are commonly used models for studying the effects and effectiveness of food components, and their mechanism of action. For the vasculature, cultured cell models are widely used (e.g. human umbilical vein endothelial cells, human umbilical aorta smooth muscle cells). However, vascular tissue models are rarely used, probably because of the lack of availability of suitable tissue. There are a number of reasons why cultured tissue models are more physiologically representative than single cell culture models. The main reason is that vascular tissues are complex and comprise multiple cell types (including endothelial cells, smooth muscle cells and macrophages) and an intercellular matrix that also performs important functions. The development of a cultured vascular tissue model would be a valuable experimental tool for exploring vascular responses to food components.

In this report, we describe the culture of human aortic tissue obtained during elective open surgery with abdominal aortic aneurysm (AAA) patients, and experiments to test the possibility of using this tissue as an experimental model to investigate the effects of dietary components on certain vascular tissue functions. AAA aetiology involves dilation of all layers of the artery wall and is associated with inflammation [16, 17]. High plasma cholesterol concentrations have been associated with an increased risk of AAA in large screening studies [18]. The aims were to (i) successfully culture AAA tissue, (ii) investigate within-patient and between-patient variation and develop an appropriate cultured tissue experimental design, (iii) determine the effects of EPA, DHA, and EGCG on the expression of various genes involved in cholesterol metabolism and trafficking.

Materials and methods

Materials

5,8,11,14,17-Eicosapentaenoic acid (≥99%), cis-4,7,10,13,16,19-docosahexaenoic acid (≥98%), and Lactate dehydrogenase (LDH) based In Vitro Toxicology Assay Kit (TOX7) were purchased from Sigma Chemical (St. Louis, MO). EGCG was extracted from green tea by Dr Paul

Needs (Institute of Food Research, Norwich) and was shown to be ≥95% by HPLC. Medium 199 was purchased from Invitrogen (Paisley, UK). RNA later, proteinase K, and RNase Mini Kits were purchased from QIAGEN (Crawley, UK), Taqman one-step RT-PCR Master Mix Reagent and predesigned TaqMan gene expression assays were purchased from Applied Biosystems (Foster City, USA). All other chemicals and solvents were of the highest analytical grade.

Sampling and treatment

Tissue samples, ranging in length from 3 to 7 cm, were collected from the Norfolk & Norwich University Hospital (NNUH) Human Tissue Bank according to local procedures and in accordance with international ethical guidelines on the use of human tissues in scientific research, and the Human Tissue Act (UK). The use of NNUH Human Tissue Bank tissue samples for research purposes had been approved by the Cambridge 1 Research Ethics Committee (REC Reference: 08/H0304/85) and the specific use of the tissue samples in this project was approved by the East Norfolk and Waveney Research Governance Committee, Norwich, UK [Proposal Ref: 2009HIST04S (91-06-09)]. Tissue samples were only used if the patient had provided informed consent for the spare resected AAA tissue to be used for scientific research purposes. Samples of aortic tissue were obtained from five patients undergoing surgery for open repair at the NNUH.

The tissue sample obtained from each patient was cut into several pieces using sterile scissors, and subsequently each larger piece was cut into ~2 mm wide slices. Contiguous 2mm slices coming from the same larger piece of tissue were allocated to different wells in order to reduce the effects of tissue heterogeneity (figure 1). This process was repeated for each of the larger tissue pieces until each culture well contained several pieces of

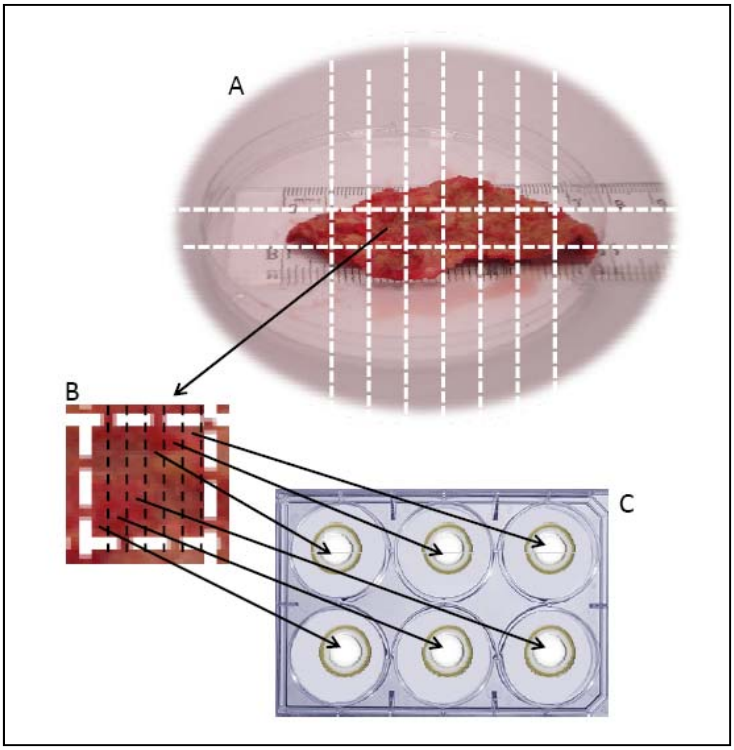

Figure 1. Scheme for sample preparation. Tissue was cut into several pieces as indicated by the white dotted grid in panel a. Each piece was subsequently cut into ~2 mm wide slices, as indicated by the black dotted grid on panel b. Contiguous 2mm slices coming from the same larger piece of tissue were allocated to different wells on a stainless steel tissue grid support (panel c). This process was repeated for each of the larger tissue pieces until each culture well contained several pieces of aortic tissue from across the original tissue sample. Atypical slices were discarded.

aortic tissue from across the original tissue sample. Atypical slices (e.g. exhibiting excessive calcification) were discarded. Tissue slices were placed on a stainless steel tissue grid support mounted on the bottom of a 6-well culture plate and cultured in GIBCO medium 199 containing 1% non essential amino acids, 2 mmol/L glutamine, 1 U/L penicillin, 1 g/L streptomycin and 5% fetal calf serum. The tissue was maintained overnight in a humidified cell culture incubator with 5% CO_2 at 37^{o}C, then medium was removed and the different treatments were supplied in fresh media. EPA, DHA and EGCG stock solutions were prepared by dissolving the pure compound in 100% filtered ethanol. Equivalent concentrations of ethanol (<0.01% v/v) were added to not supplemented wells in order to control for potential vehicle interference. Bioactives were added to fetal bovine serum, and after 5 minutes incubation the medium was also added. For the tissue sample from each patient, 5 different treatments were applied: (1) vehicle in control media, (2) 60 μM EPA, (3) 60 μM DHA, (4) 25 μM EGCG, (5) 25 μM EGCG + 60 μM EPA. After 24 h of incubation in the presence of the treatments, the tissue pieces were placed in 1.5 ml tubes containing RNA Later (1 ml), incubated at +4°C for 24h, and then frozen at -80°C.

Assessment of tissue viability

The relative number of cells surviving in an AAA tissue that had been prepared and maintained in culture in the manner described above was estimated using a lactate dehydrogenase (LDH) based In Vitro Toxicology Assay Kit (Sigma, TOX7). Untreated tissue samples were taken from culture at various time points, rapidly frozen on dry ice and stored at -80°C. Weighed pieces of frozen tissue (approx. 15 mg) were transferred to 1.5 ml tubes and dispersed in 300 μl kit LDH Assay Lysis Solution using a micro Ultra Turrax homogeniser. The homogenate was incubated at 37°C for 30 min to complete cell lysis and then centrifuged at 13000 x g for 5 min. Aliquots of the supernatant were then assayed for LDH activity according to the kit manufacturer's instructions and the resultant absorbance measured at 450 nm on an MRX-II 96-well plate reader (Dynex Technologies, Worthing, West Sussex, UK). LDH activity relative to tissue sampled at time zero was calculated. Tissue explants and the surface of cell culture dishes were also examined microscopically during culture to identify signs of cell proliferation.

RNA extraction and quantification

Total RNA was extracted from tissue using RNase Mini Kits. Weighed tissue (typically 250 mg) from each treatment was cut into smaller pieces and homogenized in kit RLT buffer (2 ml) containing 1% β-mercaptoethanol using a PRO 400 homogenizer (Pro Scientific Inc, Oxford, USA). The homogenizer was rinsed with RNase-free water (4 ml). Proteinase K solution (65 μl ≡ 40

mAU) was added to the combined homogenate and washings and the mixture was incubated at 55°C for 20 min. After centrifugation (5 min at 5000 x g), the supernatant was then processed according to the kit manufacturer's instructions. RNA was eluted from RNase Mini columns using 2 x 150 µl RNase-free water, quantified and assessed for purity on a nanodrop ND-1000 (Thermo Fisher Scientific Inc, Wilmington, USA).

q-PCR analysis

The protocol used was in accordance with the MIQE Guidelines: Minimum information for Publication of Quantitative Real-Time PCR Experiments [19]. All samples were prepared for q-PCR in microamp optical 96-well plates in a total volume of 20µl per well using a CAS-1200 automated PCR robot (Corbett Life Science, St. Neots, UK) with dedicated software. All reactions were carried out using Taqman One-step RT-PCR Master Mix Reagent on a Step-One-Plus real-time PCR system with Step One Software v2.0. q-PCR conditions were as follows: 48°C 30 min; 1 cycle of 95°C 20 min. 40 cycles: (95°C 15 sec; 60°C 1 min). TaqMan gene expression assays used were: sterol regulatory element binding proteins 1 and 2 *Srebp1* (Hs00231674_m1), *Srebp2* (Hs 01081784_m1); acyl-coenzyme A cholesterol transferase *Acat2* (Hs255067_m1); low-density lipoprotein receptor *Ldlr* (Hs00181192_m1); hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase *Hmgcr* (Hs 00168352_m1); Glyceraldehyde phosphate dehydrogenase *Gapdh* (Hs 99999905_m1), and beta-actin β-actin (Hs 9999993_m1). Data were analyzed using a standard curve generated by a serial dilution of total RNA from untreated tissue and then normalized against the expression of invariant endogenous control genes (GAPDH and β-actin).

Results

Culture of human AAA tissue

The AAA tissue samples that were monitored for LDH activity showed only a small reduction in activity after 24h of culture (90.4±7.7%) compared to pre-cultured tissue. Tissue maintained in culture for longer periods still exhibited a high LDH activity, e.g. 60.7±4.8% after 4 days. In addition, microscopic observation of the cell culture dish revealed the appearance of clusters of loosely adherent new cells growing around the tissue explants for all the AAA tissue samples that were cultured, and it was also observed that the numbers of these cells increased with time. These results suggest that under the culture conditions used here the viability of the tissue is maintained and cell proliferation is stimulated.

Between patient variation in baseline (not treated) gene expression

The variability of inter-individual gene expression was evaluated by comparing basal gene expression in not treated samples derived from the different patients. As shown in figure 2, the expression of all the selected genes appeared significantly different between patients for all examined genes (18-, 5-, 185-, 198-, and 317-fold for *Srebp1*, *Srebp2*, *Ldlr*, *Acat2* and *Hmgcr*, respectively; n=4 patients; p<0.0001).

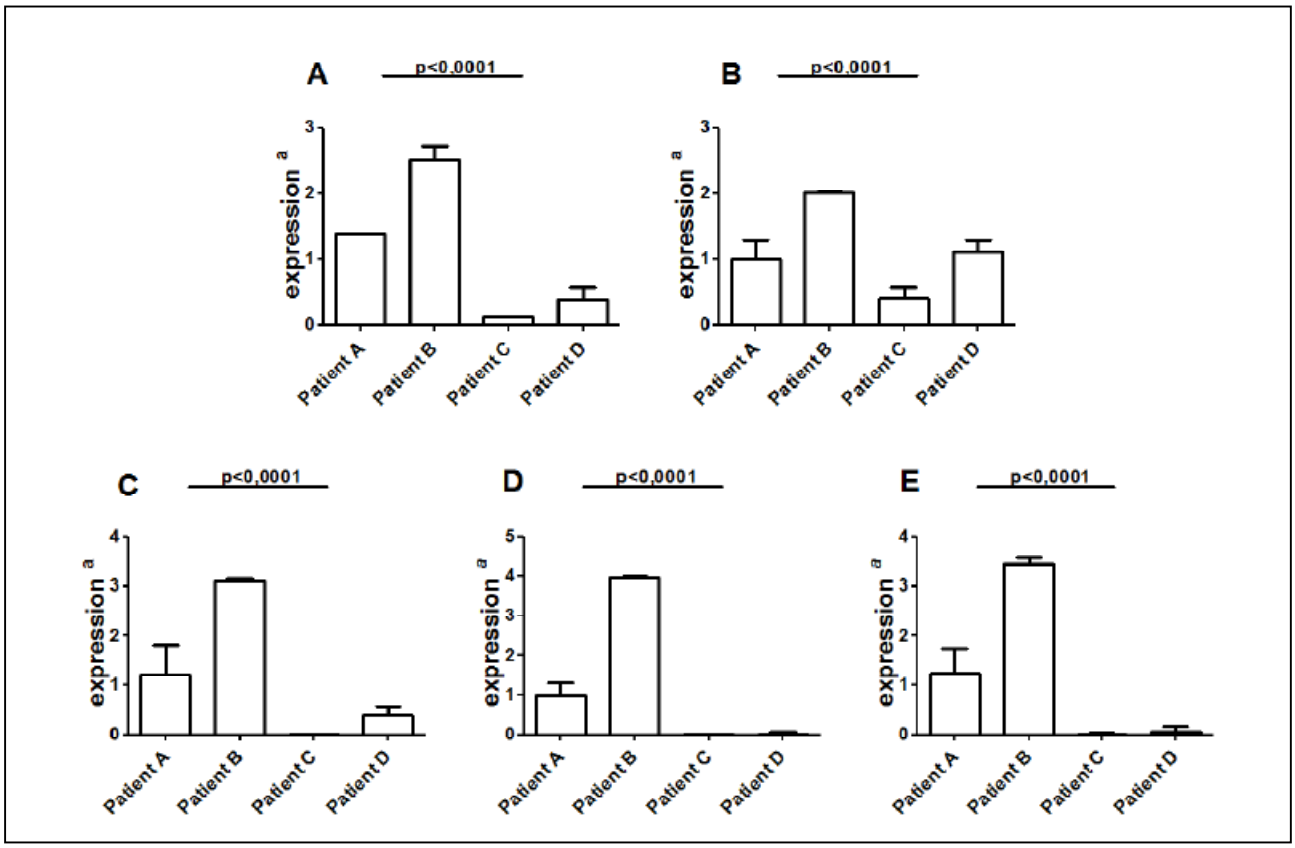

Figure 2: Inter-individual variability of basal gene expression in tissue derived from different patients. Basal expression of *Srebp1* (A), *Srebp2* (B), *Acat2* (C), *Hmgcr* (D) and *Ldlr* (E) was evaluated in untreated samples from 4 different patients. Gene expression analysis was performed as reported in Methods. [a]Genes were normalized against *Gapdh* (Δcq) and the average of all the not treated (NT) samples was used as calibrator (ΔΔcq). Data are means +/- SD of three technical replicates. Statistical analyses were by one way ANOVA (p<0.0001 for all genes).

Within patient variation in baseline (untreated) gene expression

To test the possible existence of not only inter-, but also intra-individual variability in gene expression, six samples derived from the tissue of the same patient were evaluated for *Srebp2* gene expression under basal conditions (no treatment) (Figure 3). The huge intra-individual variability observed (3-fold for *Gapdh* normalized data; n=6 samples analysed in triplicate; p<0.0001) was not related to technical problems, since technical replications on the same sample were almost identical.

Furthermore, normalization with two different reference genes, *Gapdh* and β-actin, generated a substantial change in the gene expression profiles.

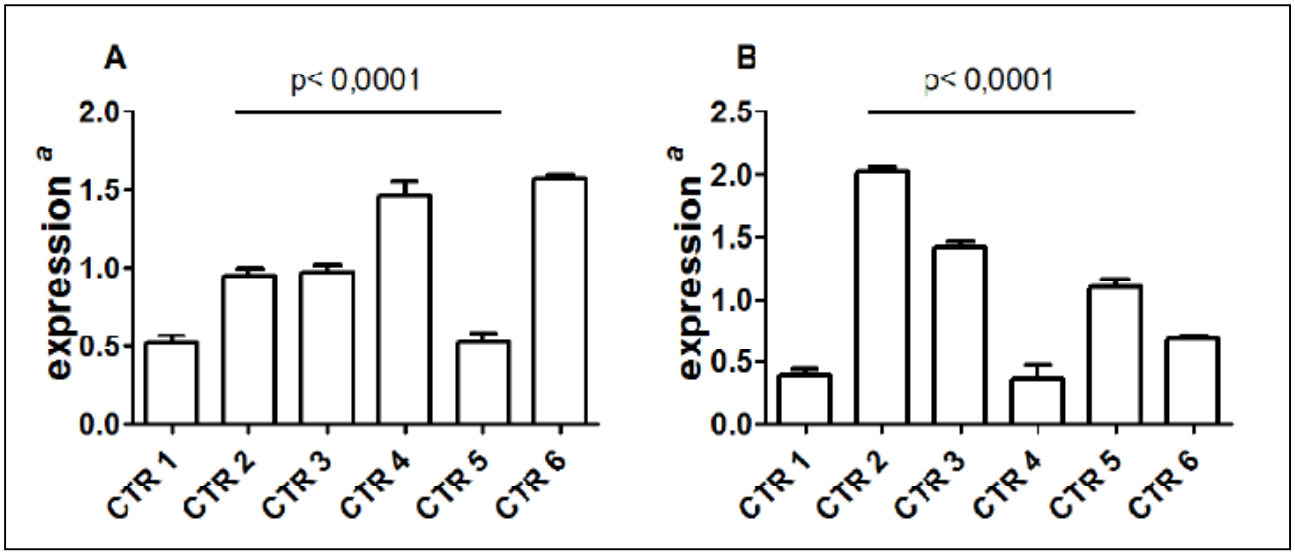

Figure 3. *Srebp2* gene expression in 6 untreated samples derived from the same patient. The quantification of *Srebp2* transcripts was performed three times in each sample. [a]Δcq was compared against *Gapdh* (panel A) or β-actin (panel B), then normalized against the average relative gene expression of Srebp2 in all the samples. Data are reported as mean +/- SD. Statistical analysis was performed using one-way ANOVA ($p<0.0001$ in both comparisons).

Effects of EPA, DHA and EGCG on gene expression

The effect of the different treatments on gene expression was quantified for all the patient tissue samples. As shown in figure 4, none of the treatments caused significant differences in the mean expression of any of the 5 genes tested (compared to not treated samples). The high standard deviation values indicate that there is considerable variation in the measurements, which is consistent with the data in the two preceding subsections.

Discussion

Because the complexity of vascular tissue responses cannot be emulated in single cell culture models, the initial aim of the present study was to investigate the effect of three different bioactives, namely EGCG, EPA and DHA, on the expression of genes related to cholesterol metabolism (*Srebp1*, *Srebp2*, *Acat2*, *Ldlr* and *Hmgcr*), utilizing cultured explants of AAA as a model system. Absorbed bioactives are delivered to all tissues through the blood stream, and arterial vessels are directly in contact with the bioactives in the blood. Therefore, a cultured aortic tissue model would be expected to more closely replicate possible *in vivo* effects of bioactives, compared to a single cell-type culture model.

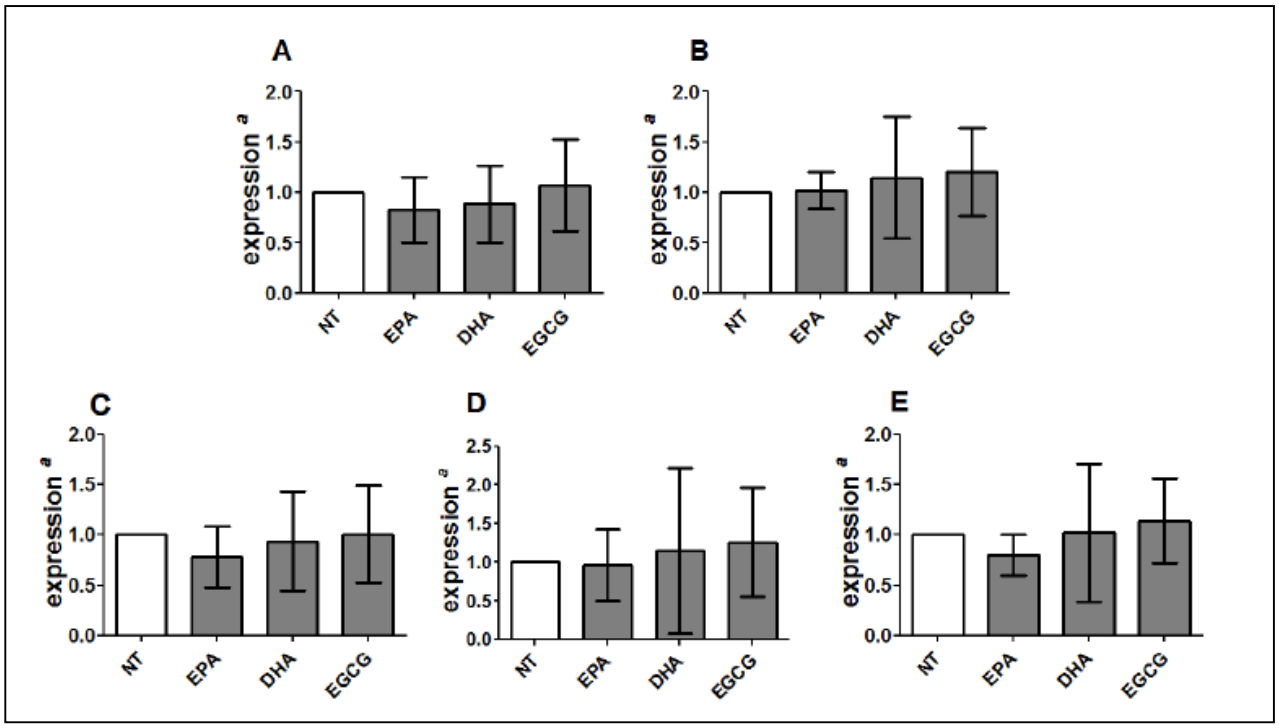

Figure 4: Modulation of gene expression by tea catechins and n-3 PUFAs. *Srebp1* (A), *Srebp2* (B), *Acat2* (C), *Hmgcr* (D) and *Ldlr* (E) expression is reported in not treated (NT, white bars) and treated samples (grey bars). [a]Value for each gene was obtained using Δcq against *Gapdh*, and ΔΔcq was in comparison with the corresponding NT sample. Data are the means ± SD. Statistical analysis was performed using one-way ANOVA (n.s.).

The main finding of this research was that there was substantial within-subject and between-subject variation in gene expression. The within-subject variation in gene expression was high even though great care was taken to ensure that each culture well contained a representative set of tissue pieces from across the original tissue sample. In diseased tissue such as that of aneurytic aorta, it is not surprising that there is quite considerable within tissue variation in gene expression, but it was somewhat surprising that the effects of this variation could not be smoothed out by using multiple tissue pieces from across the original tissue sample. This observation likely means that there are small regions of the tissue in which genes are highly expressed, and other regions of the tissue in which gene expression is virtually absent. Normalization using two different reference genes resulted in different intra-sample profiles of *srebp2* gene expression. Since reference genes can be differentially expressed in different cell types [20, 21], our data may be explained by the presence of different proportions of cell transcriptotypes in the different culture wells containing tissue slices from the same patient. This could be related to inflammatory cell infiltration [22]. Alternatively, a different Chol accumulation in the different areas of the lesion [23, 24] could have modulated the expression of genes related to Chol metabolism. It is well know that the sterol concentration regulates its own biosynthesis [25]. A previously published report has demonstrated that there can

be considerable heterogeneity in gene expression between cells isolated from the same piece of tissue. Trogan et al. [26] used laser capture micro-dissection to demonstrate considerable between cell variations in gene expression in macrophages isolated from atherosclerotic regions of apolipoprotein-E mice.

The inter-sample variation described here (up to 317-fold for the 5 cholesterol metabolising and trafficking genes) was of a magnitude that was likely to seriously limit the potential utility of such a model for research purposes. High intra-subject and inter-subjects variations have been reported previously by Hennig et al. for samples from both Barrett's mucosa and normal squamous epithelium [27].

Although we conducted experiments with cultured AAA tissue to examine the potential for the major tea catechin EGCG and fish oil n-3 PUFAs to modulate the expression of a set of genes involved in cholesterol metabolism and trafficking, we did not observe any significant changes in expression in response to the treatments. However, because the variability of expression of the target genes within and between tissues and subjects was shown to be very substantial, these data do not allow any conclusions to be drawn regarding the potential efficacy of EGCG, EPA or DHA to modulate the expression of these genes.

Conclusions

In conclusion, substantial within tissue variation in gene expression was observed in abdominal aortic aneurysm tissues obtained from patients who had undergone open surgery, and the magnitude of the variation precludes the use of such tissue in cultured tissue models for the purposes of investigating the effects of treatments on gene expression.

Acknowledgements

This research was funded by a Bologna University Marco Polo grant to EB, by the Biotechnology and Biological Sciences Research Council UK (JRB & PAK) and by the Italian MIUR (RFO; VT & AB). The authors are thankful to Loma Holmes and Mandy Burrows (NNUH) for assistance in obtaining tissue samples, Wendy Hollands for assistance with ethics paperwork, and to the Norfolk and Norwich University Hospital Foundation Trust Human Tissue Bank and donating patients for providing tissue samples for this research.

II. Effect of n-3 LC-PUFA supplementation on the lipid environment: a HR-MAS NMR and GC study in cultured rat cardiomyocytes

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INTRODUCTION

Cardiovascular diseases are responsible for significant morbidity and mortality throughout the world. It is well recognized that a significant dietary intake of polyunsaturated fatty acids (PUFA) has profound benefits on health and prevention of chronic disease states, although the overall responsible mechanism remains partially unclear [28, 29].

Supplementation with the main n-3 long chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (20:5n-3, EPA), and docosahexaenoic acid (22:6n-3, DHA) has been widely reported as protective for cardiovascular health, and fish oil feeding has been associated to reduced

Abbreviations: 1D: monodimensional; 2D: bidimensional; Ac: acetate; Ala: alanine; ARA: arachidonic acid; C: cholesterol; CE: cholesteryl esters; Cho: choline; ChoCC: choline-containing compound; COSY: COrrelation SpectroscopY; CPMG: Carr-Purcell-Meiboom-Gill; Cr: creatine; DHA: docosahexaenoic acid; DPAn-3: docosapentaenoic acid; EPA: eicosapentaenoic acid; Etn: ethanolamin; FA: fatty acid(s); FCS: fetal calf serum; FFA: unesterified fatty acids; GC: gas chromatography; Gln: glutamine; Glu: glutamate; Gly: glycine; GPC: glycerophosphocholine; HR-MAS: high resolution magic angle spinning; HS: horse serum; Lac: lactate; LB: lipid body(ies); LC-PUFA: long chain polyunsaturated fatty acid(s); Lys: lysine; MUFA: monounsaturated fatty acid(s); Myo: *myo*-inositol; NMR: nuclear magnetic resonance; PCho: phosphocholine; PEtn: phosphoethanolamine; PL: phospholipid(s); PtdCho: phosphatidylcholine; PUFA: polyunsaturated fatty acid(s); Scy: *scyllo*-inositol; TAG: triacylglycerol(s); Tau: taurine; TLC: thin layer chromatography; TOCSY: Total Correlation SpectroscopY; UDP: uridine diphosphate; α-CH of aminoacids (α-CH).

mortality in several studies [30-32]. Notwithstanding, the effects of these fatty acids (FA) on cardiac cell lipid environment are almost unknown.

In this study we have coupled gas chromatography (GC) analysis and high-resolution magic-angle spinning (HR-MAS) nuclear magnetic resonance (NMR) spectroscopy to evidence the modifications occurring in the lipid environment and in the metabolic profile of cultured cardiomyocytes supplemented with n-3 PUFA. Chromatographic methods have become the standard for analyzing FA composition [33]. Particularly, GC is sensitive and specific for FA analysis, while HR-MAS NMR spectroscopy takes a picture of the whole metabolic profile detecting lipids, macromolecules, and small metabolites. Using HR-MAS NMR the sample can be analyzed directly without any manipulation, and many different compounds such as macromolecules, lipids, and small metabolites can be detected at the same time [34, 35]. The study of cellular metabolism using NMR spectroscopy has been successfully carried out for years in cell extracts solution, either hydrophilic or lipophilic. More recently, the development of HR-MAS techniques has made the direct analysis of cells increasingly preferred for metabonomic studies [36]. HR-MAS NMR spectroscopy is currently employed for the analysis of the biochemical profile of complex systems such as biofluids, biological tissues, or cells. In fact, it can provide the detection of many different metabolites, and information on the metabolic changes occurring in response to external stimuli, e.g. drug exposure, or disease [35]. To our knowledge, HR-MAS NMR investigations, alone or combined with GC analysis, involving cardiomyocytes are still virtually absent. In our opinion, they could offer baseline data for future investigation on the effects of FA supplementation to mammalian cells.

MATERIALS AND METHODS

Materials. FA, Ham F10 media, fetal calf serum (FCS), horse serum (HS), gentamicin, amphotericin B, 2′,7′-dichlorofluorescein were from Sigma (St. Louis, MO, USA). Hexane, diethyl ether, and formic acid were purchased from Carlo Erba (Milan, Italy). All other chemicals and solvents were of the highest analytical grade.

Cardiomyocytes cell cultures. Primary cultures of neonatal rat cardiomyocytes were obtained from the ventricles of 2-4 day-old Wistar rats according to Yagev *et al.* [37]. Cells were seeded at the density of $1.5x10^6$ cells/ml in 100-mm i.d. Petri dishes in Ham F10 nutrient mixture supplemented with 10% v/v FCS, 10% v/v HS, gentamicin (1%), amphotericin B (1%), and grown at 37°C, 5% CO_2 and 95% humidity. The study protocol was approved by the Animal Care Committee of the University of Bologna (Italy) (prot. n. 58897-X/10). Forty-eight h after seeding, cardiomyocytes

were randomly divided in control and FA supplemented groups. EPA and DHA were dissolved in ethanol, added to FCS to allow binding to albumin, and supplemented at 60 μM concentration. Control medium was added with the same volume of ethanol (≤0.1% v/v) to avoid interference due to vehicle. Media were changed every 48 h, and cardiomyocytes were grown till complete confluence in a monolayer (day 8 from seeding).

Cardiomyocytes FA composition. At confluence cardiomyocytes were washed three times with ice-cold phosphate buffered saline, scraped off, and cell total lipids were extracted according to Folch *et al.* [38]. Washings were analyzed by GC to ensure that supplemented FA had been completely removed and did not interfere with following analyses. Total cell lipids were separated by thin layer chromatography (TLC) using plates coated with silica gel G. Plates were developed in hexane/diethyl ether/formic acid (8:2:0.1 by vol). Spots were made visible under ultraviolet light by spraying with 2′,7′-dichlorofluorescein (0.2% w/v in ethanol), and identified by comparison with authentic co-chromatographed standards. Spots corresponding to the phospholipid (PL) and triacylglycerol (TAG) fractions were scraped off, extracted with methanol or dimethylether, respectively, and methylesterified according to Stoffel *et al.* [39]. Prior to methyl esterification, pentadecanoic acid was added as internal standard. FA composition (as methyl esters) was determined by GC (GC 8000, Fisons, Milan, Italy) using a capillary column (SP 2340, 0.2 μm film thickness) at a programmed temperature gradient (160-210°C, 8°C/min), as previously reported [40]. The gas chromatographic peaks were identified on the basis of their retention time ratios relative to methyl stearate and predetermined on authentic samples. Gas chromatographic traces and quantitative evaluations were obtained using a Chrom Card Software computing integrator (Thermo Electron Scientific, Milan, Italy).

Nuclear Magnetic Resonance Spectroscopy. At confluence, after washing with ice-cold phosphate buffered saline, cells were scraped off in deuterated water. Fifty μl of the cell suspension were introduced in a 50 μl MAS zirconia rotor (4 mm OD), closed with a cylindrical insert to increase sample homogeneity, then transferred into the probe cooled to 4°C. ^{1}H and ^{13}C HR-MAS NMR spectra were recorded with a Bruker Avance 400 spectrometer operating at 400.13 and 100.61 MHz, respectively. The instrument was equipped with a $^{1}H,^{13}C$ HR-MAS probe, which temperature was controlled by a Bruker Cooling Unit. The whole experiments were performed at 4°C to prevent cell degradation processes [41]. Samples were spun at 4000 Hz. After an about 20 min set up, three different types of monodimensional (1D) proton spectra were acquired by using: *i*) a composite pulse sequence (zgcppr) [42], with 1.5 s water-presaturation during relaxation delay, 8 kHz spectral width, 32k data points, 32-64 scans; *ii*) a water-suppressed spin-echo Carr-Purcell-

Meiboom-Gill (CPMG) sequence (cpmgpr) [43], with 1.5 s water presaturation during relaxation delay, 1 ms echo time (τ), and 360 ms total spin-spin relaxation delay (2nτ), 8 kHz spectral width, 32k data points, 128 scans; and *iii*) a sequence for diffusion measurements based on stimulated echo and bipolar-gradient pulses (ledbpgp2s1d) [44] with big delta 200 ms, eddy current delay T_e 5 ms, little delta 2*2 ms, sine-shaped gradient with 32 G/cm followed by a 200 μs delay for gradient recovery, 8 kHz spectral width, 8k data points, 256 scans. Two-dimensional (2D) ^{1}H,^{1}H-COrrelation SpectroscopY (COSY) spectra [45, 46] were acquired using a standard pulse sequence (cosygpprqf) and 0.5 s water presaturation during relaxation delay, 8 kHz spectral width, 4k data points, 32 scans per increment, 256 increments. 2D ^{1}H,^{1}H-TOtal Correlation SpectroscopY (TOCSY) spectra [47, 48] were acquired using a standard pulse sequence (mlevphpr) and 0.5 s water-presaturation during relaxation delay, 100 ms mixing (spin-lock) time, 4 kHz spectral width, 4k data points, 32 scans per increment, 128 increments. NMR spectra of specimens were analyzed using MestReC software (Mestrelab Research, Santiago de Compostela, Spain). A line-broadening apodization function of 5 Hz was applied to lead diffusion-edited HR-MAS ^{1}H FIDs prior to Fourier transformation. NMR spectra were referenced with respect to FA terminal -CH_3 signal at δ 0.89 ppm, manually phased, and a Whittaker baseline estimator was applied to subtract the broad components of the baseline.

Statistical analysis. Three batches for each type of samples (control, EPA supplemented and DHA supplemented cardiomyocytes) derived from three independent cell cultures were analyzed. Statistical analysis was performed by the Student's t test comparing control and LC-PUFA supplemented cells.

RESULTS

HR-MAS NMR. The metabolic pattern of un-supplemented, control cardiomyocytes can be derived by the analysis of the spectra shown in figure 1. The first spectrum (figure 1a) is a conventional presaturated 1D spectrum detecting lipids, macromolecules, and small metabolite contribution. Figure 1b shows the spectrum obtained using a CPMG spin-echo sequence. This allows to separate signals according to their different T_2, and to enhance the resonance of small metabolites with respect to macromolecules. The small metabolites are labeled in figure 1b: alanine (Ala), choline-containing compounds (ChoCC), creatine (Cr), glutamate (Glu), glycine (Gly), *myo*-inositol (Myo), phosphoethanolamine (PEtn), *scyllo*-inositol (Scy), taurine (Tau), ; α-CH of amminoacid (α-CH). The diffusion-edited spectrum (figure 1c) displays broad resonances arising

from macromolecules. Particularly, mobile FA chains [signals at 0.89 ppm (-C**H**$_3$), 1.33 ppm

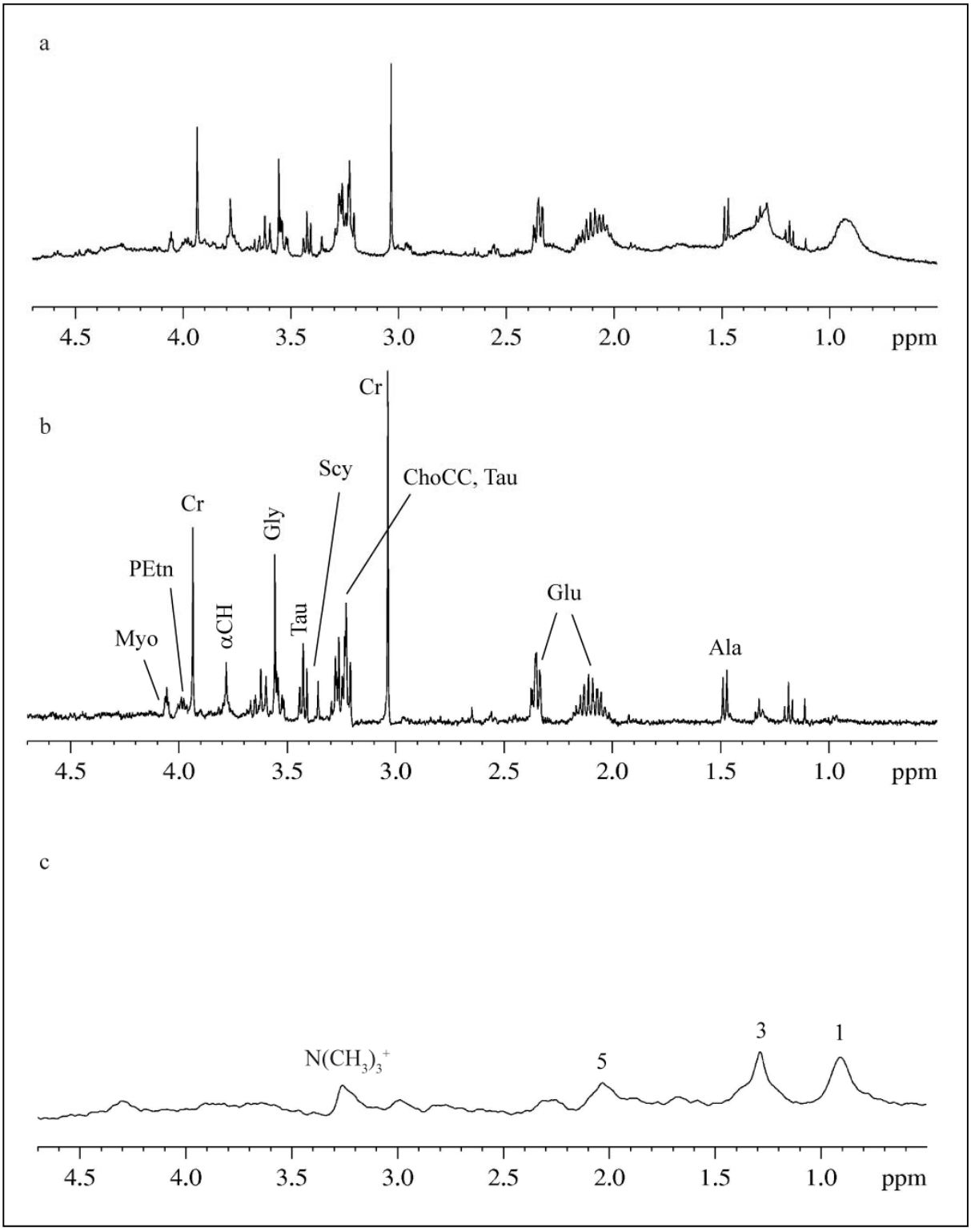

Figure 1. Representative *ex vivo* HR-MAS ^{1}H NMR spectra of neonatal rat cardiomyocytes: water-presaturated pulse sequence with composite pulse (a), CPMG spectrum (b), and diffusion-edited spectrum (c).

The mayor metabolites are labeled: alanine (Ala), choline-containing compounds (ChoCC), creatine (Cr), glutamate (Glu), glycine (Gly), phosphoethanolamine (PEtn), *scyllo*- (Scy) and *myo*-inositol (Myo), taurine (Tau), α-CH of aminoacids (α -CH). Lipid components are labeled: 1, -CH_3; 3, acyl chain methylene- $(CH_2)_n$; 5, CH_2C=C; $N(CH_3)_3^+$, signal for PL components.

($C\underline{\mathbf{H}}_2)_n$, and 2.03 ppm $C\mathbf{H}_2C{=}C$], and phosphatidylcholine (PtdCho) [signal at 3.26 ppm $N(C\underline{\mathbf{H}}_3)_3^+$] are clearly detectable. Membrane PL do not contribute significantly to the NMR signals due to their low mobility and short relaxation times. Control cardiomyocytes ^{1}H HR-MAS NMR spectra were used for the comparison with spectra obtained from cells supplemented with EPA and DHA.

In figure 2 the conventional water-presaturated ^{1}H spectra of control (2a), EPA supplemented (2b), and DHA supplemented (2c) cardiomyocytes are reported. In the spectra of n-3 LC-PUFA supplemented cardiomyocytes it is possible to recognize the characteristic signals from unsaturated FA. The resonances of FA chains are identified as: 1, methyls ($-C\underline{\mathbf{H}}_3$) at 0.89 ppm (saturated FA chains), and 2, $-C\underline{\mathbf{H}}_3$ at 0.96 ppm (n-3 EPA, and DHA); 3, acyl chain methylenes $(C\underline{\mathbf{H}}_2)_n$ (1.33 ppm); 4, $C\underline{\mathbf{H}}_2$-C-CO (1.58 ppm); 5, $C\underline{\mathbf{H}}_2C{=}C$ (2.02 ppm); 6, $C\underline{\mathbf{H}}_2CO$ other than DHA (2.25 ppm) and 7, $C\underline{\mathbf{H}}_2CO$ of DHA, (2.33 ppm); 8, =C-$C\underline{\mathbf{H}}_2$-C= (2.78 ppm); 9, $-C\underline{\mathbf{H}}{=}C\underline{\mathbf{H}}-$(5.33 ppm) [49-51]. Signals at 2.02 ppm are assigned to methylene protons of the $C\underline{\mathbf{H}}_2$-CH=CH moiety of both monounsaturated FA (MUFA) and PUFA, while the signal at 2.78 ppm is characteristic of PUFA [49, 50]. Both MUFA and PUFA are also identified by the signals at 5.33 ppm, due to the protons of the $-C\underline{\mathbf{H}}{=}C\underline{\mathbf{H}}-$ moiety.

The comparison of ^{1}H NMR spectra of supplemented and control samples shows that the PUFA signals are dominant in EPA and DHA supplemented cardiomyocyte spectra, whereas they are almost absent in those of control cells. Moreover, the signal at 2.33 ppm, characteristic of DHA $C\underline{\mathbf{H}}_2$-CO and $C\underline{\mathbf{H}}_2$-C-CO protons, permits the distinction between DHA and EPA supplemented samples.

In figure 3 the ^{1}H HR-MAS NMR diffusion-edited spectra of control (3a), EPA supplemented (3b), and DHA supplemented (3c) cardiomyocytes are reported. According to literature, lipid components are identified as above. Signals 10 and 11, due to bonded glycerol (4.10 and 4.30 ppm) are clearly detected, especially in the case of DHA supplemented cells.

The comparison of these spectra shows an increase of signals coming from mobile lipids in PUFA supplemented cells with respect to the unsupplemented ones. Low PL signals (particularly that of $N(CH_3)_3^+$ at 3.26 ppm) are present, together with macromolecules broad components, in the control cell spectrum (see figure 3a). In supplemented cells, these signals are obscured by the high intensity of signals from PUFA, mainly bonded to glycerol in TAG. Signals from CH_2 of bonded glycerol in TAG are clearly seen at 4.30 and 4.10 ppm (see figure 3, signal 10, 11), whereas the CH resonance at 5.26 ppm is overlapped by the CH=CH protons of FA chains. Signals attributable to cholesterol (C) or cholesteryl esters (CE), the most characteristic of which is that of 18-CH_3, at about 0.7 ppm, are not detected.

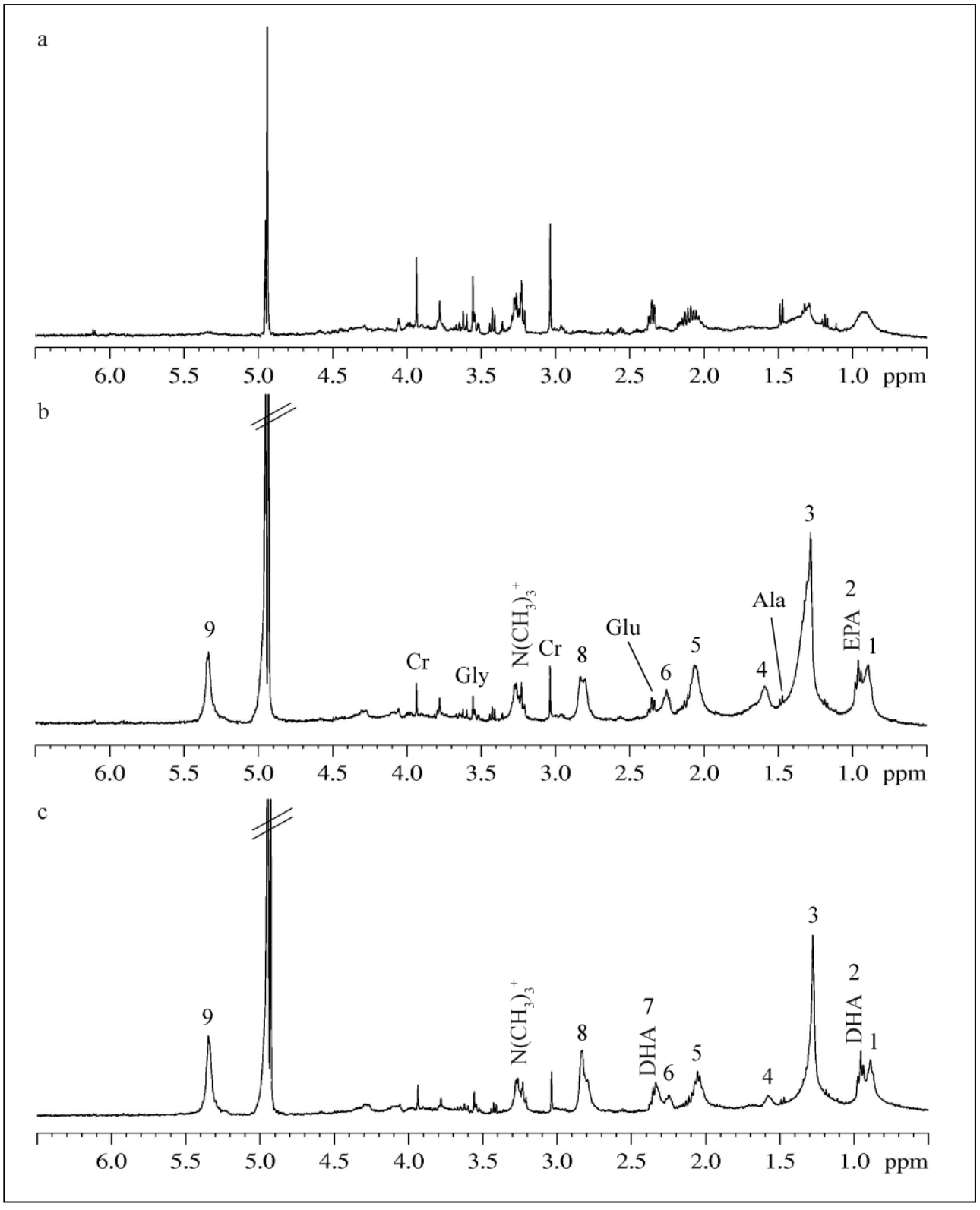

Figure 2. Water-presaturated *ex vivo* HR-MAS ^{1}H NMR spectra of control (a), EPA (b), and DHA (c) supplemented cardiomyocytes.

Lipid components are labeled as in the previous figure and: 2, DHA and EPA -CH_3; 4, CH_2-C-CO; 6, CH_2CO other than DHA; 7, CH_2CO and CH_2-C-CO of DHA; 8, =C-CH_2-C=; 9, CH=CH.

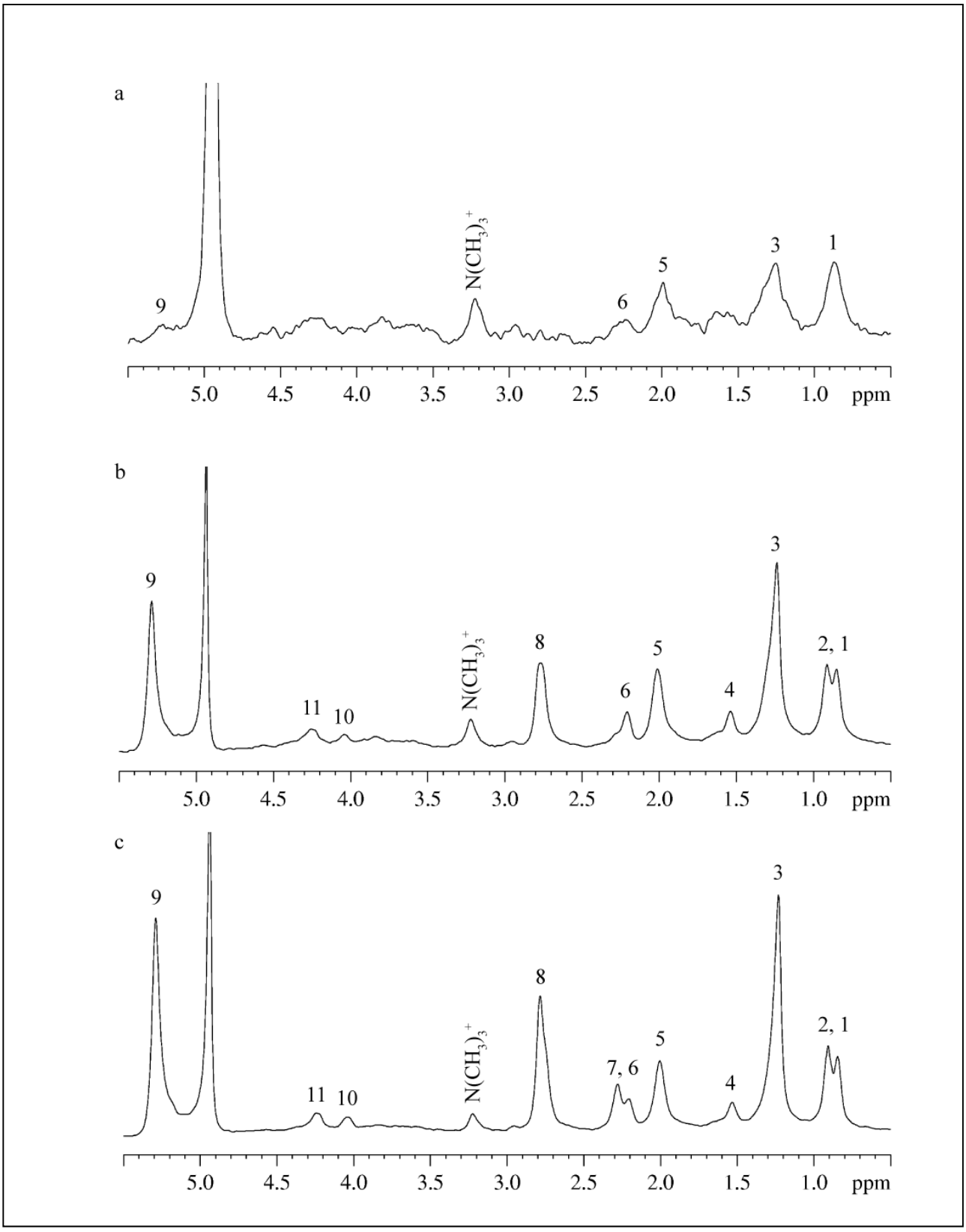

Figure 3. ^{1}H HR-MAS NMR diffusion-edited spectra from control (a), EPA (b), and DHA (c) supplemented cardiomyocytes.

Lipid components are labeled as in previous figures and: 10 and 11, bonded glycerol.

The evaluation of the variation of the mobile lipid components can be obtained by the integration of the resonances present in HR-MAS led diffusion-edited spectra (figure 4). Supplemented cardiomyocytes show a general increase of lipid signals, particularly unsaturated FA, which are almost absent in control cells (lack of signals at 2.78 ppm and 5.33 ppm). Instead, the PL fraction ($N(CH_3)_3^+$ signal at 3.26 ppm) does not seem significantly affected by supplementation (see figure 3). In EPA and DHA supplemented cardiomyocytes glycerol bonded signals (at 4.10 and 4.30 ppm), absent in control cells, are detected, indicating that NMR-visible FA are involved in TAG. The comparison between theoretical (6:4) and experimental (7.4:4) TAG integral ratio between the CH_2CO (2.25 ppm) and glycerol (4.30 and 4.10 ppm) signals in EPA supplemented cell spectra points to the presence of almost completely glycerol-esterified FA chains, and a slight excess of unesterified FA (FFA). The same approach, if applied to DHA samples, is less straightforward. In this case two signals, at 2.33 and at 2.25 ppm, derive from CH_2CO protons (DHA and other FA, respectively), and the former receives also a contribution from CH_2-C-CO protons of DHA. Moreover, the signal at 2.25 ppm accounts for about 25-30% of the total integral (2.25+2.33). The theoretical ratio between the integrals of the signals at 2.25+2.33 ppm and that of glycerol (4.30 and 4.10 ppm) is 12:4 in the case of glycerol-triesterified with DHA (signal at 2.25 ppm absent) and 6:4 in the case of glycerol-triesterified with FA other than DHA (signal at 2.33 ppm absent). On these basis, in DHA supplemented cells we calculate a [DHA/(FA other than DHA)] ratio of about 3:2 and a theoretical ratio of (12x0.6+6x0.4)= 9.6:4 for FA involved in TAG. This compares well with the experimental value of 10.1:4, and indicates that the NMR visible lipid signals are due to almost completely glycerol-esterified FA chains also in DHA supplemented cells.

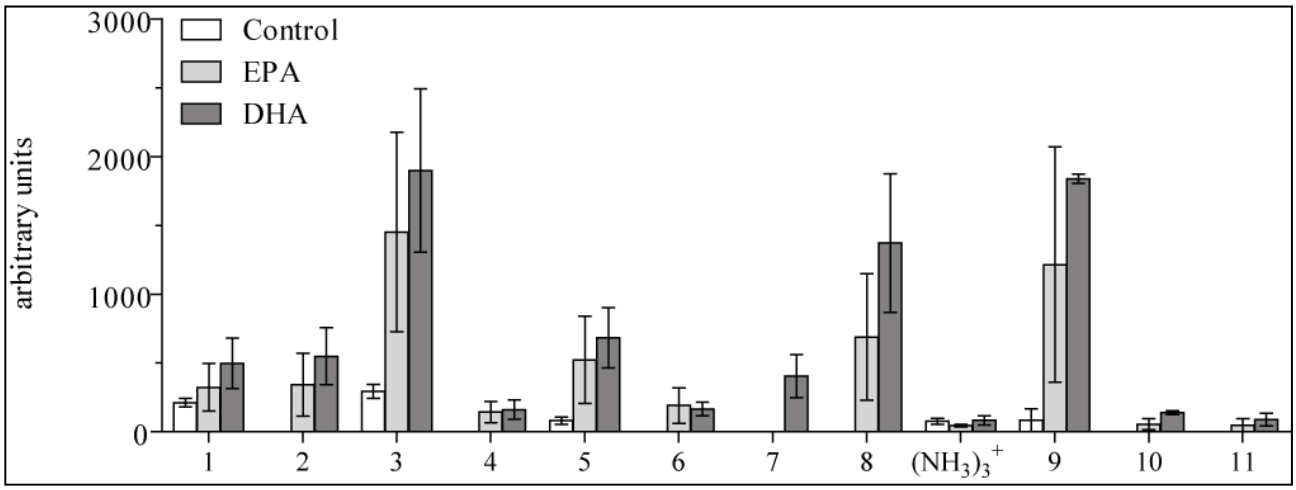

Figure 4. Relative amount from HR-MAS diffusion edited spectra of lipid components from control, EPA, and DHA supplemented cardiomyocytes.

X axis represents ppm, and Y-axis intensity; the spectra are scaled respect to $-CH_3$. Data are reported as means ± SEM. Lipid components are labeled: 1, $-CH_3$ (0.89 + 0.96 ppm); 2, $-CH_3$ from EPA and DHA (0.96 ppm); 3, acyl chain methylene- $(CH_2)_n$; 4, CH_2-C-CO; 5, CH_2C=C; 6, CH_2CO (2.25 ppm); 7, CH_2CO and CH_2-C-CO from DHA (2.33 ppm); 8, =C-CH_2-C=; 9, CH=CH; 10 and 11, bonded glycerol (4.10 and 4.30 ppm).

Figure 5 reports the CPMG HR-MAS ^{1}H NMR spectra of control (5a), EPA (5b) and DHA (5c) supplemented cardiomyocytes. In the spectra some residual signals from EPA and DHA are present, indicating that some protons in PUFA chains are characterized by long transverse relaxation times T_2. Hence the chains are not only slowly diffusing (as appears from diffusion edited spectra), but also free to rotate. This situation is usually indicative of the presence of lipid bodies (LB) in the cell [49, 50, 52]. As above reported, the NMR data show that these lipids are essentially constituted by mobile TAG.

The analysis of the CPMG spectra shows that supplementations do not have relevant effects on the small metabolites (polyols, osmolites, and aminoacids) profile. They were assigned by direct inspection of 1D and 2D spectra (COSY and TOCSY spectra, here not reported), and by comparison with literature data [53, 54] The main metabolites detected by HR-MAS NMR in cardiomyocytes are ethanol, lactate (Lac), Ala, acetate (Ac), Glu, Cr, Tau, Gly, choline (Cho), ethanolamine (Etn) phosphocholine (PCho), glycerophosphocholine (GPC), Sci and Myo, PEtn, uridine-diphosphate (UDP), adenine, trace of glutamine (Gln), and lysine (Lys).

GC. In the PL fraction (figure 6a), EPA supplementation to cardiomyocytes leads to an enhancement of the relative molar content of both EPA itself and its metabolic elongation derivative docosapentaenoic acid (22:5n-3, DPA) [28]. No further conversion of n-3 DPA to DHA is detected. Following DHA supplementation, the relative molar content of this FA increases, without any appreciable retroconversion to DPA or EPA. The increased incorporation of n-3 PUFA is accomplished to a decrease in palmitoleic (16:1n-7), oleic (18:1n-9), and arachidonic acid (20:4n-6, ARA) relative molar contents.

In the TAG fraction (figure 6b), EPA is not incorporated as itself but after conversion in its derivative DPA [55], while DHA is incorporated without any further metabolization. In both cases, a concomitant decrease in the relative molar content of saturated FA and ARA is detected.

The total amount of FA esterified in the PL fraction is slightly lower in DHA supplemented cells than in controls (figure 7a). On the contrary, the low amount of FA esterified in the TAG fraction of control cells significantly increases after EPA and DHA supplementation, as shown in figure 7b.

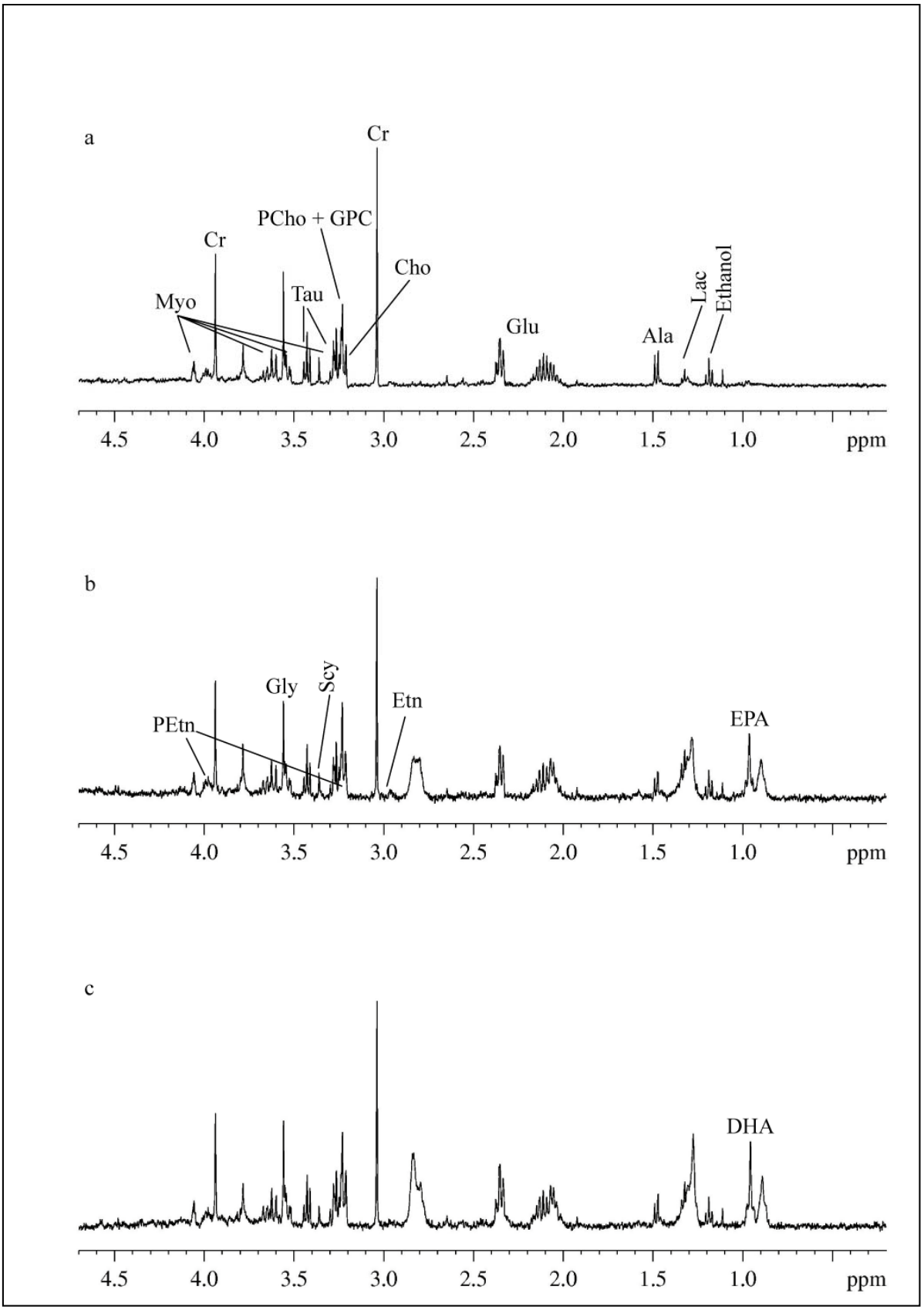

Figure 5. ^{1}H CPMG *ex vivo* HR-MAS ^{1}H NMR spectra of control (a), EPA (b), and DHA (c) supplemented cardiomyocytes.

The major metabolites are labeled as in previous figures and: choline (Cho), ethanolamine (Etn), glycerophosphocholine (GPC), lactate (Lac), phosphocholine (PCho).

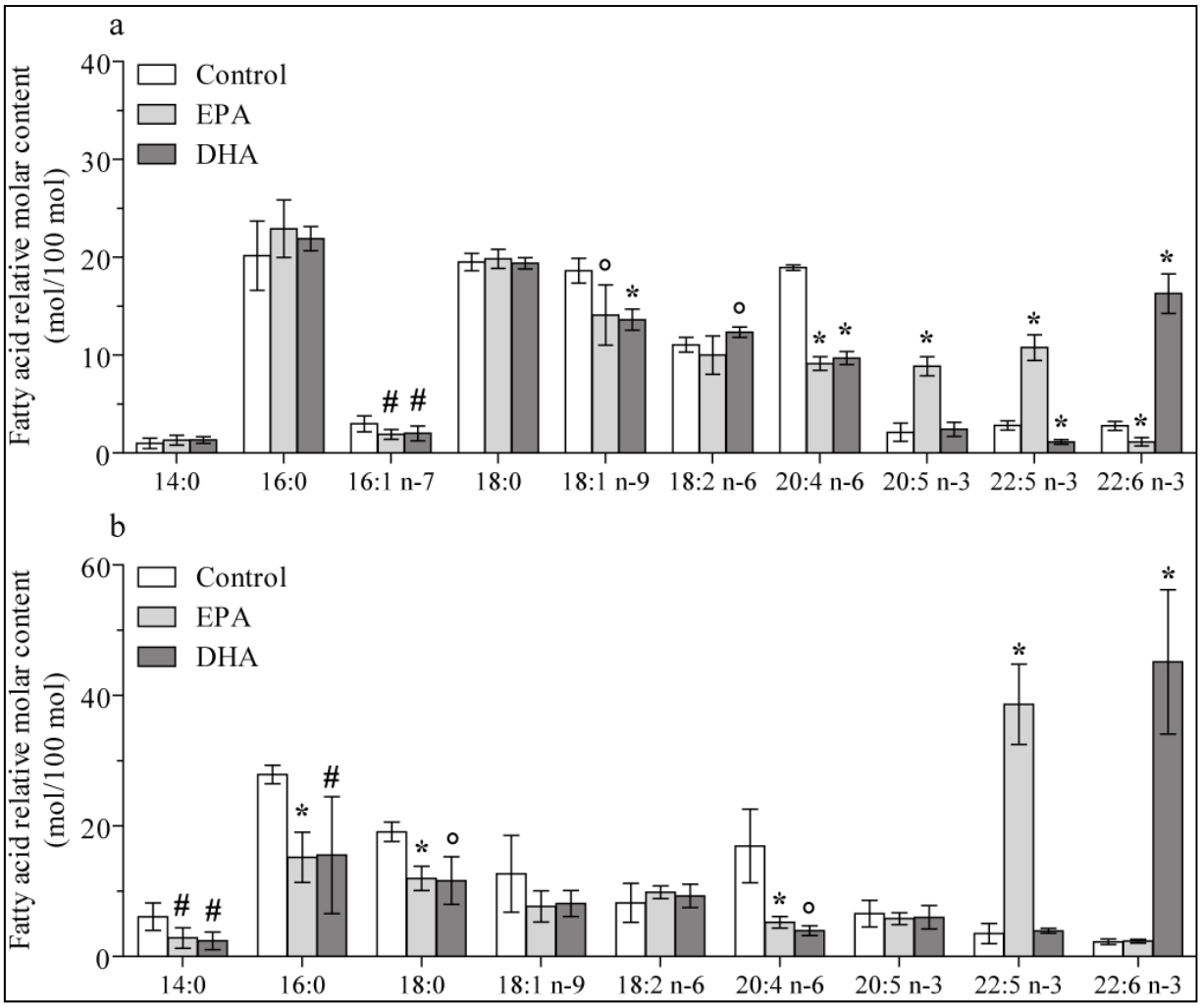

Figure 6. Fatty acids composition of PL (a) and TAG (b) derived control, EPA, and DHA supplemented cardiomyocytes.

Data are expressed as moles/100 moles and are means ± SD of at least three independent cell cultures. Statistical analysis was by the Student's t test comparing control and n-3 LC-PUFA supplemented cells: # P<0.05; ° P<0.01; * P<0.001

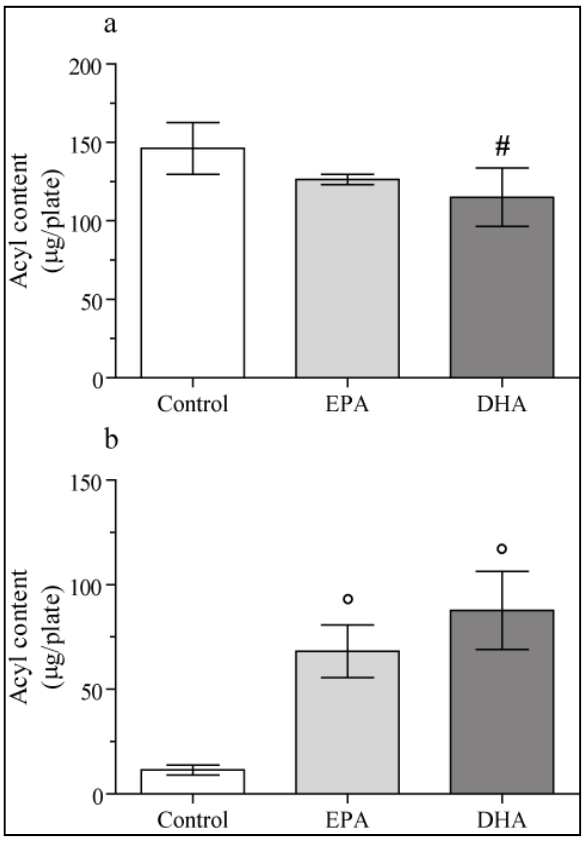

Figure 7. Acyl content of PL (a) and TAG (b) derived from control, EPA, and DHA supplemented cells.

Data are expressed as µg/plate and are means ± SD from at least three independent cell cultures. Statistical analysis was by the Student's t test comparing control and n-3 LC-PUFA supplemented cells: # P<0.05; ° P<0.01; * P<0.001.

DISCUSSION

Cells contain a considerable amount of polar lipids, PL and C, and neutral lipids such as TAG and CE. Taken together, these account for roughly 4-16% of total and some 20-50% of dry cell mass [56]. Although NMR is considered a universal detector, lipid ^{1}H NMR resonances are not always observed in tissues that are rich in C and PL. This directly implies that ^{1}H NMR visible lipids must have unique structural and biochemical properties that set them apart from the bulk of tissue lipids. This apparent paradox is resolved by considering the physical basis of NMR, which underscores the importance of sufficient molecular mobility in the immediate chemical environment for a given molecule to become detectable by NMR [52]. Lipid resonances must arise from the isotropically tumbling, relatively non restricted molecules. Since it is known that LC-PUFA mainly incorporate into membrane PL, the use of NMR alone does not allow a complete overview of the lipid environment. For this reason, we coupled GC analysis to NMR. As expected, signal related to PL is very low in NMR analysis, regardless the experimental condition, whereas GC analysis, besides detecting PL, shows that EPA and DHA supplementation causes a wide modification of PL FA composition. In several studies, the biological effects of n-3 LC-PUFA have been ascribed to the incorporation of these FA into PL in cellular membranes, where they may alter membrane fluidity and functionality, and may affect cellular eicosanoid synthesis [57-60]. According to GC data, EPA and DHA are actively incorporated in the PL fraction displacing ARA, which relative molar content is reduced by more than 50%. It is conceivable that the modifications occurred in PL after EPA and DHA supplementation could cause important changes not only in membrane structure, but also in membrane and cell functionality. The total amount of FA esterified in PL is decreased in supplemented cardiomyocytes, particularly in DHA supplemented ones (Figure 7a). The larger packing free volume associated with LC-PUFA-rich membranes could explain in part the reduction of the acyl content in the PL fraction. In fact, in saturated or monounsaturated membranes the acyl chains pack quite uniformly, while in membranes containing DHA the packing is distorted by the steric restrictions associated with a higher surface area of the FA [61]. As an alternative, EPA and DHA could have triggered a remodeling mechanism causing an increase in lyso-PL. Actually, DHA and EPA have been shown to alter cell membranes increasing the activation of phospholipase A2 [62].

The most important difference between control and n-3 LC-PUFA supplemented cardiomyocytes highlighted by HR-MAS NMR spectroscopy is the increase of signals coming from mobile lipids, identified as TAG. Regarding TAG, two alternative sources for ^{1}H NMR visible mobile lipid resonances have been proposed, i.e. membrane-associated globular microdomains [63, 64] and intracellular LB [65, 66]. Many *in vitro* cell studies and *in vivo* diffusion measurements have

suggested the importance of LB as contributors to ^{1}H NMR detectable lipid resonances [52, 65-67]. It is therefore conceivable that the observed increase of NMR-visible TAG in supplemented cells is associated to an enhanced presence of LB, as already reported for human cardiomyocytes [68]. The observed increase of mobile TGs could represent a metabolic response to n-3 LC- PUFA supplementation, which leads to an increased lipid storage. The increase in the total amount of FA incorporated in TAG, as well as the higher relative molar content of n-3 LC-PUFA in this fraction after supplementation, is confirmed by GC. Our data are in agreement with Finstad *et al.* [69], who evidenced in monocytic U937-1 cells an increase in TAG content after treatment with 60 μM EPA. This TAG increase was accompanied by the accumulation of LB.

The second hypothesis has been considered the more probable in the last years [70, 71]. The sequestration of mobile lipids in LB provides a deposit of stored energy that can be accessed in a regulated fashion according to metabolic need [72]. In particular, the myocardium stores FA, the major energy substrate for normal heart function, as TAG in LB [73].

Incorporation of n-3 LC-PUFA into TAG-rich LB could also be the basis for a yet unknown mechanism important for cell regulation. First, the storage of FA in LB may function to protect the cells from exposure to high concentrations of FFA. Secondly, a TAG pool rich in LC-PUFA may be an important reservoir for signal molecules. Finstad *et al.* [69] suggested that EPA, DPA and DHA, when liberated from an intracellular storage pool, affect intracellular signal transduction systems differently than when released from cellular PL. Furthermore, LC-PUFA stored as TAG in LB could be used for membrane PL synthesis. In the heart, the synthesis of PL to ensure membrane homeostasis is a priority that warrants the membrane depolarization that triggers cardiac contraction [73]. It is also possible that n-3 LC-PUFA stored in TAG may influence cardiomyocytes gene expression via PPAR-dependent signal pathways. We have already demonstrated that incubation of with 60 μM EPA and DHA alters the expression of different genes [74].

The accumulation of LB has been correlated with the reduction in cell number, inhibition of proliferation and induction of apoptosis in monocytic U937-1 cells [69]. In a previous study, supplementing neonatal rat cardiomyocytes with the same EPA and DHA concentration, we observed a modest increase in cell growth and no significant alteration in cell cycle distribution. Furthermore, n-3 LC-PUFA supplementation significantly protected cardiomyocytes from apoptosis [74]. The different cell type used in these studies can explain the different results, underlining that cell response to LC-PUFA supplementation can be deeply different.

The signal from C or CE is not detected in cardiomyocyte NMR spectra, probably due to their low mobility. Similarly, the TLC spot corresponding to CE was hardly detectable, regardless the

supplementation. This is in agreement with Finstad *et al.* [75] who, supplementing different cell lines with labeled EPA, evidenced that only the 1% of the FA was recovered in the CE fraction.

Regarding the small molecules, the HR-MAS NMR metabolic characterization of cardiomyocytes exhibits only negligible variations of the metabolic profile among differently treated cells. Indeed, the metabolome of unsupplemented and supplemented cells is characterized by the same small metabolites (mainly Ala, Lac, Ac, Glu, Cr, Tau, Gly, Cho, PCho, GPC, Sci, Myo, PEtn, Gln, UDP, adenine and Lys) in almost the same relative ratios. N-3 LC-PUFA supplementation to neonatal rat cardiomyocytes causes a huge variation in the cell lipid environment, but a negligible variation in the metabolome.

In conclusion, the simultaneous use of ^{1}H HR-MAS NMR spectroscopy and GC permits to derive information on the cell lipidome and metabolome. This work shows the complementary potential of these techniques. Furthermore, in our knowledge it is the first report on changes in the lipid components of cardiomyocytes after n-3 LC-PUFA supplementation. The understanding of the modification of the lipid environment represents a further step towards the clarification of n-3 LC-PUFA role in cardiovascular disease prevention.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

III. N-3 PUFA regulation of genes related to cholesterol metabolism is related to genotype

INTRODUCTION

Although cholesterol (Chol) is essential for membrane structure and as precursors of hormones, vitamin D, bile acids and other bioactive molecules [76], it is perceived as dangerous since its increased total plasma concentration is an important risk factor for cardiovascular diseases (CVDs) [77]. Human Chol concentration mainly depends on *de novo* endogenous biosynthesis and on dietary intake, with a ratio estimated as ~70:30 [78]. Endogenous synthesis is subject to feedback control by hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoAR) activity, the rate-limiting enzyme in Chol biosynthesis that catalyzes the formation of mevalonate from HMG-CoA [79]. The exogenous supply is mainly controlled by the modulation of the low-density lipoprotein receptor (LDLr) [80], which mediates the clearance of Chol and cholesteryl ester-containing low-density lipoprotein (LDL) particles from the blood [81].

Prevention trials using statins have demonstrated that lowering Chol results in a substantial decreased risk of CVD events [82]. Statins are widely used for reducing hypercholesterolemia although their side effects have been largely demonstrated [83, 84]. It would be therefore crucial to identify nutritional agents able to reduce cholesterolaemia, and to accomplish them to a low-Chol diet for an effective cholesterol-lowering nutritional strategy.

The feedback control of HMG-CoAR and LDLr occurs through regulation of gene transcription or by regulation of protein level through translation or degradation [85]. Both *Hmgcr* and *Ldlr* encoding genes exhibit the sterol response element (SRE) sequence, and are recognized as SREBP-regulated genes [86, 87]. The sterol regulatory element binding proteins (SREBPs) are helix-loop-helix transcription factors involved in the transcription of gene related to Chol and lipid biosynthesis. Three members of the SREBP family have been described in several mammalian species: SREBP-1a and 1c produced from a single gene (*Srebf-1*) located on human chromosome 17p11.2, and SREBP-2 from a separate gene (*Srebf-2*) located on human chromosome 22q13. SREBP-1a and SREBP-2 increase the expression of genes involved in Chol synthesis, and SREBP-1c of genes related to fatty acid (FA) and triglyceride (TG) synthesis [88]. The endoplasmic reticulum (ER) houses the precursor of SREBP and associated proteins, the cholesterol-sensor protein SCAP (SREBP Cleavage Activating Protein) and the ER retention protein, Insig [89]. Chol

biosynthetic enzymes, such as HMG-CoAR, also reside in the ER [90] along with the Chol etherifying enzyme acyl-CoA: cholesterol acyltransferase (ACAT) [91] Excess cellular Chol is converted to cholesteryl esters (CE) by ACAT-2 or is removed from cell by cellular Chol efflux at the plasma membrane [92]. Sterol-sensing domains are present in several membrane proteins, including HMG-CoAR, ACAT, and the SREBP cleavage–activating protein [92].

Polyunsaturated fatty acids (PUFAs) are among nutritional candidates for lipid synthesis regulation. Fish oil, rich in n-3 PUFAs, has proven to lower serum levels of triglycerides (TG), Chol, free fatty acids, and to elevate high-density lipoprotein cholesterol (HDL-chol) [12-14]. It has been suggested that some of the beneficial effects of n-3 PUFA are due to changes in membrane fatty acid composition and subsequent alterations in hormone signalling. However, fatty acids, their CoA derivatives and their metabolites are able to regulate gene expression, up-regulating the expression of genes encoding proteins involved in fatty acid oxidation while simultaneously down-regulating genes encoding proteins of lipid synthesis [93].

In this study we have evaluated in rats the modulation of genes related to Chol homeostasis by a 3 month administration of a diet enriched inn n-3 PUFA. In particular we have evaluated the expression of genes encoding for SREBP-1, SREBP-2, HMG-CoAR, LDLR and ACAT2 in the liver.

We used SH rats since they are the most commonly used animal models for the metabolic syndrome (MS) [94]. Actually, SH rats develop insulin resistance, hyper-triglyceridemia, abdominal obesity, hypertension, and hypercholesterolemia [95]. It is reported that naturally occurring variation in the gene encoding the SREBP-1 isoforms might contribute to inherited variation in lipid metabolism in the SH versus other strains of rats [96]. We also used Wistar Kyoto (WK) rats as control.

To evaluate the effect of the different diets on lipemia, as well the incorporation of the supplemented PUFA, plasma lipid profile and total lipid fatty acid composition in plasma and liver were also determined in all rats. To evaluate possible modification of cholesterol content in liver, nuclear resonance spectroscopy (NMR) analysis was performed on this organ.

Our findings confirm that dietary n-3 PUFA are absorbed, and incorporated in hepatic tissue. In SH animals PUFA supplementation recovered the imbalanced plasma lipids status. At molecular level, n-3 PUFA exerted effects in both strains, but in a different way, evidencing the importance of genotype in the mechanisms of n-3 PUFA cholesterol lowering action.

MATERIALS AND METHODS

Materials

Diets were from Mucedola (Milan, Italy). Chloroform and n-hexane were from Carlo Erba SpA Co. (Milan, Italy). Methanol, potassium chloride, sodium sulphate anidre, TRIS borate EDTA buffer, Chloroform-d 99.8 atom % D, and Methanolic- chloridric acid 3N were purchased from Sigma Chemical Co. (Milan, Italy). 25 bp and 1 Kb Plus DNA Ladder, SYBR Safe DNA gel stain 10,000X in DMSO, and Ultra Pure Agarose were from Invitrogen (Paisley, UK). Lipoprotein measurements commercial kits were from (Roche Diagnostics SpA, Milan, Italy). QIAshredder, RNeasy mini kit, Quantitect reverse transcription kit, QuantiTect SYBR Green PCR Kit, and premade primers Quanti Tect Primer Assay 200: *Srebf-1* (QT00432684); *Srebf-2* (QT00403305); *Acat-2* (QT00412461); *Ldlr (*QT00177744); *Hmgcr* (QT00182861); *Gapdh* (QT00199633) were from Qiagen (Hilden, Germany). *β actin* was a custom primer purchased from IDT, Integrated DNA Technologies (San Diego, California)

Methods

Animals and diet. N-3 PUFA solution used for diet preparation contained eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as ethyl esters, in the ratio 0.9:1.5. N-3 PUFA solution was added in appropriate amounts (0.1% w/w) to control diet during its preparation. Protein, lipid and carbohydrate content of control diet were in the normal range of adequacy for rats (g/100 g diet): proteins about 21g; lipids about 8g; carbohydrates about 61.5g, and contained appropriate amounts of vitamins and other minerals.

14 male WK rats and 14 male SH rats, aged 14 weeks, were used. Animals were housed in individual cages under strictly controlled conditions of temperature (20 ± 2 °C) and humidity (60–70%), with a 12-hour dark-light cycle, and were weighed each week. Water was provided ad libitum. After a 15 day period of acclimatation at control diet, both WK and SH rats were randomly divided into two groups, one fed the control diet (WK-St and SH-St) and the other fed the PUFA supplemented diet (WK-PUFA and SH-PUFA). . After 90 days of dietary treatment, rats were weighted, anesthetized and killed. Blood samples were collected in heparinised test tubes, and livers were quickly excised and frozen at -80°C in RNA Later.

Plasma was obtained by centrifugation at 800 X g for 5 min. Then samples were stored -20°C until analysis.

The Animal Care Committee of the University of Bologna approved the study

Lipid profile and fatty acid composition. The lipid profile (plasma triglycerides, total and HDL-cholesterol) was analysed using enzymatic and colorimetric methods with commercial kits (Roche Diagnostics SpA, Milano, Italy) according to manufacturer. The concentrations were determined spectrophotometrically using a Hitachi 911 auto analyser. Low-density lipoproteins (LDL-chol) were estimated indirectly by using formula: (LDL-chol)=TG-(HDL-chol)-(TG/5) [97, 98] Atherogenic index was calculated using the following formula: Atherogenic index= [TG-(HDL-chol)]/HDL-chol.

Total lipid fatty acid composition was determined on plasma and liver by gas chromatographic analysis. Whole lipids were extracted from 300µl plasma or 0,3 g hepatic tissue according to *Folch et al.* [38]. Fatty acid methyl esters were prepared from all samples according to *Stoffel et al.*[39]. Methyl esters dissolved in n-hexane were gas chromatographed on a Carlo Erba model 4160 (Milan, Italy) equipped with a capillary column (30 m×0.25 mm i.d.) filled with a thermo stable stationary phase (SP 2340, 0.10–0.15 µm film thickness), at a programmed temperature (160–210 °C, with a 8 °C/min gradient), with He as carrier gas at a flow rate of 2 ml/min as previously reported [40, 99]. Gas chromatographic traces and quantitative evaluations were obtained using a Chrom Card Software (Thermo Electron Scientific, Milan, Italy) computing integrator.

Statistical analysis was by the Student's *t*-test [100].

Liver cholesterol concentration. Total lipids were extracted from 1 g liver according to *Folch et al.* [38]. The solvent was then dried under nitrogen, and the lipid fraction resuspended in deuterated chloroform. Cholesterol concentration was evaluated by High Resolution Magic Angle Spinning (HR-MAS) NMR. 1D ^{1}H HR-MAS spectra were recorded with a Bruker Avance400 spectrometer equipped with a $^{1}H/^{13}C$ inverse probe operating at 400.13 and 100.61 MHz, respectively. In the ^{1}H NMR spectra, free Chol signal was evidenced at 0.65ppm and 1,01ppm, while cholesteryl ester signals were not detectable (figure 1). Since retinol (signal at 1.13ppm) was present in the same amount in both experimental diets, we could assume its liver concentration as a constant. Therefore retinol concentration was used for free cholesterol signal normalization.

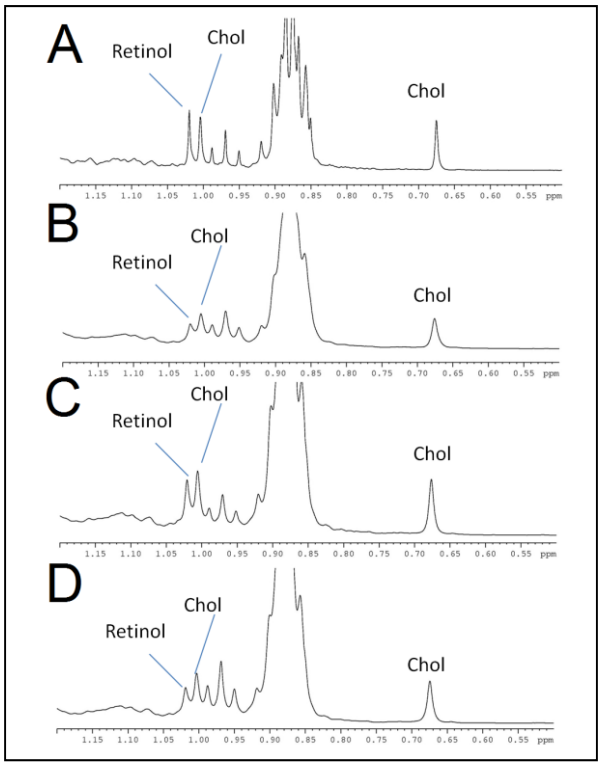

Figure 1: HR-MAS spectra of hepatic tissue from WK and SH rats fed the different diets.

A) WK-CT B) WK PUFA C) SH-CT; D)SH PUFA

Statistical analysis of NMR data was by the Students't test.

RNA extraction, retro-transcription and gene expression analysis. 30 mg of hepatic tissue were mechanically disrupted by sterile scissors and homogenized using QIAshredder (Qiagen, Hilden, Germany) according to manufacturer. RNA was extracted using RNeasy mini kit (Qiagen, Hilden, Germany) and elution was done in a final volume of 50 µl. Samples integrity were assessed by nanodrop nd-100. All used samples had absorbance A $_{260/280}$ in the range of purity.

Retro transcription was performed on 200ng of extracted RNA in a 20µl total reaction volume using Quantitect reverse transcription kit (Qiagen, Hilden, Germany) as follow. Samples were incubated 2 minute 42°C with gDNA Wipeout Buffer to eliminate possible genomic DNA contamination. After this samples were quickly put on ice and reverse transcription mix were added as shown by manufacturer. Optimized blend of oligo-dT and random primers dissolved in RNAse free water supplied by the kit were used as primers. Mix contains also RNAse inhibitors. Reaction conditions were as follow: 15 min 45°C, 3 min 95°C and 5 min 4°C. Obtained cDNA were quickly stored at -20°C. Relative gene expression analysis was performed on 6000 Rotor gene (Corbett, Sydney, Australia) by two step real-time q-PCR assays using SYBR® Green detection (Qiagen: QuantiTect SYBR® Green PCR Kit). Amplification was done in a 25 µl final volume including 1 µl of cDNA as template. The PCR Master Mix was prepared according to Qiagen protocol and amplification conditions were as follows: 15 min at 95 °C followed by 45 cycles (94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s). Melt curve were obtained every time 15sec at 95°C. Amplicons length were assessed using 2% agarose gel electrophoresis using SYBR green 1X Invitrogen (Paisley, UK). The primer pairs used were (Qiagen: Quanti Tect Primer Assay 200). The sequences are confidential and available data are reported in table 1. All primer set were bioinformatically validated by QIAGEN. Actin-β primer was a custom primer: Forward: GGGAAATCGTGCGTGACATT (20 bp) Reverse: GCGGCAGTGGCCATCTC (17 bp). Amplicon length was 76 bp on the recognized sequence

NM_031144. Primer specificity was evaluated using melt curve that showed unique specific peak in all cases. Q-PCR validation was carried out by standard curve. Reaction efficiency and R value were in the range of 80-100% and 0,99 respectively, for all examined genes.

Three expression technical replicate was performed on RT duplicate. Seven biological repeats for each experimental group were used. Relative quantification of the mRNA levels of all genes in exam was determined using the Rotor-gene 6000 software 1.7 comparative quantification analysis, in which reaction efficiency is calculated on each reaction tube. All samples used had >85% efficiency.

Gene	QIAGEN code	Amplicon length	Recognized sequence	Between exons position
Srebf-1	QT00432684	82	XM_213329 XM_001075680	6-7
Srebf-2	QT00403305	86	XM_216989	8-9
Acat-2	QT00412461	72	NM_001006995	8-9
Ldlr	QT00177744	111	NM_175762	3-4
Hmgcr	QT00182861	87	NM_013134	10-11
Gapdh	QT00199633	149	NM_017008	1-3

Table 1: Qiagen Quanti Tect Primer available information.
For each employed gene, gene code, amplicon length, recognised sequence on gene bank, between exons amplicon position are reported.

Srebf-1 primer is between exons 6 and 7, so can recognize both the isoform –a, and -c, but SREBP-1c is the predominant SREBP-1 isoform in the liver [101], so our findings are ascribed to this isoform.

GAPDH was chosen as reference gene since it is reported that fish oil supplementation has no effect on its expression [102]. Actin-β was previously used in gene expression study involving PUFA supplementation by *Caplan et al.* and by *Prasad et al.* [103, 104].

Statistical analysis of gene expression data was performed by REST 2009 software in Rotor Gene (RG) Mode that use Taylor's series to find statistical differences, as shown by *Pfaffl et al.* [105]. Gene expression was normalized on the reference genes glyceraldehyde-3-phosphate-

dehydrogenase (GAPDH) and actin-β. All sample groups were referred to WK standard diet rat calibrator group.

RESULTS

Plasma lipid profile

The lipid profile of WK and SH rats fed the different diets are reported in table 2.

Comparing control diet fed groups, total cholesterol, HDL-chol and TG were lower in SH than in WK rats, while LDL-chol was higher. In both PUFA fed groups, total cholesterol and triglyceride were significantly lower than in the corresponding control. In SH rats the LDL fraction was also reduced by PUFA. While HDL fraction was not affected. As a result, the atherogenic index higher in control SH than in control WK rats, was normalized by the PUFA diet

Parameter (mg/dl)	WK-CT	WK-PUFA	SH-CT	SH-PUFA
TOT-chol	$107.60 \pm 2.70^{a,b,c}$	$88.40 \pm 2.30^{a,d}$	$91.60 \pm 4.83^{b,e}$	$73.40 \pm 3.65^{c,d,e}$
HDL-chol	$41.80 \pm 2.49^{a,b}$	37.20 ± 2.28^{c}	$26.00 \pm 3.74^{a,c}$	31.20 ± 5.85^{b}
LDL-chol	26.24 ± 6.19^{a}	25.48 ± 3.04^{b}	$41.48 \pm 9.57^{a,b,c}$	23.88 ± 6.14^{c}
TG	$197.80 \pm 14.20^{a,b,c}$	$128.60 \pm 12.44^{a,d}$	$120.60 \pm 12.50^{b,e}$	$91.60 \pm 11.28^{c,d,e}$
Atherogenic index	1.44 ± 0.29^{a}	1.38 ± 0.14^{b}	$2.59 \pm 0.60^{a,b,c}$	1.41 ± 0.41^{c}

Table 2: Plasma lipid profile in WK and SH rats fed the different diet.

Data are means +/- SD of 7 animals per group. Statistical analysis was by the one way ANOVA using Tukey's as post test. Similar superscript letters indicate statistical significance (at least $p<0.05$)

Plasma fatty acid composition

The fatty acid composition of plasma total lipid is reported in figure 2A. EPA (20:5n-3) and DHA (22:6 n-3) concentration increased in both PUFA-fed groups, evidencing the absorption of the supplemented fatty acids. In WK rats this increase was accomplished to a decrease in linoleic acid (18:2 n-6, LA) content, while in SH ones to a decrease in arachidonic acid (20:4 n-6, AA) content.

Dietary EPA and DHA were also incorporated in the hepatic tissue (figure 2B), mainly at the expense of AA.

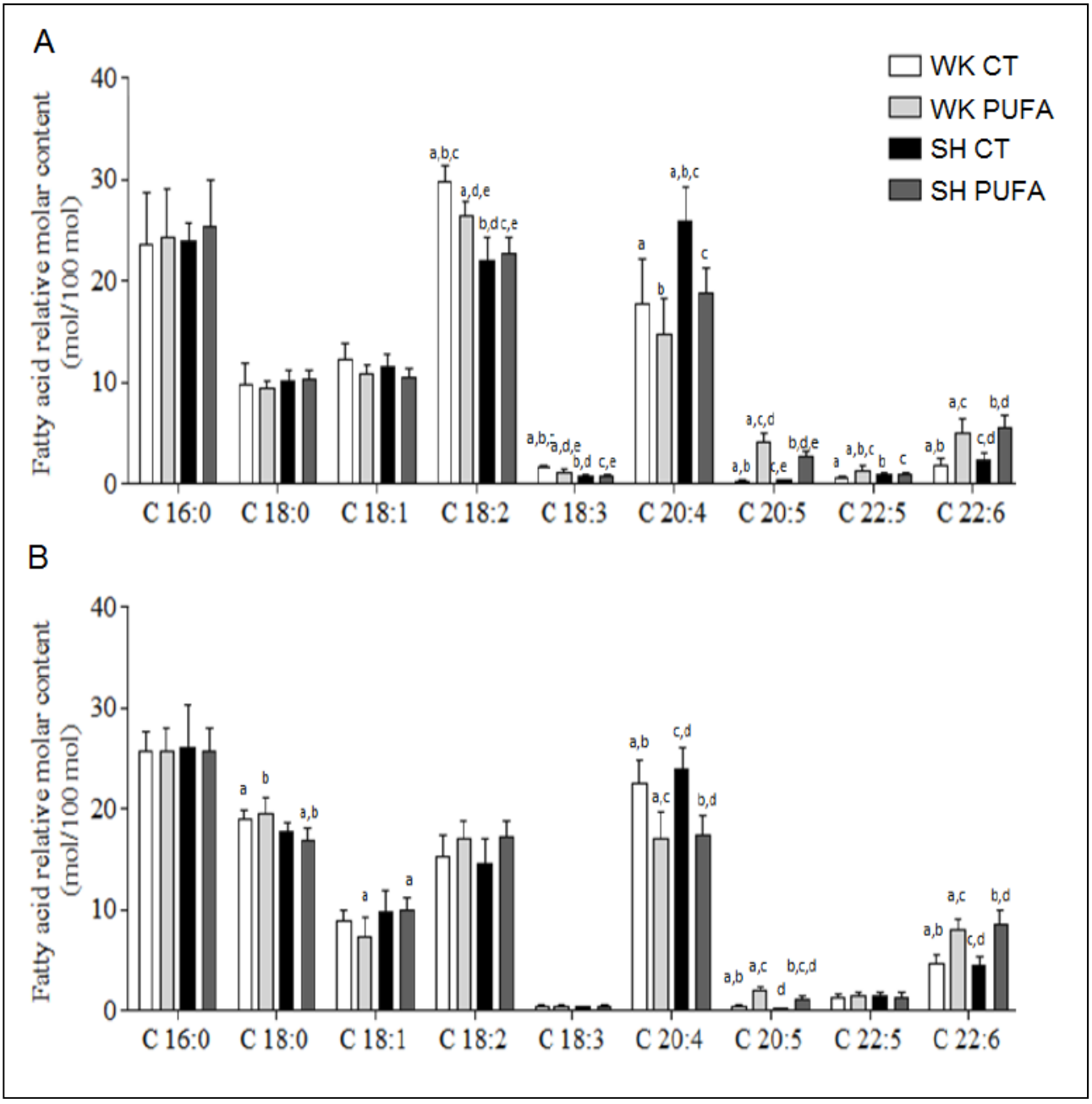

Figure 2. Plasma (A) and liver (B) total lipid fatty acid composition in WK and SH rats fed the different diets.

Data are means +/- SD of 7 animals per group. Statistical analysis was by the Students't test. Similar superscript letters indicate statistical significance (at least $p<0.05$).

NMR free hepatic cholesterol revelation.

In control condition, free cholesterol content (Figure 3) was higher in the liver of SH than WK rats. N-3 PUFA enriched diet increased liver cholesterol concentration in WK animals only.

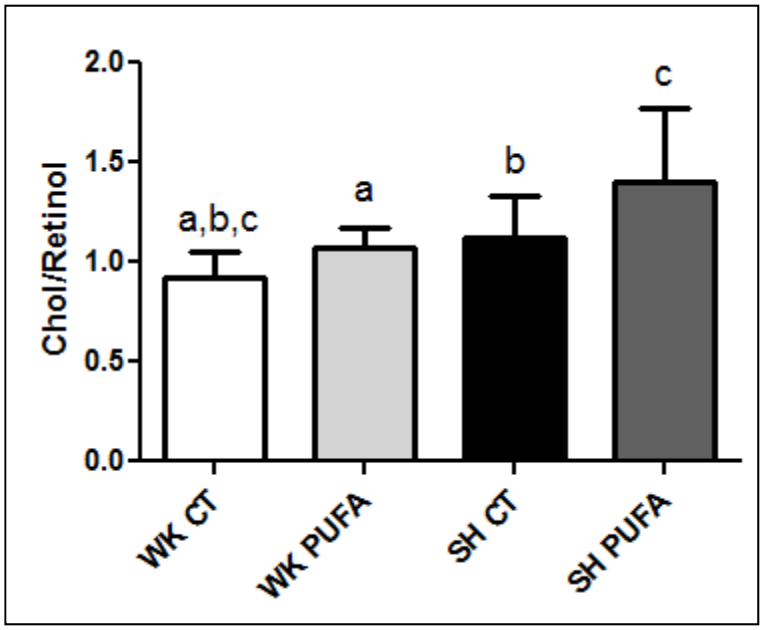

Figure 3. Free cholesterol concentration in liver of WK and SH rats fed the different diets.

Cholesterol is reported as the ratio between the intensity of cholesterol and retinol signals. Data are means +/- SD of 7 animals per group. Statistical analysis was by the Students' t test . Similar superscript letters indicate statistical significance (at least $p<0.05$).

Gene expression analysis.

In rats fed the control diet no differences were observed in both *Srebf-1* and *Srebf-2* gene expression (figure 4 A and B respectively). PUFA treatment down regulated *Srebf-1* expression in SH animal only, without any effect on *Srebf-2*.

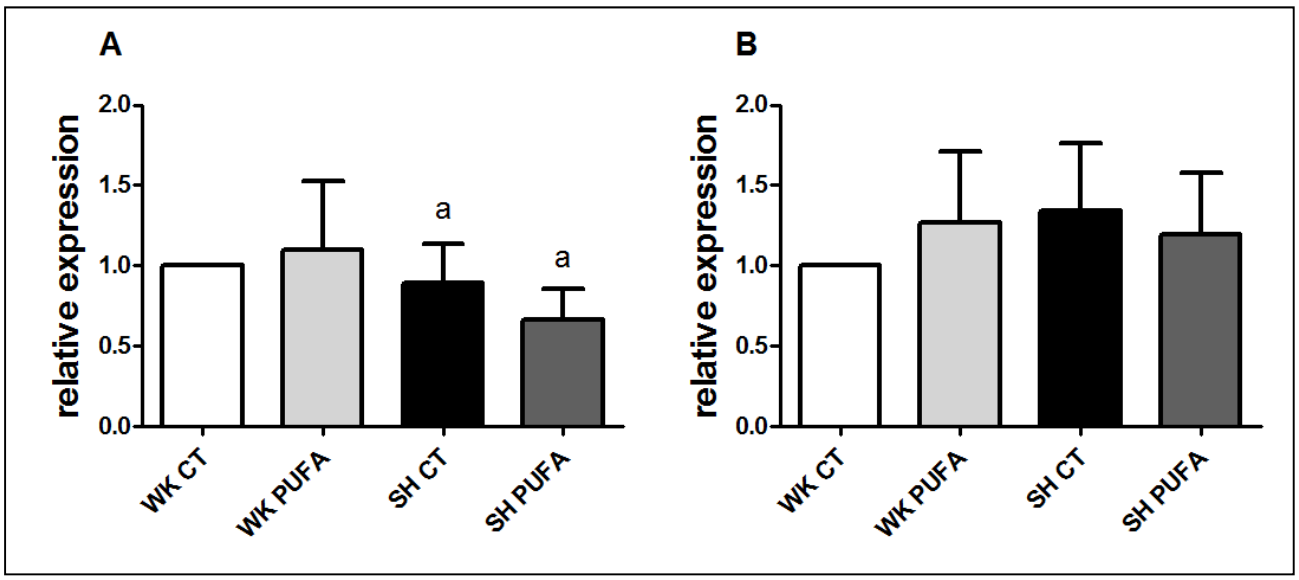

Figure 4. *Srebf-1* (A) and *Srebf-2* (B) gene expression in the liver of WK and SH rats fed the different diets.

Data are median +/- standard error. Significant differences were evaluated with Taylor's series statistical analysis. Similar superscript letters indicate statistical significance (at least $p<0,05$).

No difference was observed in *Hmgcr* expression between WK and SH animals fed the control diet (figure 5A). PUFA treatment down-regulated *Hmgcr* expression in both groups.

In control condition, *Ldlr* gene expression (figure 5B) appeared up-regulated in SH rats. The n-3 PUFA enriched diet decreased *Ldlr* expression in SH animals, without any effect in WK ones.

Acat-2 gene expression (figure 5C) appeared up-regulated in control SH rats compared to the corresponding WK. The n-3 PUFA rich diet further increased *Acat-2* expression in SH animals, without any effect in WK ones.

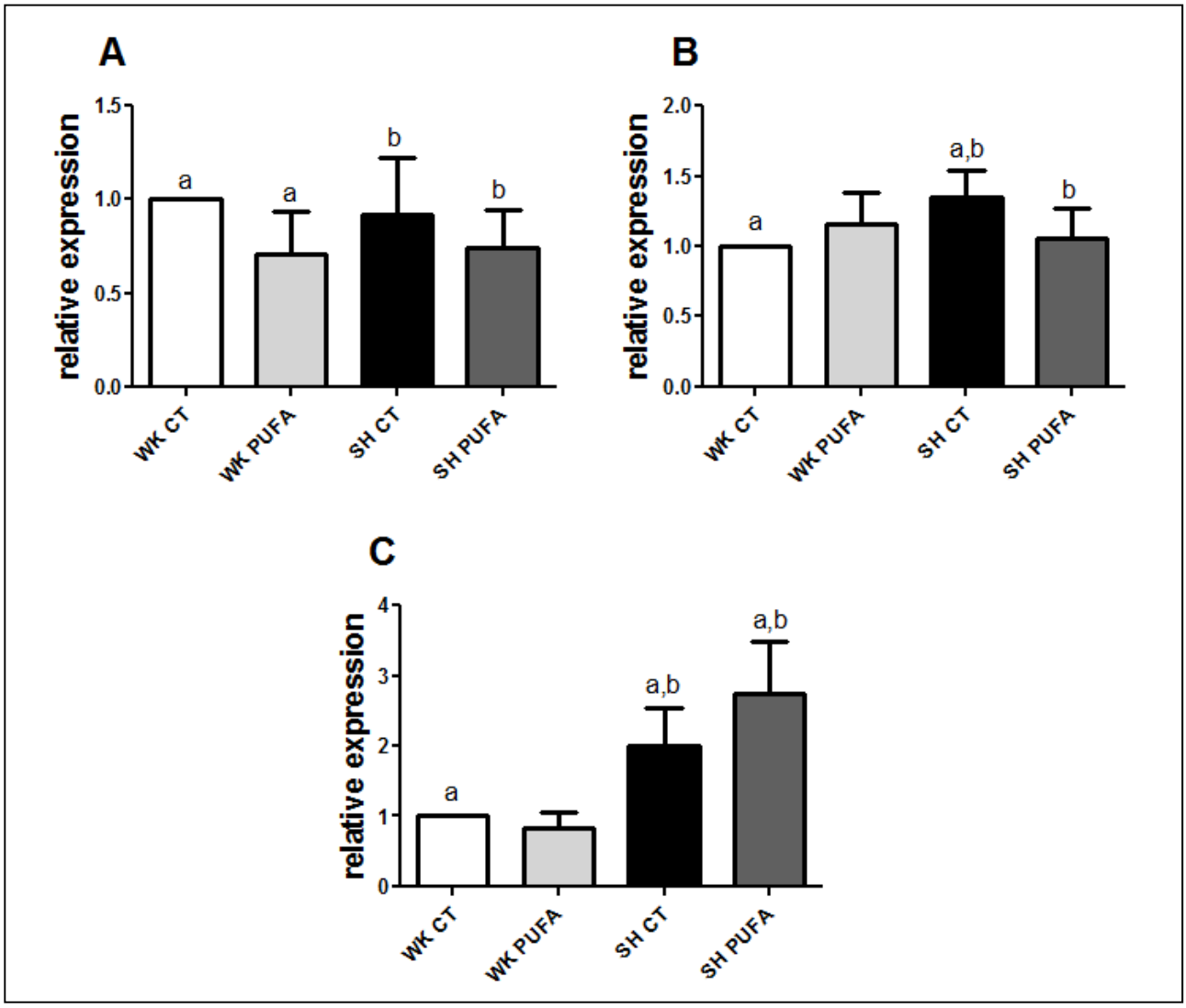

Figure 5. *Hmgcr* (A), *Ldlr* (B), and *Acat-2* (C) gene expression in the liver of WK and SH rats fed the different diets

Data are median +/- standard error.Significant differences were evaluated with Taylor's series statistical analysis. Similar superscript letters indicate statistical significance (at least $p<0.05$).

DISCUSSION

PUFA play a key role in membrane composition and function, in cell signaling, and in the control of gene expression. A very high omega-6/omega-3 ratio, as in today's Western diet, could promote the onset of many diseases, including CVD. On the contrary, increased levels of n-3 PUFA, and a lower omega-6/omega-3 ratio, appear to exert protective effects [106]. Understanding how dietary lipids influence chronic diseases is complicated by the fact that they exert many physiological roles. Dietary fats are substrates for energy metabolism, membrane formation and signaling molecules. Furthermore, PUFAs also regulate gene expression.

The main aim of this study was to evidence in liver the effect of EPA and DHA supplementation on the expression of genes related to cholesterol metabolism. We compared the effect of a PUFA-rich diet in control rats and in SH rats, which are commonly used as animal model for the metabolic syndrome.

As indicated by plasma and liver fatty acid composition, the supplemented fatty acids were absorbed, and incorporated in liver. The liver plays a central role in whole body lipid metabolism, and responds rapidly to changes in dietary fat composition [107]. The incorporation of dietary EPA and DHA mainly at the expense of AA reflects the metabolic competition between PUFA families, as previously reported by H. *Mu et al*. and *Simopoulos et al*. [108, 109].

According to *Aziz et al.* [110], control diet fed SH animals did not show high blood level of total cholesterol. However their HDL-chol level was lower and LDL-chol higher than WK animals, resulting in a higher atherogenic index. Therefore SH rats can be considered a good model to assess imbalanced cholesterol metabolism. N-3 PUFA supplementation normalized the imbalanced blood parameter, lowering the atherogenic index and confirming the positive effect of n-3 PUFA on LDL and HDL-cholesterol levels.

Gene expression analysis underlined that in control condition some genes are differently transcribed in WK and SH animals. Furthermore, the response to the dietary treatment appeared different in the two groups of rats.

SREBP-1 is a master regulator of lipid biosynthesis, and it is reported that naturally occurring variation in the gene encoding the SREBP-1 isoforms might contribute to inherited variation in lipid metabolism in the SH versus other strains of rats [96]. We did not observe a different *Srebf-1* expression between WK and SH rats fed the control diet, but after PUFA supplementation *Srebf-1* expression level decreased in SH rats only. This is consistent with the observed reduction in plasma TG level.

PUFA supplementation decreased plasma TG level also in WK rats, although no modifications occurred in *Srebf-1* expression. Other mechanisms, i.e. a different proteolytic cleavage of SREBP-1 to its nuclear active form, might be involved in n-3 PUFA TG lowering effects. Actually, the activating cleavage of SREBP is inhibited by n-3 PUFA [111-113]. N-3 PUFA are suppressors of SREBP-1 nuclear abundance [114], but their effect on *Srebf-1* mRNA is still controversial. The different PUFA concentration used may explain in part the discrepancies in literature. *Nakatani et al.* [115] evidenced that EPA and DHA reduce the amount of mature active SREBP-1 in a dose dependent manner. At low concentration SREBP-1 proteolytic cascade is inhibited, while at high concentration a decrease of *Srebf-1* mRNA is also observed.

Our data seem to indicate that not only PUFA dose, but also the constitutive expression of the *Srebf-1* gene could influence the effect of PUFA supplementation. SH rats harbor a valine-to-methionine substitution in the COOH terminal portion of the SREBP-1 protein that is not present in 44 other strains of laboratory rats [96]. This reflects SNPs in the *Srebf-1* gene, which could also be related to a different response to PUFA supplementation.

Furthermore, it is reported that SH rats have increased fasting levels of insulin [116], and insulin stimulates the transcription of SREBP-1c [117]. A diet enriched in n-3 PUFA significantly lowers insulinemia [118], so it is conceivable that in PUFA fed SH rats a reduction in insulin level had contributed to the decrease in *srebf-1* expression.

In addition, plasma TG level can be also reduced by the inhibition of TG secretion via VLDL [119], and possibly by accelerating VLDL and chylomicron degradation by lipoprotein lipase (LPL) [120]. In humans, n-3 PUFA increase LPL plasma activity and gene expression [121]. Further studies are needed to clarify the effect of n-3 PUFA on plasma TG level in both WK and SH animals. .

Concerning *Srebf-2* expression, we did not observe any differences due to PUFA supplementation. *Xu et al.* [102] reported that PUFA suppress the in vivo proteolytic release of SREBP-1 and -2, although the effect on SREBP-2 is transient. To date, no evidences about n-3 PUFA effects on Srebf-*2* mRNA abundance are reported in literature. Since in this study the expression of *Hmgcr, Ldlr ,* and *Acat-2,* which are all regulated by SREBP-2, was affected by n-3 PUFA, our data seem to confirm a post-transcriptional regulation of SREBP-2 by EPA and DHA.

HMGCR is one of the key enzymes that regulate endogenous cholesterol production. *Hmgcr* DNA sequence encode SRE responsive element, so is strongly regulated by nuclear SREBP2 [122]. In both WK and SH rats *Hmgcr* gene expression was down-regulated by PUFA treatment. This data are consistent with the decrease in plasma cholesterol level due to treatment.

Control diet fed SH rats evidenced an *Ldlr* up-regulation coupled with high LDL-chol level. This underlines the imbalance in lipid processing that characterizes SH rats. In PUFA fed SH

animals *Ldlr* mRNA was similar to corresponding WK. As for *Hmgcr,* this could be ascribed to a reduction in SREBP-2 post-transcriptional activation.

Another justification could emerge analyzing the relationship between Ldlr activity and cholesterol esterification. This topic has been largely discussed.

It has been suggested that dietary fatty acids and cholesterol regulate hepatic LDL receptor activity via cholesteryl ester and free cholesterol regulatory pools [123]. These regulatory pools are affected by the activity of ACAT, the rate-limiting enzyme of cholesterol esterification. The increase in hepatic cholesteryl ester is negatively correlated with LDL receptor activity in hamsters [124]. Moreover, human interventional studies underline that ACAT inhibitors cause a significant elevation in LDL cholesterol [125, 126].

In our study, *Acat-2* expression was up-regulated in SH rats at control diet, and the PUFA diet further enhanced the expression of this gene. We can therefore hypothesize an increased cholesteryl ester concentration in the liver of SH animals, contributing to the observed decrease in plasma LDL level. Unfortunately, in our conditions NMR analysis was unable to detect cholesteryl esters. This could be due to overlapping signals or to a low concentration of cholesteryl esters compared to the other lipids fractions. Further studies are therefore needed to clarify this issue.

The results on cholesterol esterification need further elucidation. in our condition.

In conclusion, in this study we evidenced that SH rats are characterized by a deep imbalance in lipid metabolism, which is related to a different expression of genes implied in cholesterol metabolism. N-3 PUFA supplementation recovered the impaired blood parameters observed in SH animals, confirming the positive effect of these fatty acids. It is worth noting that the molecular effects of n-3 PUFA were different in WK and SK rats. Although further studies are needed, this observation could be useful while considering n-3 PUFA supplementation in humans with familial forms of hypercholesterolemia.

IV. Phytosterols supplementation reduces metabolic activity and slows cell growth in cultured rat cardiomyocytes

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Introduction

Phytosterols or plant sterols (PSs) are minor constituents and essential phytochemicals that are present in plant foods such as nuts, peanuts, sesame seeds, soybean seeds, and grains as well as their products. The most common PSs in foods are sitosterol (SS), campesterol, and stigmasterol, representing about 50-65, 10-40, and 0-35% of the total phytosterol fraction, respectively[127]. PSs are well known cholesterol-lowering agents[128], and several theories have been proposed to explain their action[129]. Unlike cholesterol, PSs are poorly absorbed, and the small absorbed amount is actively re-excreted in bile, resulting in low serum levels of these sterol molecules.

List of abbreviation: 19-OH: 19-hydroxycholesterol; ACAT: acyl-CoA:cholesterol acyltransferase; GC/MS: coupled system gas chromatography/mass spectrometry; IS: internal standard; LDH: lactate dehydrogenase; MTT: 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI: propidium iodide; POP: oxidised phytosterol; PS: phytosterol; SPE: solid phase extraction; SS: sitosterol; TMS: trimethylsilyl.

Notwithstanding the low plasma concentration, due to their similarities to cholesterol, PSs can be incorporated into cell membranes and affect its composition and functionality[130].

Beside the cholesterol-lowering effect, PSs have been shown to inhibit the growth and development of tumours[131] at concentration levels within the physiological human blood range. Normal PS blood concentration in humans is about 10-15 μM and it doubles when a vegetarian or a PS-rich diet is consumed[132, 133]. Although to date clinical studies have demonstrated no obvious side effects of dietary PSs, apart from a reduction of carotenoid blood level[134], the similarity between anti-neoplastic and cholesterol-lowering concentrations deserves attention. Few studies have been addressed to the effect of PSs on non-neoplastic cells. Awad *et al.*[132] showed in an *in vitro* study that SS inhibited smooth muscle cell proliferation. Rubis *et al.*[135] evidenced SS strong cytotoxic properties at very low concentration (2 μM) in human abdominal aorta endothelial cells. These effects, keeping a balance in proliferation and apoptosis of endothelial cells, have been considered positive in atherosclerosis prevention[136]. Notwithstanding, they further rise concerns about the possibility of PS detrimental effects on other non-neoplastic cell types, and therefore on their long-term use as nutritional hypolipidaemic agents.

To further clarify this important issue, in this study we have evaluated the effect of the supplementation of a mixture of soybean-derived PSs, in which the main component was SS, using primary cultures of neonatal rat cardiomyocytes as a model of non-neoplastic cells. We choose primary cultures of cardiomyocytes for two main reasons. First, we wanted to avoid the use of immortalised cell lines derived from neoplastic cells. Second, the heart is considered the target organ for PS subchronic toxicity[137]. Due to the recommendation of high PS intake for long time in primary and secondary cardiovascular prevention[138], the clarification of PS effect on cardiac cells is extremely important. We supplemented cardiomyocytes with two different PS concentrations, both within the range of plasma concentration considered effective for cholesterol-lowering[139]. To exclude that the observed effects, if any, could be accounted to phytosterol oxidation products (POPs), which are negatively perceived in terms of health[140], POP content in the PS mixture used for supplementation was also determined.

Experimental methods

Materials

Horse serum, foetal calf serum, Ham F10, and other biochemicals were from Sigma-Aldrich (St. Louis, MO, USA), as well as the PS mixture (derived from soybean) used for supplementing cells. Chemicals and solvents, unless specified, were of analytical grade and purchased from Carlo Erba Reagenti (Rodano, Italy), Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA). Dihydrocholesterol (assay: 94.8%) employed as internal standard for the quantification of phytosterols, and campesterol, stigmasterol and sitosterol used for the identification of PS compounds, were from Sigma-Aldrich. 19-hydroxycholesterol (19-OH) from Steroloids (Newport, RI, USA) was employed as internal standard to quantify POPs in the phytosterol mixture adopted for cell supplementation. Deionised water was obtained from a water purification system Elix 10 from Millipore (Bedford, CT, USA).

Methods

Quantification of PSs and POPs in the PS mixture. 10 mg of the soybean PS mixture powder employed for cell supplementation were dissolved in 10 mL *n*-hexane/*i*-propanol 4:1 (v/v). 0.2 mg of dihydrocholesterol in *n*-hexane/*i*-propanol 4:1 v/v (c = 1.9 mg/mL) were added as internal standard to 1 mL of the PS solution. After silylation performed according to Sweeley *et al.*[141] and the addition of 4 mL of *n*-hexane, samples were centrifuged at 1000·g for 3 min. One µL of the resulting solution was analysed using a GC/MS apparatus mod. GCMS-QP2010 Plus (Shimadzu, Tokyo, Japan). Gas chromatographic conditions were as follows: injector temperature: 310°C; oven temperature from 265 to 310°C at 0.80°C/min, finally held at 310°C for 10 min; gas carrier (helium) velocity: 30.0 cm/sec. The separation was carried out on a fused silica capillary column Zebron ZB-5 (30 m × 0.25 i.d., 0.25 f.t.) coated with 95%-dimethyl-5%-diphenyl-polysiloxane (Phenomenex, Torrance, CA). Instrumental conditions employed for MS detector were as follows: acquisition mode: total ion current; ion source temperature: 230°C; interface temperature: 210°C; detector voltage: 0.95 kV; scan range from 40 to 600 m/z; scan speed: 1250; solvent delay time: 10 min. Data were filed and processed by the software GCMSsolution ver. 2.50 SU1 from Shimadzu. PS identification was achieved by comparing peak mass spectra with those obtained from a standard mixture containing campesterol, stigmasterol, and SS and with the data reported by Pelillo *et al.*[142]. Quantification of identified PSs was done relative to dihydrocholesterol.

To evaluate the content of POPs in the soybean PS mixture, 10 µg of 19-OH in *n*-hexane/*i*-propanol 4:1 (v/v) was added as internal standard (c = 0.1 mg/mL) to 3 mL of the aforementioned

PS solution in *n*-hexane/*i*-propanol. After drying and the addition of 1 mL of *n*-hexane/ethyl acetate 95:5 (v/v), POPs were purified by solid phase extraction[143] using aminopropyl bonded phase cartridges (STRATA NH_2, 500 mg stationary phase, 3 mL reservoir volume) from Phenomenex. To evaluate the recovery of procedure, 9 µg of dihydrocholesterol in *n*-hexane/*i*-propanol 4:1 v/v (c = 0.07 mg/mL) were added to the POP containing fraction, which was then silylated[141], and centrifuged at 1000·g for 3 min after the addition of 300 µL of *n*-hexane. One µL of the resulting solution was analysed by GC/MS under the same conditions as formerly reported. POPs were identified by comparing the peak mass spectra with those reported by Dutta[144]. Quantification of identified POPs was done relative to 19-OH.

Cell culture.

Heart cells were obtained from the ventricles of 2-4 d-old Wistar rats as reported[145]. The study was approved by the Ethical Committee for Animal Care of the University of Bologna (Italy) (prot. n. 58897-X/10). Apart from cell growth determination, due to technical reason, cells were seeded at 1 x 10^6 cells/mL concentration. Twenty four h after seeding (T0) cardiomyocytes were divided at random in control, grown in control medium (Ham F10 plus 10% foetal calf serum plus 10% horse serum), and supplemented, grown in the same medium supplemented with PSs (3 or 6 µg/mL). PS stock solution (600 µg/mL) was prepared dissolving the mother solution (derived from soybean and containing 45% SS, 35% campesterol, and 5% stigmasterol, as determined) in 100% ethanol. Control cells received a similar amount of ethanol (<0.01% v/v) to avoid interference due to the vehicle. Media were changed every 2 d, the last change being 48 h before the experiment. Before each determination, at each time point, viable cells were counted by trypan blue dye exclusion. On day 6 (T144) control cardiomyocytes were at complete confluence, as assessed by cell counting and by protein determination with the method of Bradford[146].

Cell count: trypan blue exclusion test.

Trypan blue exclusion test was performed by adding 25 µL of 0.1% trypan blue solution to 100 µL of cells suspended in PBS. The cells that excluded the dye were counted on a haemocytometer as described[147].

Identification and quantification of PSs in cardiomyocytes by GC/MS.

On day 6 (T144), cells were washed three times in PBS, and scraped off in cold n-hexane/isopropanol (4:1 v/v). Phytosterols were recovered from lipids after a cold saponification[148], using hydrocholesterol as internal standard. Before GC/MS analysis, the

trimethylsilyl (TMS) derivatives of PSs were obtained as reported by Sweeley *et al.*[141]. One µL of the derivatised sample was analysed under the same conditions as reported above. PS identification was achieved by comparing peak mass spectra with a standard mixture containing campesterol, stigmasterol, and sitosterol and with the data reported by Pelillo *et al.*[142]. Quantification of identified phytosterols was done relative to dihydrocholesterol.

Cytotoxicity.

According to the cytotoxicity assay techniques, cytotoxicity was evaluated as cell metabolic activity using the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay[149]. At T144, after a washing with 0.9% NaCl, 1 mL of MTT reagent diluted in RPMI-1640 medium modified without phenol red (1 mg/mL) was added to each dish, and the cell cultures were incubated for 3 h at 37°C. After removal of the medium, the cells were lysed with isopropanol for 15-20 min. Formazan production, which is proportional to cell vitality, was determined spectrophotometrically at 560 nm.

Cell growth curve

Cells were seeded in 24-well plates at a concentration of 2 x 10^5/well. Two-four replicates were performed for each time point. Viable cells were counted each day for the following 6 days (T0, T24, T48, T96, T120, and T144).

Cell cycle. Cells were seeded in duplicate in 6-well plates. At each time point (T0, T12, T24, T48, T72, T96, and T144) cardiomyocytes were washed with PBS, harvested and resuspended in 500 µL of a solution (0.1% sodium citrate, 0.1% Triton X-100 in PBS) containing PI 50 µg/mL, as described[150]. Cell cycle analysis was performed by cytofluorimetric detection of DNA content with a FACS Calibur Cytometer (Becton Dickinson, Mansfield, MA, USA) after staining with PI.

Apoptosis.

Cells were plated in duplicate in a 6-well plate. At each time point (T0, T12, T24, T48, T72, T96, and T144), after counting for viable cells, cell number was normalised to 5 x 10^5, and apoptosis was verified using two different methods. In the first one (propidium iodide uptake) cells were washed with PBS, harvested and resuspended in 500 µL of propidium iodide (PI) buffer[150]. PI-stained cells were analysed by flow cytometry as described above; apoptotic cells were counted as the percentage of sub-G1 cells. In the second one (Annexin-V binding) cells were washed with PBS, harvested and resuspended in 100 µL of Annexin-V-Fluos and PI-labelling solution (Roche, Penzberg, Germany) for 15 minutes. The stained cells were analysed by flow cytometry with a

FACS Calibur Cytometer (Becton Dickinson, Mansfield, MA, USA); apoptotic cells were counted as the percentage of Annexin-V-positive, PI-negative cells.

Cell membrane damage: lactate dehydrogenase (LDH) release in media.

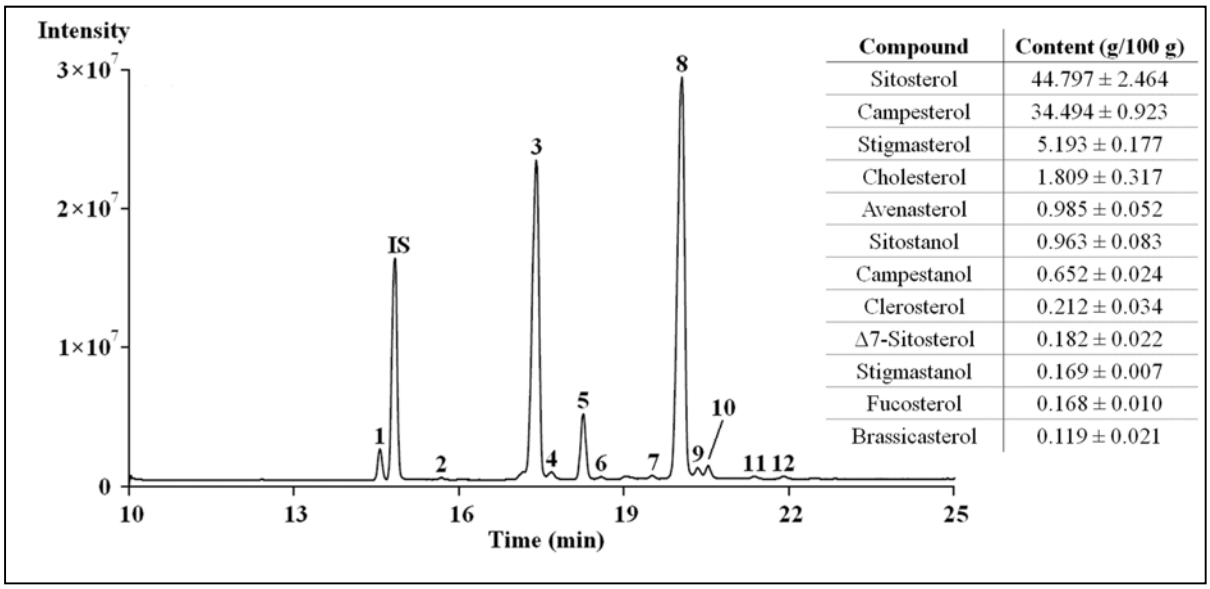
Cells were seeded in duplicate in 6-well plates. To evaluate the possible damage to cell membrane integrity, the release of the LDH enzyme in the media was determined. At each time point (T24, T48, T96, and T144) LDH activity in the media was determined spectrophotometrically by measuring NADH levels at 340 nm[151].

Statistical Analysis.

The reported data are the means of at least three samples obtained from independent cell cultures. Statistical analysis was carried out by one-way ANOVA using Tukey's as post test.

Results

The composition of the supplemented PS solution was analysed prior to experiments and is reported in figure 1. The total POP content in the PS mixture was low (0.124 ± 0.012 g/100 g) and the amount of single POPs identified is shown in figure 2. As regards the performance of the analytical methods herein employed, the determination of PSs after cold saponification ensured a good reproducibility, with a relative standard deviation associated to total phytosterol amount which was usually less than 5%. POP purification in cell feeding mixture by solid phase extraction (SPE) showed a high recovery percentage (85.9 ± 1.5%) and an acceptable reproducibility (≈9%) as concerns the amount of total oxidised sterols detected.



Compound	Content (g/100 g)
Sitosterol	44.797 ± 2.464
Campesterol	34.494 ± 0.923
Stigmasterol	5.193 ± 0.177
Cholesterol	1.809 ± 0.317
Avenasterol	0.985 ± 0.052
Sitostanol	0.963 ± 0.083
Campestanol	0.652 ± 0.024
Clerosterol	0.212 ± 0.034
Δ7-Sitosterol	0.182 ± 0.022
Stigmastanol	0.169 ± 0.007
Fucosterol	0.168 ± 0.010
Brassicasterol	0.119 ± 0.021

Figure 1: Gas chromatographic trace of trimethylsilyl (TMS) ethers of sterols contained in the PS mixture. Peak identification: IS, internal standard (cholestane); 1, cholesterol; 2, brassicasterol; 3, campesterol; 4, campestanol; 5, stigmasterol; 6, stigmastanol; 7, clerosterol; 8, sitosterol; 9, sitostanol; 10, avenasterol; 11, fucosterol; 12, Δ7-sitosterol. For analytical conditions see Methods.

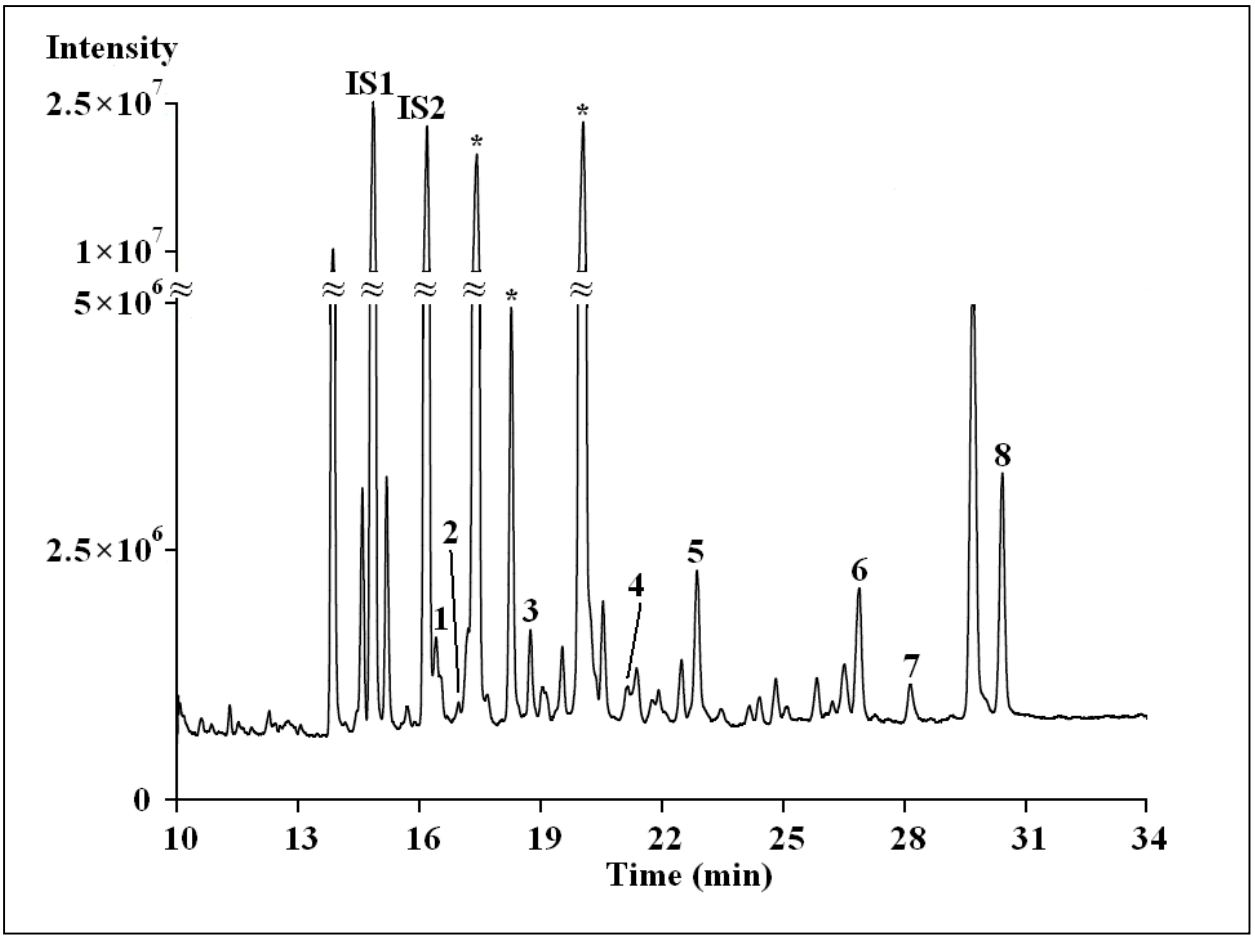


Figure 2. Gas chromatographic trace of trimethylsilyl (TMS) ethers of phytosterol oxidation products (POPs) purified from the PS mixture. Peak identification: IS1, dihydrocholesterol; IS2, 19-hydroxy cholesterol; 1, 7α-hydroxy campesterol; 2, 7α-hydroxy stigmasterol; 3, 7α-hydroxy sitosterol; 4, sitotrienol; 5, 7β-hydroxy sitosterol; 6, 7-ketocampesterol; 7, 7-ketostigamsterol; 8, 7-ketositosterol. Peaks marked with an asterisk were non-oxidised sterols. Other peaks were not identified. For analytical conditions see Methods**.**

Incorporation of supplemented PSs by cardiomyocytes was verified at T144. On that day, control cardiomyocytes were at complete confluence, as determined by microscopy. To evaluate if PS supplemented cardiomyocytes also reached confluence at T144, cells were counted and protein content was determined, evidencing no differences among groups (table 1). As shown in figure 3, cardiomyocytes actively incorporated the supplemented PSs, in a dose-dependent manner (r^2 = 0.92).

Treatment	Cell number (cells/dish)		Protein content (mg/dish)	
	Mean	SD	Mean	SD
Control	5,158,333	914,429	0.98	0.02
PS 3 µg/mL	5,637,500	2,203,974	1.05	0.05
PS 6 µg/mL	5,197,500	1,255,998	0.99	0.06

Table 1. Cell number and protein content at T144 in the different experimental conditions. Data are mean value and standard deviations of at least three samples obtained from independent cell cultures. No differences in cell number and protein among the different experimental conditions were determined by the one-way ANOVA analysis.

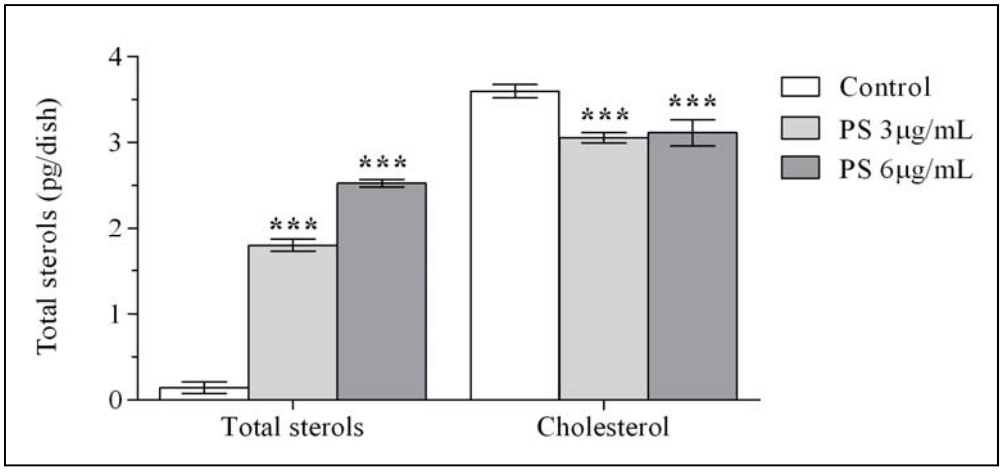


Figure 3. Total PS and cholesterol content of unsupplemented and supplemented cardiomyocytes. PS and cholesterol concentration was determined as described in Methods. Results are expressed as µg/dish and are means of at least three samples obtained from independent cell cultures, with standard deviations represented by vertical bars. Statistical analysis was by the one-way ANOVA (PSs: $p<0.001$; cholesterol $p<0.01$) using Tukey's as post test (PSs: control vs. 3 µg/mL $p<0.001$; control vs. 6 µg/mL $p<0.001$; 3 µg/mL vs. 6 µg/mL $p<0.001$. Cholesterol: control vs. 3 µg/mL $p<0.05$; control vs. 6 µg/mL $p<0.01$; 3 µg/mL vs. 6 µg/mL n.s.).

Cholesterol content significantly decreased in PS supplemented cardiomyocytes compared to controls, with no difference between the two supplemented groups. According to the composition of the supplemented PS solution, SS appeared to be the most incorporated one, followed by campesterol and stigmasterol (figure 4). Figure 5 illustrates the different chromatographic traces of PSs extracted from cardiomyocytes grown in non-supplemented and supplemented media.

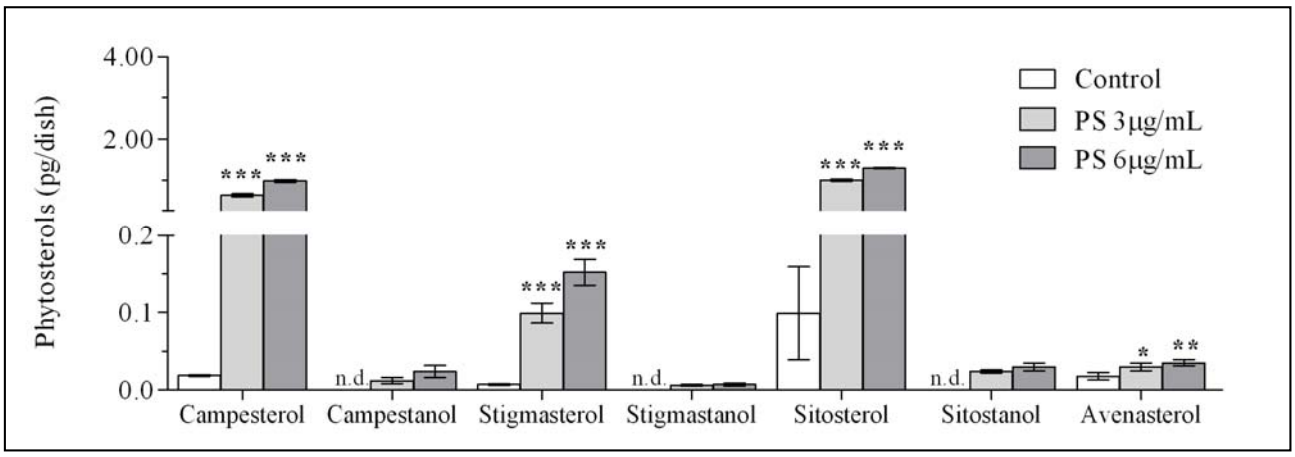


Figure 4. Distribution of the different PSs in unsupplemented and supplemented cardiomyocytes. PS incorporation was determined as described in Methods. Results are expressed as µg/dish and are means of at least three samples obtained from independent cell cultures, with standard deviations represented by vertical bars. Statistical analysis was by the one-way ANOVA (campesterol $p<0.001$; stigmasterol $p<0.001$; sitosterol $p<0.001$; avenasterol $p<0.01$) using Tukey's as post test for evaluating differences between the two PS concentrations used (campesterol $p<0.001$; stigmasterol $p<0.01$; sitosterol $p<0.001$).

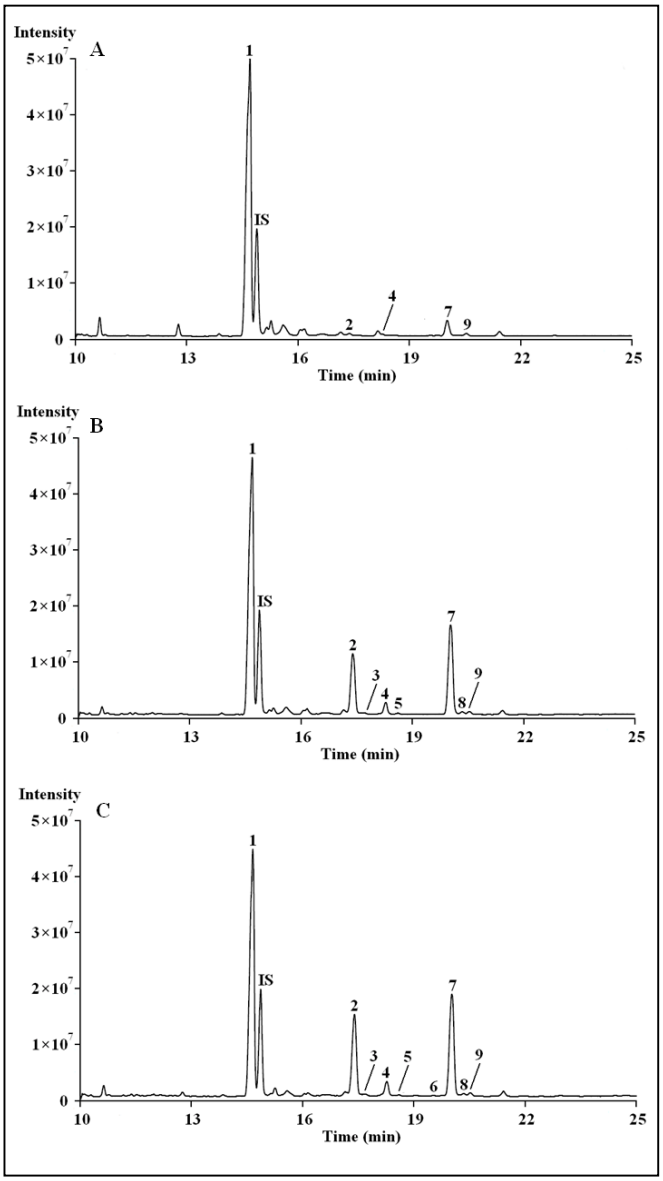


Figure 5. Gas chromatographic traces of trimethylsilyl (TMS) ethers of sterols recovered from the lipid fraction of unsupplemented and PS supplemented cardiomyocytes. Cardiomyocytes were grown in unsupplemented (trace **A**), 3 µg/mL PS supplemented (trace **B**) and 6 µg/mL PS (trace **C**) supplemented media. Peak identification: IS, internal standard (cholestane), 1, cholesterol; 2, campesterol; 3, campestanol; 4, stigmasterol; 5, stigmastanol; 6, clerosterol; 7, sitosterol; 8, sitostanol; 9, avenasterol. For analytical conditions see Methods

To verify the possible interference between the observed PS incorporation and cardiomyocyte metabolic activity, the MTT assay was also performed at T144. MTT conversion to formazan appeared reduced in supplemented cardiomyocytes compared to controls, independent of PS concentration in the media (figure 6).

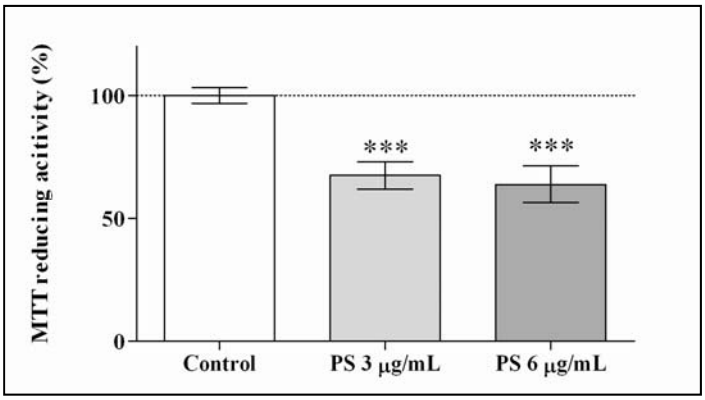


Figure 6. Metabolic activity in unsupplemented and PS supplemented cardiomyocytes. MTT conversion to formazan, as a measure of metabolic activity, was determined as reported in Methods and expressed as percentage of value obtained in unsupplemented cells (assigned as 100%). Data are means of at least three samples obtained from independent cell cultures, with standard deviations represented by vertical bars. Statistical analysis as by the one-way ANOVA ($p<0.001$) using Tukey's as post test (control vs. 3 µg/mL $p<0.001$; control vs. 6 µg/mL $p<0.001$; 3 µg/mL vs. 6 µg/mL n.s.).

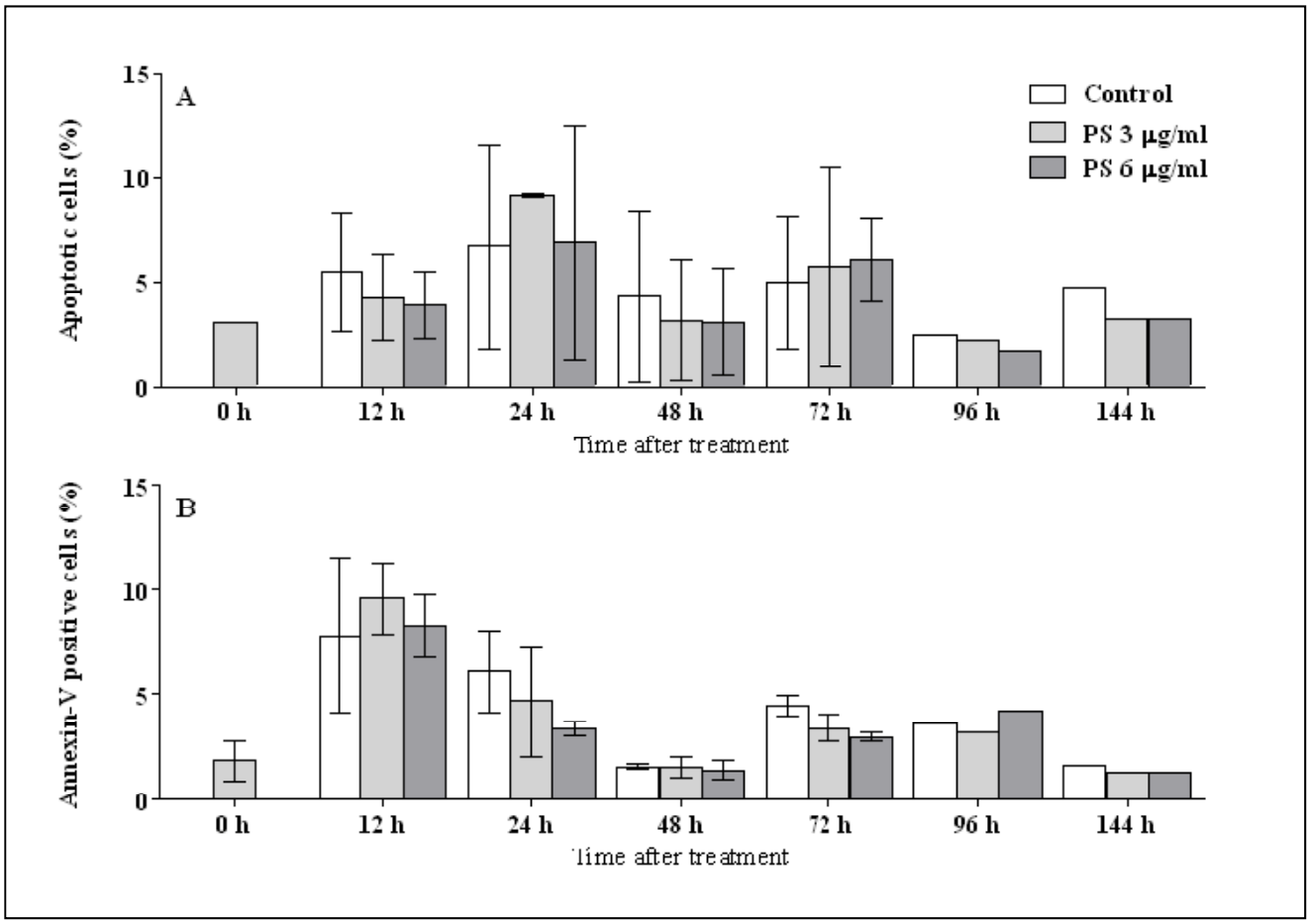


Figure 7. Apoptotic effects of PS supplementation evaluated using Annexin-V at different time points. Data are means of at least three samples obtained from independent cell cultures, with standard deviations represented by vertical bars. No differences in the number of apoptotic cells were detected among groups at any time point by the one-way ANOVA.

The pro- or anti-apoptotic effects of PSs were investigated using two different independent methods, Annexin-V (figure 7) and propidium iodide uptake (*data not shown*). Both methods evidenced no differences in the number of apoptotic cells at any time point. LDH release in the media was also evaluated, and no differences were detected among groups at any time point (*data not shown*).

Regarding the cell cycle, the distribution of cell populations was similar in both control and PS supplemented cells (figure 8), but significant differences were observed in the growth curve. Performing this analysis, for technical reason cells were seeded at a lower concentration to prevent them from reaching confluence in an early stage, therefore hiding possible differences in cell growth. As evidenced in figure 9, supplemented cardiomyocytes grew more slowly than controls.

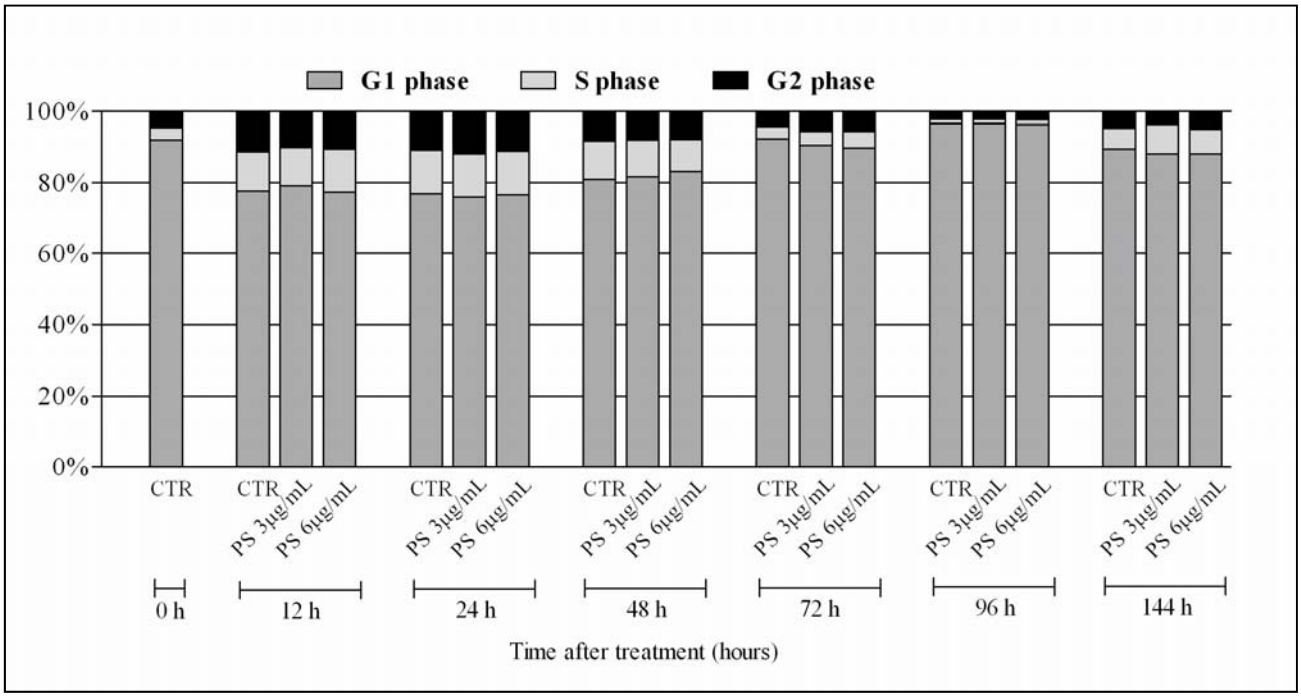


Figure 8. Cell cycle distribution in control and PS supplemented cardiomyocytes at different time points. The bar graph shows the distribution of cells among the different phases of the cell cycle: G0/G1 phase - quiescent cells, S phase - DNA replicating cells, and G2/M phase - cells with two full complements of DNA or at mitotic phase. Data are means of at least three samples obtained from independent cell cultures, with standard deviations represented by vertical bars. No differences in the cell cycle distribution were detected among groups at any time point by the one-way ANOVA.

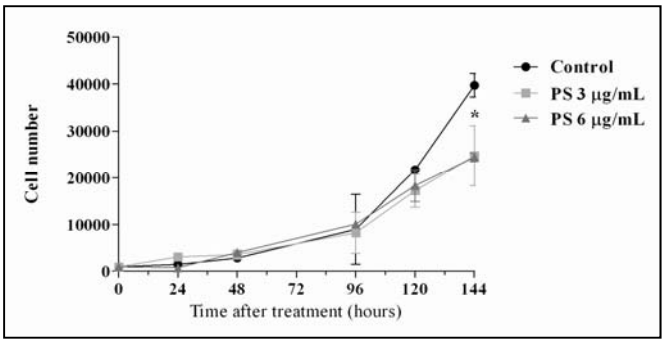


Figure 9. Cell growth analysis in unsupplemented and PS supplemented cardiomyocytes. Cell growth analysis was performed as reported in Methods, and cells counted at different time points. Data are means of at least three samples obtained from independent cell cultures, with standard deviations represented by vertical bars. Statistical analysis was by the one-way ANOVA (T144 $p<0.05$).

Discussion

In neoplastic cells, PS anti-proliferative activity is accounted to their incorporation in cell membrane[152], but the effects of PS incorporation in normal cells have been described in few studies, and never in cardiac cells. In the herein reported study, following supplementation, a dose-dependent incorporation of PSs by cardiomyocytes was clearly evidenced. It was accompanied by a decrease in cholesterol content. The replacement of cholesterol by PSs could have caused the observed slowing down of cell growth and the reduction in metabolic activity, which could rely on PS increase, cholesterol decrease, or both. High sterol concentration within the cells is cytotoxic, and the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) represents one of the most important mechanisms for reducing excess sterols, preventing cytotoxicity[153, 154]. Previous works showed that PSs are relatively poor substrates for ACAT[155, 156]. According to our results, Melchert *et al.*[157] evidenced that the decrease of cell cholesterol content, due to the inhibition of cholesterol synthesis by statins, reduces growth and MTT reduction in cardiac myocytes, without significant changes in propidium iodide staining. Furthermore, replacement by PSs of cholesterol molecules increases ion permeability [158] and modifies the activity of enzymes such as Na^+, K^+-ATPase[159] in biomembranes.

Another possible explanation of the presented results could be related to PS oxidation. Although POPs were present in low amount in the PS mixture used for cell supplementation, PS oxidation processes could take place in cells *in vitro*, producing not only harmful epoxy forms but also generating free radicals[135]. In this light, PS negative effects could be also dependent on POP production and POP scavenging capacity in different cell types. Further studies are in progress to clarify this point.

Although it has been reported that SS induces apoptosis in cancer cells through caspase activation[160], we did not observed apoptosis in cardiomyocytes. Recently Bao *et al.*[161] evidenced that SS did not activate the caspase-dependent pathway in non-neoplastic macrophages. The observed death of these cells was caspase-independent, involving necroptosis and autophagy, and was induced by SS concentration higher than those used in the present study.

The lower MTT conversion observed in PS supplemented cells suggests that the study compounds more efficiently target cell metabolism, including cytochrome activity, than membrane integrity. This is in agreement with our data on LDH release. The reduction of MTT into formazan pigment predominantly depends upon mitochondrial reductase activity, and is also correlated with the mitochondrial metabolic capacity[162]. Mitochondrial metabolism provides the heart with very high amount of ATP per day[163], and the importance of intermediary metabolism to sustain the

function of the heart has long been appreciated. The decrease of MTT reduction activity in PS supplemented cardiomyocytes suggests that cardiac energy metabolism is switched from β-oxidation of fatty acid to glycolysis[164], and Taegtmeyer [165] has proposed that metabolic changes often antedate functional contractile changes in the heart. The reduced cell metabolic activity could also explain the results obtained on cell growth curve.

Conclusions

The herein reported study is the first report on the effect of PSs in cardiac cells, and although PS supplementation appeared less deleterious than in neoplastic cells our results deserve important consideration. It is important to note that we grew cardiomyocytes in PS supplemented media, so reproducing more closely the *in vivo* situation when consuming a PS-rich diet. In other studies higher PS concentrations were supplemented to cells for a short time period. Although at present it is difficult to translate the obtained results to the health of heart tissue, the slowed cardiac cell growth and metabolic activity could represent a potential hazard. As already suggested by Lizard[166], and in the light of our data further investigations are needed to evaluate the biological impact of long-term exposure to physiologically relevant PS concentrations in humans, due to their wide use in the prevention and treatment of hypercholesterolaemia.

Acknowledgment

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

V. Hypocholesterolemic effect of a new bifidobacteria mix on rat induced for hypercholesterolemia.

INTRODUCTION

The association between cardiovascular disease (CVD) and elevated serum cholesterol (chol) was established more than 50 years ago. Since then CVD has been considered a preventable health hazard. In spite of this, lifetime risk for CVD remains high and it continues to be an important health problem [167]. When LDL cholesterol (LDL-chol) levels are too high, chol tends to slowly accumulate in the artery inner wall, encouraging the development of atherosclerosis [168-171].

Approximately 25% of cholesterol within the human body derives from the diet, while about 75% is endogenously synthesized [172]. The human diet provides ~400 mg of cholesterol, but only 50% is absorbed in the intestine. The liver secretes ~1 g cholesterol daily [173]. Cholesterol absorption efficiency and absorbed dietary cholesterol significantly regulate cholesterol synthesis and elimination. These factors are important determinants of within-population variation in the serum levels of total, LDL, and HDL cholesterol (HDL-chol) [174].

Therapeutic interventions with lipid-lowering drugs (statins, fibrates) act by reducing levels of total cholesterol (TOT-chol) and LDL-chol, and by increasing HDL-chol levels [175, 176]. Like other drugs, they may have side effects [177], and should be assumed for a very long period or during the whole life. For this reason the development of new non-pharmacological approaches to reduce the absorption of cholesterol, interacting with steps that regulate the digestion and absorption of dietary lipids could be a very important topic.

Probiotics are accounted among active non pharmacological compounds able to low cholesterol absorption. Probiotics are live microbial food supplements, which benefit the health of consumers by maintaining or improving their intestinal microbial balance [178]. In recent years many researchers have demonstrated that gut microflora can exert positive effects in several pathological conditions such as heart diseases, pseudomembranose colitis, lactase deficiency and many others [179]. Microflora composition could be affected by improving the presence of some species introducing specific probiotic strains by diet. Different strains have different ability to colonize and persist in the gut. This is tightly linked to the specific rate of growth, division, interaction with other strains, and presence/quality of substrate to be fermented [180, 181].

Probiotics are a very heterogeneous group of bacteria that account for a large specie-specific number of effects. Some strains are known to exert, more than other, positive outcome against cholesterol accumulation with a mechanism not always completely understood. Different strains, and also different strains combination, exert diverse effects [182-185].

Among species, *Bifidobacteria* seems to be particularly effective against cholesterol adsorption. For exempla, *Bifidobacterium longum* exhibited a more significant effect in lowering serum total cholesterol than a mixed colture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* both in rats and humans [186].

Bifidobacteria have been shown to constitute 95% of the total gut bacterial population in healthy breast-fed newborns. The content decreases in the adult, although it remains relatively stable representing 3–6% of the fecal flora, until advanced age when the population of *bifidobacteria* appears to decline [187, 188]. Preparations containing *bifidobacteria* are safe, commonly used and well tolerated. Two mechanisms are purposed to explain the hypocholesterolemic effect:

a. co-precipitation with bile salt [189]

b. microbial uptake and co-precipitation of cholesterol by bacteria [190, 191].

Different *Bifidobacterium* strains have different effectiveness in cholesterol control [192, 193], this leading to focus on the search of more efficient strain combinations.

The aim of this study was to evaluate in rats the effectiveness of a *Bifidobacteria* mix in reducing cholesterol. The mix contained three different strains of *Bifidobacterium*, namely ***B. breve*** (WC 0463), ***B. breve*** (WC 0420), and ***B. bifidum*** (WC 0417).

The experiment was carried out on Wistar Kyoto rats. During the first part of the study, hypercholesterolemia was induced in rats by means of a cholesterol-enriched diet. After 15 days, rats were divided into three groups:

1. hypercholesterolemic diet 1% plus probiotics mix (i-chol 1%+mix)

2. hypercholesterolemic diet 1% alone (i-chol 1%)

3. standard diet (st-diet).

The probiotic mix was administered to rats by gastric gavage, in an amount providing $0{,}33 \times 10^9$ cfu/die of each strain.

Blood was taken at the beginning of the study (T0), after the hypercholesterolemic period (T15), and at the end of the treatment (T45). The following parameters were measured on serum: TOT-, LDL-, HDL-chol, triglyceride (TG) level, and the atherogenic index were calculated. Results obtained indicated that the treatment with the *Bifidobacterium* mix partially counteracts the effects of the hypercholesterolemic diet.

MATERIALS AND METHODS

Materials

Cholesterol/ Cholesteryl Ester Quantification Kit; HDL and LDL/VLDL Cholesterol Quantification Kit; and Triglyceride Quantification Kit were purchased by BioVision Inc. (CA, U.S.A.); Phosphate Buffered Saline were from Lonza Walkersville Inc (Maryland, U.S.A.).

The probiotic mix was a kind gift of Prof. Diego Matteuzzi (University of Bologna) and Prof. Maddalena Rossi (University of Modena).

Methods

Diets: Diets were prepared by Mucedola (Milano, Italy) according to *Reeves et al.*[194]. To obtain the hypercholesterolemic diets, 1% or 3% cholesterol were added to the standard diet AIN-93M.

Animals: 24 male, 30 day old Wistar-Kyoto rats were used. Animals were housed in individual cages under strictly controlled conditions of temperature (20 ± 2 °C) and humidity (60–70%), with a 12-hour dark-light cycle. Food and water were provided *ad libitum*. During the whole experiment rat were weighted weekly and food consumption was evaluated daily.

Before starting the experimental period, each animal was weighed and rats statistically different from the average were discarded and substituted. After a 5 day of acclimatation at standard diet, followed by 12h fasting, rats were weighted and blood samples were collected from the retro orbital plexus (T0).

During the following 15 day animals were fed a 3% hypercholesterolemic diet (AIN-93M diet + 3% chol). At the end of this period, after 12h fasting, rats were weighted and blood samples were collected from the retro orbital plexus (T15). Animals were randomly divided into three groups of 8 rats, each receiving the following diets:

1. AIN-93M + 1% chol hypercholesterolemic diet plus probiotics mix (i-chol 1%+mix)
2. AIN-93M + 1% chol hypercholesterolemic diet alone (i-chol 1%)
3. AIN-93M standard diet (st-diet)

The probiotic mix was administered to rats by gastric gavage, in an amount providing $0{,}33 \times 10^9$ cfu/die of each strain in isotonic water. A similar amount of water was administered by gastric gavage to rats not receiving the probiotic mix.

After 30 days, subsequent to 12h fasting, rats were weighted, sacrificed and blood samples were collected (T45). Immediately after withdrawal, serum was isolated by natural blood sedimentation and frozen -20°C.

The study was approved by the Ethical Committee for Animal Welfare of the University of Bologna.

Biochemical measurements

Total cholesterol evaluation: Cholesterol/ Cholesteryl Ester Quantification Kit (BioVision Inc., CA, U.S.A.) was used. Reaction mix was prepared adding cholesterol esterase, cholesterol oxidase and DMSO eluted probe according to manufacturer. Cholesterol esterase was used to hydrolyze cholesterol esters into free cholesterol. Cholesterol is oxidized by cholesterol oxidase to yield hydrogen peroxide (H_2O_2). The probe purchased in the kit reacts with H_2O_2 to produce resorufin, a colored substrate that is directly proportional to cholesterol content and is relievable spectrophotometrically at λ=570 nm.

In a 96 multi-well plate, 2µl of serum were mixed with 48 µl cholesterol assay buffer. A calibration curve was obtained in the same multi-well plate using different concentrations of pure cholesterol. Then 50 µl reaction mix were added to each well. After 1h incubation at 37°C in the dark, samples were read at λ=570 nm using a spectrophotometer Infinite M200 multi plate reader TECAN (Männedorf, Switzerland). All values were corrected from background, and cholesterol concentration in wells containing the samples was calculated by comparison with the generated standard curve.

Cholesterol concentration in the samples was calculated as

C =A / V (µg/µl)

Where: **C=** chol concentration in the sample; **A** = cholesterol in the well (µg); **V** = sample volume in the well (µl)

LDL- HDL-cholesterol evaluation. The HDL and LDL/VLDL Cholesterol Quantification Kit (BioVision Inc., CA, U.S.A.) was used. HDL and LDL/VLDL separation was obtained mixing 100µl Precipitation Buffer to 100µl serum. After 10 minutes incubation samples were centrifuged 10 minutes at 2000 x g. The supernatant, representing HDL fraction, was removed and collected in a new tube. The resulting pellet, representing the LDL fraction, was re-suspended in 200µl PBS.

Analysis was performed as described above for total cholesterol evaluation. Sample wells preparation were done adding 18µl LDL fraction to 32 µl assay buffer or 15 µl HDL fraction to 35 µl assay buffer. After 1h incubation at 37°C in the dark, samples were read at λ=570 nm using a spectrophotometer Infinite M200 multi plate reader TECAN (Männedorf, Switzerland).

All values were corrected from background. LDL-chol, and HDL-chol concentration in wells containing the samples were calculated by comparison with the generated standard curve.

LDL- and HDL-chol concentrations in the samples were calculated as

C =A / V (µg/µl)

Where: **C=** concentration in the sample; **A** = concentration in the well (µg); **V** = sample volume in the well (µl)

TG content evaluation. The Triglyceride Quantification Kit (BioVision Inc., CA, U.S.A.) was used. TG are converted to free fatty acids and glycerol by lipase enzyme. The glycerol is then oxidized to generate a product which reacts with the probe to generate a substrate relievable at λ = 570 nm. Reaction mix was prepared adding Triglyceride Assay Buffer, Triglyceride Enzyme Mix and DMSO eluted probe according to manufacturer. In a 96 multi-well plate, 5µl serum were mixed with 45 µl cholesterol assay buffer. A calibration curve was obtained in the same multi-well plate using different concentrations of pure Triglyceride Standard. 2 µl of lipase was added to each standard and sample well, mixed, and incubated 20 min at room temperature to convert triglyceride to glycerol and fatty acid. 50 µl reaction mix were then added to each well. After 50 minutes incubation at room temperature in the dark, samples were read at λ=570 nm using a spectrophotometer Infinite M200 multi plate reader TECAN (Männedorf, Switzerland). All values were corrected for background, and TG concentration in sample wells were calculated by comparison with the calibration curve. TG concentration in the samples was calculated as

C = **Ts** / **Sv** (mM)

Where **Ts** is TG concentration in the sample well (nmol) and **Sv** is the sample volume in the well (µl).

Statistical analysis. Data are reported as means ± SD. Statistical analysis was done by the one way ANOVA with Tuckey as post test and by the Students' t test.

RESULTS

Rat body weight was monitored during the whole trial, and was compared to an external control group of rats of the same age, fed a standard diet and grown in the same condition (ext-ctr) **(figure 1)**. Although no significant differences were detected between experimental rats and external controls, experimental rats gained weight with an irregular pattern. This finding was consistent with the measured food consumption, which showed significant differences between rats.

Since during the first phase of the trial all rats received the same hypercholesterolemic diet, all animals were considered together. Serum TOT-, LDL-, and HDL-chol, and TG at T0 and T15 were compared, as reported in **table 1**, as well as the HDL/LDL ratio (atherogenic index). The diet induced a significant increase in LDL-chol ($p<0.01$) with an even more significant decrease in HDL-chol ($p<0.001$). The decrease in TOT-chol, which is was also significant, was the consequence of the strong decrease in HDL fraction. The HDL / LDL ratio (normal value >3) was greatly reduced at T15. A significant decrease in TG level was also count at T15.

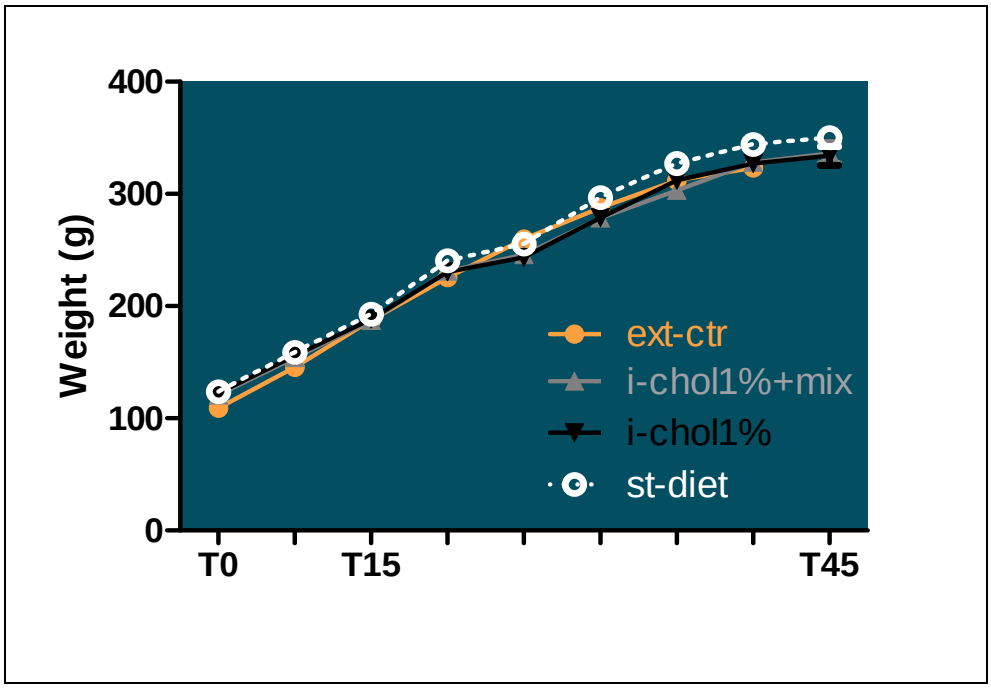

Figure 1: Change in rat body weight gain during the trial.
Data are means +/- SD. Statistical analysis was carried out using the one way ANOVA with Tuckey as post test (n.s.).

	T0	T15
TOT-chol (mg/dl)	99.33 ± 10.03	45.46 ± 6.51*
LDL-chol (mg/dl)	18.52 ± 1.07	22.31 ± 3.86°
HDL-chol (mg/dl)	65.22 ± 3.10	21.85 ± 4.50*
TG (µmol/dl)	67,80 ± 13,12	6.64 ± 1,29*
HDL/LDL	3.54 ± 0.25	1.00 ± 0.22*

Table 2: Serum TOT-, LDL-, HDL-chol, TG level, and HDL/LDL ratio at T0 and T15.
Data are means +/- SD. Statistical analysis was by the Students' t test: ° $p<0.01$; * $p<0.001$.

To verify if different food consumption and weight gain occurred during hypercholesterolemia induction could be related to differences in blood parameters, rats were divided into 3 subgroups according to weight gain:

W1: weight gain 60-70 g

W2: weight gain 70-80 g

W3: weight gain 80-90 g

At T0 no significant differences in TOT-chol, LDL-chol, HDL-chol, and TG levels were detected among the weight groups. At T15, TOT-chol and TG decreased and LDL-chol increased in all weight groups, without any significant differences among the groups. On the contrary, although a decrease in HDL-chol was observed in all weight groups, it appeared greater in animals expressing the lower weight gain **(figure 2).**

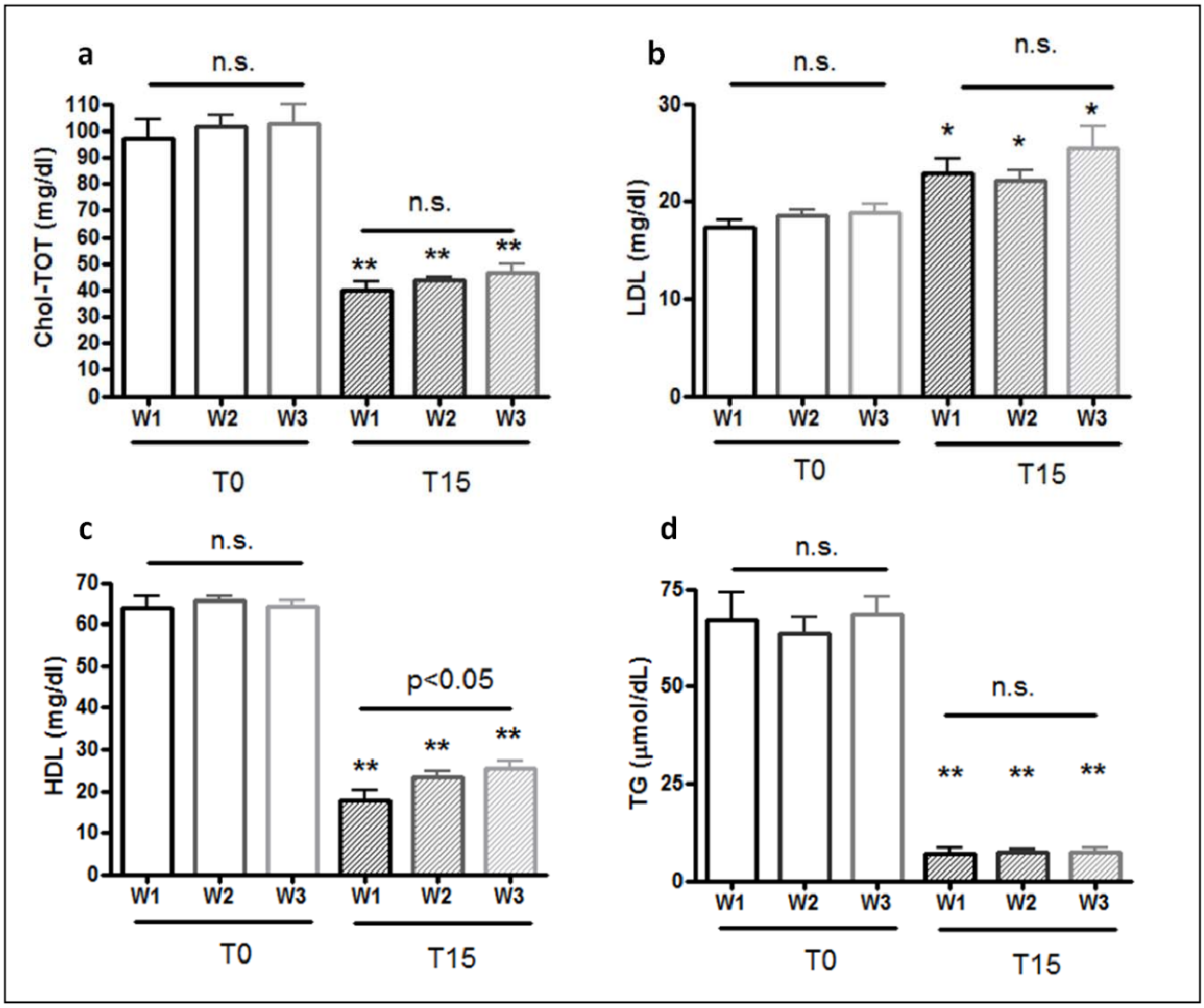

Figure 2: Serum TOT-**,** LDL-, HDL-chol and TG levels in rats grouped according to body weight gain.
(a) TOT-chol; (**b)** LDL-chol; (**c)** HDL-chol, and (**d)** TG serum levels in W1, W2 e W3 groups at T0 and T15. Data are reported as means ± DS. Statistical analysis was carried out using the one way ANOVA comparing the three weight groups at the same time point. The Student's t test was used to compare the same weight group at T0 and T15: * $p<0.01$, ** $p<0.001$.

Treatment effects:

Modifications in serum LDL- and HDL-chol level, and in HDL/LDL ratio during the whole experiment, as well as their level at T45, are shown in **Figure 3**.

Comparing T15 and T45, LDL-chol did not change in rat fed the hypercholesterolemic diet, while it decreased in rats fed the same diet but receiving the probiotic mix. An even higher reduction was observed in the standard diet fed group. In this group, HDL-chol significantly increased at T45, coming back to T0 level. In the other two groups a further significant decrease in HDL-chol was observed. The HDL / LDL ratio significantly increased in rats fed the standard diet rises significantly, reaching higher values than those observed at T0. No modification between T15 and T45 were observed in the other two groups.

Modification in TOT-chol level during the whole experiment and at T45 (**figure 4**) were consistent with the modifications observed in LDL and HDL fractions.

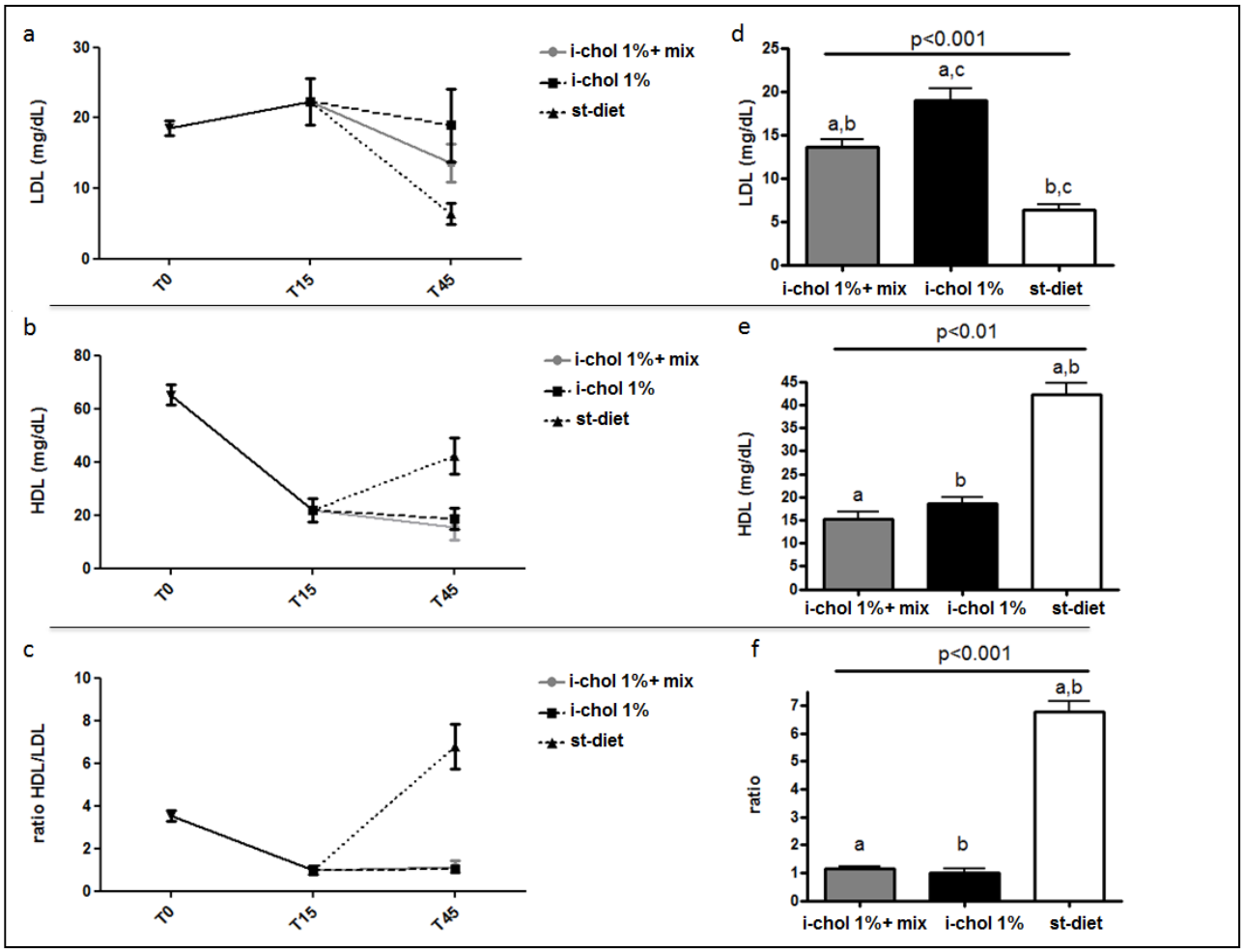

Figure 3: Serum LDL-chol, HDL-chol, and HDL/LDL ratio in rats fed the different diets.

(a) LDL-chol; **(b)** HDL-chol, and **(c)** HDL/LDL ratio Data are reported as mean ± SD. At T45, statistical analysis was carried out using the one way ANOVA with Tuckey as post test. Similar superscript letters indicate statistical significance (at least $p<0.05$).

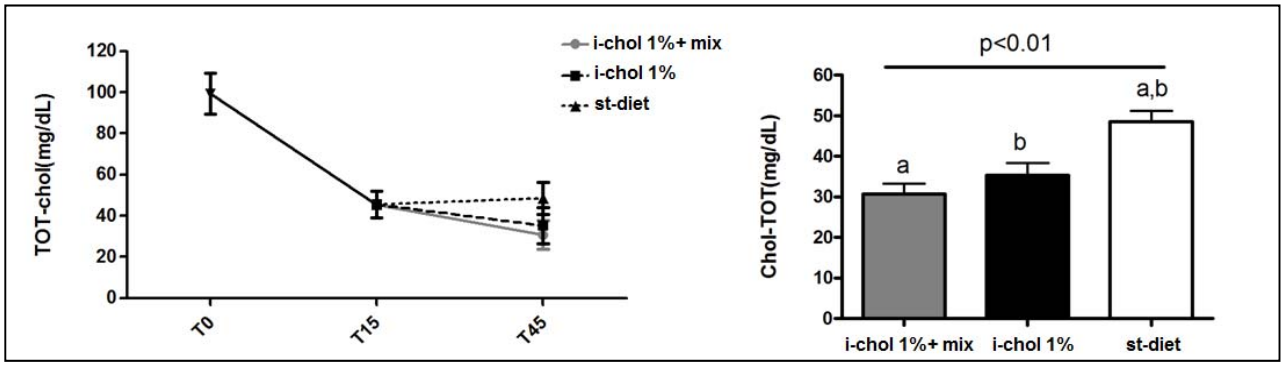

Figure 4 Serum TOT-chol in rats fed the different diets.
Data are reported as means ± SD. At T45, statistical analysis was carried out using the one way ANOVA with Tuckey as post test. Similar superscript letters indicate statistical significance (at least $p<0.05$).

TG levels increased at T45, without any significant difference among the dietary groups **(figure 5)**.

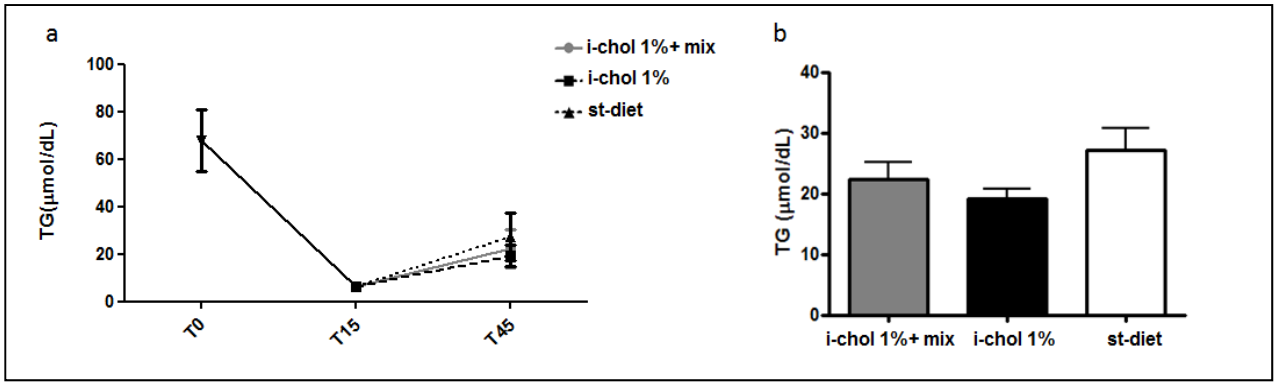

Figure 5: Serum TG (µmol/dl) in rats fed the different diets.
Data are reported as mean ± SD. At T45 statistical analysis was carried out using the one way ANOVA with Tuckey as post test (n.s.)

DISCUSSION

The first part of this study (T0-T15)was devoted to the dietary induction of hypercholesterolemia induction in normal Wistar rats. Since it is demonstrated that rats have a good resistance to hypercolesterolemia [195], and extreme dietary protocol are required to induce it, we used a diet containing a high cholesterol amount (3%).

The 3% hypercholesterolemic diet caused significant changes in lipemia toward a pathological condition, increasing LDL-chol and decreasing HDL-chol. The HDL/LDL ratio

(atherogenic index), commonly utilized as an index of cardiovascular risk [196], was strongly affected. TOT-chol level diminished at T15. TOT-chol is mostly the sum of LDL- and HDL-chol, plus a small amount related to other lipoproteins. Therefore, it is clear that the HDL-chol huge decline caused the reduction in TOT-chol level.

A reduction in HDL-chol after a strong hypercholesterolemic diet was also observed in rats by *Mahley and Holcombe* [195]. In New Zealand white rabbits, the decrease in HDL-chol observed after a 1% hypercholesterolemic diet was linked to the oxidative stress induced by a severe cholesterol-rich diet [197]. Further studies are in progress to verify if the 3% hypercholesterolemic diet used in the present study could have induced an oxidative stress.

The decrease in serum TG after the hypercholesterolemic diet could be explained by the composition of the AIN-93M diet. This diet contains ~40 g fat / kg diet, mainly mono- and polyunsaturated, and ~50 g fiber / kg diet are also present. The high content of unsaturated fat [198]and fiber [199] could explain the observed huge decrease in serum TG level.

During the first phase of the study (T0-T15), food intake and body weight gain was not homogeneous among experimental rats, although their mean body weight gain was similar to the external control. It is conceivable that the high cholesterol content modified the texture, the flavor and the taste of the pellet, so influencing food intake in some animals.

In order to evaluate the possible influence of food intake on chol and TG levels, rats were divided according to body weight gain, which reflects food intake. Among the measured parameters, only HDL-chol was affected by food intake, its decrease being lower in rats with the lower body weight gain.

At T45, rats turned to a standard diet showed a trend to normality. Compared to T15, LDL chol was strongly reduced in standard diet fed rats, while it was unchanged in animals maintained on a hypercholesterolemic diet. In rats also receiving the probiotic mix LDL-chol was 30% lower than in the hypercholesterolemic diet alone group.

At T45, HDL-chol further decreased in animals fed the hypercholesterolemic diet, regardless the administration of the probiotic mix. The standard diet raised HDL-chol level, which came back to T0 values. As a consequence, the HDL/LDL ratio significantly increased, doubling value at T0. On the contrary, it remained low in rats receiving hypercholesterolemic diet, without differences related to the treatment with probiotic**.** TOT-chol level were consistent with LDL+HDL chol summation.

The observed absence of any effect of probiotic on HDL-chol level is in agreement with *Endo et al.* [200]. Similarly, no modification of HDL-chol level was observed in male albino hypercholesterolemic rats after supplementation of *Bifidobacteria, although LDL-chol* was reduced

by 56.3% [201]. On the contrary, *B. longum* BL1 increased HDL-chol and decreased TOT-chol and LDL-chol in humans *[186]*. In women, a mix of *L. acidophilus* 145 and *B. longum* 913 in the presence of 1% oligofructose was effective in HDL-chol reduction, without affecting LDL fraction. [202].

Akalin et al.[203] evaluated the *in vivo* effect of yoghurt made from milk inoculated with *S. thermophilus* and *L. acidophilus,* showing a significant decrease in LDL-chol but no effect on both HDL-chol and TG. Also in our study, no effect related to the probiotic mix were evidenced on TG level. On the contrary, *Nguyen et al.*[204] evidenced a 10% decrease in TG serum level in hypercholesterolemic mice treated with *Lactobacillus plantarum PH04*.

Strain genotype and strain combination appear therefore the crucial point for setting an effective cholesterol lowering mix. Different Authors indicate *Bifidobacteria* as the best choice, particularly when different strains are mixed [205-207].

However, the number of living cells administered per day represents another crucial point. Number of bacteria ingested, and their permanence in the gut is essential. Prebiotics largely affect probiotics effects and residence [208]. In the present study prebiotics were not administered, but this could be a good chance for enhancing the effect of the probiotic mix.

In conclusion, our data support the hypothesized ability of *bifidobacteria* in reducing cholesterol. In particular, our probiotic mix composed by *B. breve* WC 0463, *B. breve* WC 0420, and *B. bifidum* WC 0417 reduced LDL-chol fraction by 30% in the presence of a hypercholesterolemic diet. Although the mix did not affect serum HDL-chol and TG level, as reported in many other studies, it improved one of the main cardiovascular risk. It is known that different probiotics bacterial strains possess different effectiveness on hypercholesterolemia, and it is crucial to find effective strain combinations. Our findings suggest that the *Bifidobacteria* mix used in this study could be a good coadjuvant in the treatment of hypercolesterolemia, even in subjects consuming high cholesterol diet. Humans interventional studies are anyway needed to confirm our encouraging findings. A future perspective could also be to find out suitable prebiotics able to enhance the observed probiotic effect.

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FINAL CONSIDERATIONS

Lipids cover a central role in cell life. Changes in the organization of membrane fatty acids can exert profound effects on cellular functions such as signal transduction and membrane trafficking. Modification of lipid metabolism due to genetic alterations or environmental effects (such as diet) can lead to disease development.

Cholesterol is one of the most important regulators of lipid organization, and mammals have developed sophisticated and tight regulated mechanisms to maintain cellular cholesterol levels in membranes within a narrow range. When these homeostatic mechanisms are overwhelmed, outcomes or progression of pathological conditions under cardiovascular system are facilitate.

Cardiovascular diseases (CVD) are a group of pathological condition affecting both heart and blood vessels. CVD includes coronary heart disease (i.e. heart attacks), cerebro-vascular disease (i.e. stroke), raised blood pressure (i.e. hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure.

A real representative *in vitro* model for CVD does not really exist. Cell lines or primary cultures are suitable to elucidate the basic molecular mechanism of the biological process, but cannot mimic the physiological multifactorial states that occur in a whole organism. Many *in vivo* models have been proposed. However it is difficult to quantify how closely one animal model should imitate the human syndrome. Human *ex vivo* explants are not so easy to obtain in term of number of samples and amount of tissue. Interventional studies usually stand for only the last step of investigations. These evidences underline the importance to find representative models to investigate on CVD treatments outcomes.

CVD has been strongly linked to an excessive dietary intake of saturated fatty acids and cholesterol, over production of endogenous cholesterol, and chronic inflammation due to oxidative stress. The combination of these conditions causes accumulation and oxidation of plasma low-density lipoprotein cholesterol, vascular inflammation, and endothelial dysfunction.

Therapeutic interventions with lipid-lowering drugs (i.e. statins) act by reducing levels of total cholesterol and LDL-chol, and by increasing HDL-chol levels. Like all drugs, they may have side effects, and should be assumed for a very long period or during the whole life. For this reason

the development of new non-pharmacological approaches to reduce the endogenous cholesterol production and absorption could be a very important issue.

Many nutritional compounds can act on cholesterol absorption, metabolism, and biosynthesis. There is a large amount of evidence suggesting that a high consumption of fruits, vegetables and fish can help reduce the risk of CVD. Certain phytochemicals, including flavonoids and phytosterols are the food components to which the protective effect is ascribed. A considerable amount of epidemiological data suggests that phytochemicals contained in plant derived foods are able to reduce the risk of age-related chronic illness such as CVD. The initial ideas about how these compounds provide protection following ingestion centered upon their direct antioxidant activity. Now it appears clear that phytochemicals act not only as direct anti-oxidants but also through more subtle routes such as interaction with cell signaling pathways, or modulation of gene expression. Simultaneously, in recent years many researchers have demonstrated that gut microflora can exert positive effects in CVD development and treatment. Probiotics are accounted among active non pharmacological compounds able to low cholesterol absorption.

Improve our knowledge concerning mechanisms involved in cholesterol regulation and adsorption is essential to battle the CVD development and consequences. Essential in this topic is to find even better representative models clever to imitate the human pathological condition.

For this reason we have tried to set up a new experimental model using abdominal aortic aneurysm tissues obtained from patients who had undergone open surgery. Cultured tissue models are more physiologically representative than single cell culture. The development of a cultured vascular tissue model would be a valuable experimental tool for exploring vascular responses to food components. However considerable *inter-* and *intra-* tissue variations in the expression of different genes was observed. The magnitude of these variation precludes the use of such tissue as experimental model for the investigation of the effects of bioactives in the modulation of gene expression.

Using other *in vitro* and *in vivo* models we have tested bioactives of different origin for their ability to modulate cholesterol absorption and metabolism. In particular, we have tested EPA and DHA from fish oil, phytosterols from plants, and probiotics.

Regarding n-3 PUFA, using cultured neonatal rat cardiomyocytes as *in vitro* model system, we investigated the modification occurring in the cell lipid environment after EPA and DHA supplementation by means of high resolution magic angle spinning nuclear magnetic resonance technique in combination with gas chromatography. Then, using SH rats, we evidenced that dietary

EPA and DHA are able to improve lipemia, lowering the atherogenic index. This was related to a molecular effect of n-3 PUFA, which appeared different in SH rats compared to control rats.

Phytosterols were tested in primary cultures of neonatal rat cardiomyocytes. Our first aim was to exclude any possible deleterious effect of phytosterol supplementation. Cardiac cells were chosen as experimental model since the heart has been reported as the target organ for PS sub-chronic toxicity. A dose-dependent incorporation of PSs and a decrease in cholesterol content were clearly evidenced. The replacement of cholesterol by PSs could have been the cause of the observed slowing down of cardiomyocyte growth and reduction in metabolic activity. Our findings raise the suspect that the long-term exposure to physiologically relevant PS concentrations is a potential hazard. Our future perspective is to better elucidate is a molecular mechanism is part of the well known cholesterol lowering effect of phytosterols. For this reason we have started to test changes in gene expression induced by several phytosterol on the same *in vitro* model.

We used Wistar Kyoto rat nutritionally induced for hypercholesterolemia as *in vivo* models to assess the hypocholesterolemic effect of a new probiotic mix composed by: *B. breve* WC 0463, *B. breve* WC 0420, and *B. bifidum* WC 0417. Our findings strength the hypothesized ability of *bifidobacteria* in reducing serum cholesterol level. In particular, the probiotic mix counteracted the effect of a high-cholesterol diet reducing LDL-cholesterol fraction by 30%.

Although the herein reported studies are far from being conclusive, they could represent a further step in the recognition of the effects and mechanisms of action of different bioactives commonly used for cholesterol lowering.

To find active biological compounds, elucidating their mechanism of action, is crucial for the identification of the suitable combinations of molecules able to promote health. Keeping in mind that a single bioactive cannot exert the same effect of a pharmacological drug, an overall nutritional strategy could be able to prevent the onset of diseases, therefore avoiding pharmacological therapy.