UNIVERSITY OF SANNIO Benevento

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PhD Course in Sciences and Technologies for the Environment and Health XXXVIII Cycle

PhD Thesis

METABOLOMIC AND LIPIDOMIC STUDIES IN DEGENERATIVE AND AGE-RELATED DISEASES

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ABSTRACT

This translational research program is designed to identify and validate metabolomic and lipidomic molecular biomarkers for early diagnosis, prognostic stratification, and therapeutic monitoring in degenerative and age-related disorders. A central focus has been the investigation of oxysterols as pivotal intermediates within the cholesterol–oxidative stress–target-organ injury axis. The first two studies included 14 individuals with Alzheimer's disease dementia (three of whom carried an NPC1 mutation), 22 individuals with non-Alzheimer's dementias, and 53 cognitively unimpaired controls. The third study enrolled 54 patients with arterial hypertension and 150 normotensive participants. In the first two studies, cerebrospinal fluid analyses revealed a selective increase in non-enzymatically generated oxysterols and cholesterol biosynthetic intermediates in both Alzheimer's and non-Alzheimer's dementias, delineating a metabolomic signature associated with neurodegenerative pathology. In the three familial cases of autosomal dominant Alzheimer's disease linked to a heterozygous NPC1 mutation, Miglustat treatment was associated with a reduction in cerebral amyloid burden, normalization of oxysterol profiles, and clinical stabilization. In the hypertension cohort, integrated metabolomic–lipidomic profiling disclosed significant perturbations not only in hypertensive patients but also in individuals with early normotensive phenotypes, characterized by a marked depletion of polyunsaturated fatty acids: Eicosapentaenoic Acid, Docosahexaenoic Acid, and Arachidonic Acid) and very-long-chain fatty acids. These findings suggest that lipidomic derangements may precede the overt clinical manifestation of hypertension, potentially contributing to vascular and myocardial remodeling and serving as candidate early biomarkers of cardiovascular risk. Moreover, hypertensive subjects exhibited increased 7β-hydroxycholesterol levels, which correlated closely with established indices of cardiovascular target-organ damage, including pulse wave velocity, carotid intima–media thickness, and left ventricular mass index. Collectively, these results substantiate a relevant pathogenetic contribution of lipid dysregulation to the neurodegenerative and cardiovascular processes of aging and indicate that the integration of metabolomic, lipidomic, and clinical data constitutes a rigorous methodological platform for the development of translational biomarkers and precision therapeutic strategies.

INTRODUCTION

LIPIDS GENERAL INFORMATION

Biological lipids are a group of compounds that have in common the characteristic of being insoluble in water. Phospholipids and sterols are the main structural elements of biological membranes. Other lipids, although present in relatively small amounts, play crucial roles as enzyme cofactors, electron carriers, light-absorbing pigments, hydrophobic anchors for proteins, chaperones to promote protein folding, emulsifying agents in the intestinal tract, hormones, and intracellular messengers. Fats and oils, used almost universally as energy storage forms by living organisms, are compounds derived from fatty acids [1]. Fatty acids, in turn, are derivatives of hydrocarbons and have practically the same low oxidation state (i.e. they are almost completely in the reduced state) as the hydrocarbons found in fossil fuels. The complete oxidation of fatty acids in the cell (to CO2 and H20) is a highly exergonic process. The simplest lipids constructed from fatty acids are triacylglycerols, also called triglycerides, fats, or neutral fats [1]. Triacylglycerols are composed of three fatty acids linked by ester bonds to the hydroxyl groups of a glycerol molecule. The supporting structures of biological membranes are made up of a lipid bilayer, mainly composed of phospholipids, which acts as a barrier to the passage of polar molecules and ions. Phospholipids are amphipathic, meaning one end of the molecule is hydrophobic and the other is hydrophilic. Hydrophobic interactions between lipid molecules and hydrophilic interactions between lipid molecules and water determine the arrangement of these structures into sheets, called membrane bilayers [2]. Sterols are structural lipids present in the membranes of many eukaryotic cells. The structural feature is the steroid nucleus consisting of four fused rings, three with six carbon atoms and one with five atoms (Figure 1). The steroid nucleus is nearly planar and relatively rigid. The presence of fused rings prevents rotation around the e-e bonds. Sterols are not only membrane components, but also precursors to many products with specific biological activities. For example, steroid hormones are powerful biological

signals that control gene expression. Bile acids, polar derivatives of cholesterol, act as detergents in the intestine, emulsifying dietary fats to make them easier for lipases to digest. Chromatographic methods based on differences in polarity between various classes of lipids can be used to separate complex mixtures of lipids present in tissues into their essential components [1,2].

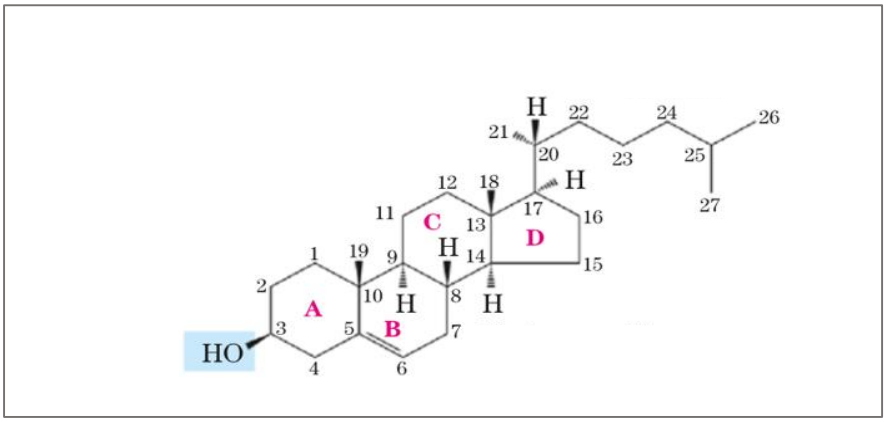

Figure 1 Cholesterol Molecule

CHOLESTEROL METABOLISM

Cholesterol homeostasis is essential for proper systemic and cellular function. Numerous diseases, including cardiovascular disease, neurodegenerative diseases, and cancer, are caused by an unstable cholesterol balance. The ongoing balance between biosynthesis, absorption, export, and esterification is represented by the cellular cholesterol level. Esterification is a process in which cholesterol is converted to neutral cholesterol esters for storage in lipid droplets or secretion as components of lipoproteins [1,3].

CHOLESTEROL SYNTHESIS

Cholesterol is a lipid molecule largely produced by the liver, with only 20% absorbed by the body through food. Endogenous production ranges from 1 to 2 grams per day and is

found in all tissues, with the highest concentrations in the brain, bile, and blood [1]. The synthesis (Figure 2) begins with acetyl-CoA at the cytoplasmic level and occurs in four main steps:

STEP 1: Condensation of three acetate units to form a six-carbon intermediate, mevalonate.

STEP 2: Conversion of mevalonate into activated isoprene units

STEP 3: Polymerization of five-carbon isoprene units to form squalene.

STEP 4: Cyclization of squalene to generate the four-ringed steroid nucleus from which cholesterol is formed.

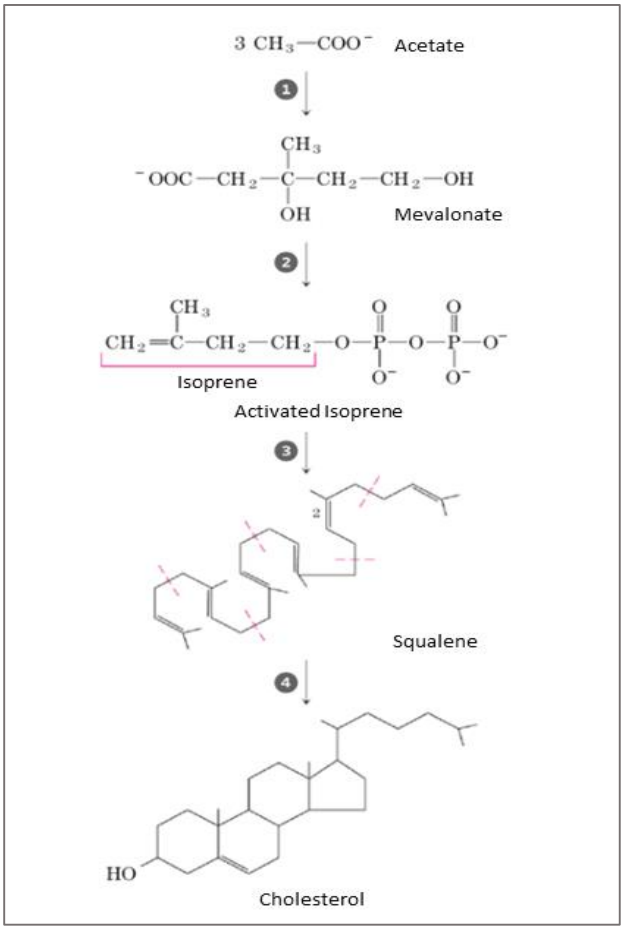

Figure 2 Biosynthetic pathway of cholesterol from acetate through the mevalonate pathway and squalene formation

STEP 1

A. The first step in cholesterol biosynthesis produces the intermediate mevalonate (Figure 3) [3,4]. Two molecules of acetyl-CoA condense to form acetoacetyl-CoA.

B. acetoacetyl-CoA, in turn, reacts with a third molecule of acetyl-CoA to generate the six-carbon compound β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). These first two reactions are catalyzed by acetyl-CoA acetyltransferase and HMG-CoA synthase, respectively. The third reaction represents the control step in cholesterol biosynthesis: the reduction of HMG-CoA to mevalonate, in which two molecules of NADPH each provide two electrons for the reaction. This reaction is catalyzed by HMG-CoA reductase, a key enzyme and major regulatory point in the cholesterol biosynthetic pathway.

C. Finally, a series of ATP-dependent reactions leads to the formation of the isopentenyl pyrophosphate molecule which will be conveyed to the second phase of the synthetic pathway.

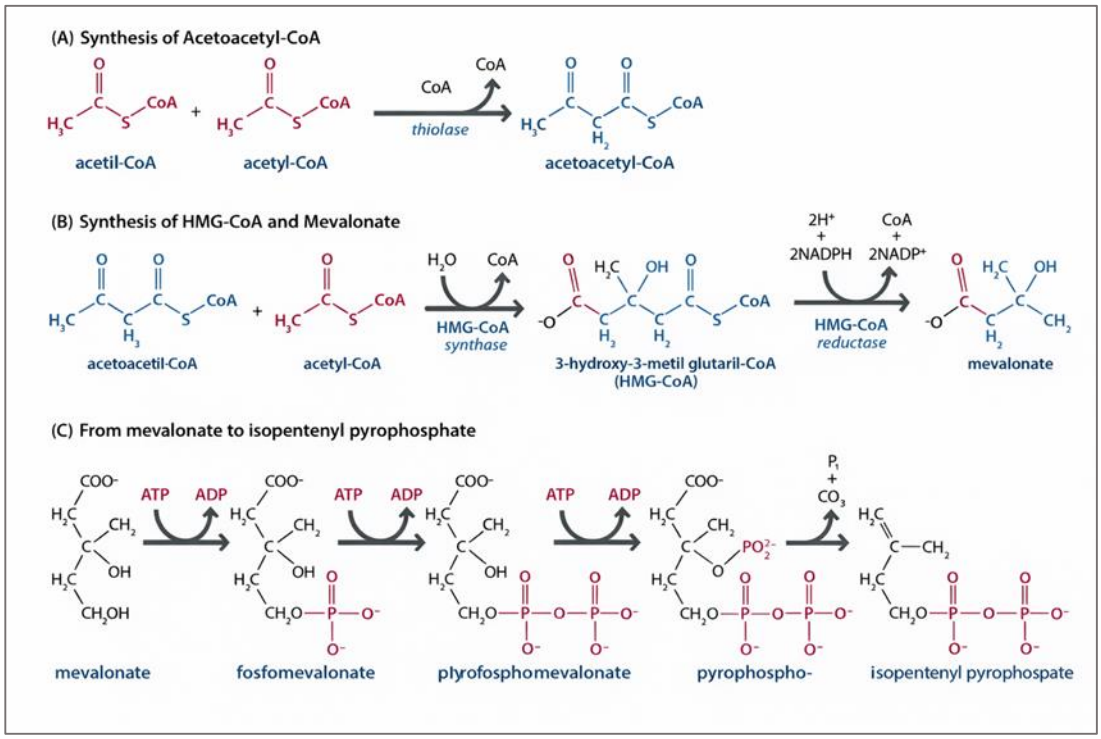

Figure 3 The first step in the cholesterol synthesis pathway. In this step of the pathway, isopentenyl pyrophosphate is synthesized from acetyl CoA

STEP 2

In this step, three phosphate groups are transferred from three ATP molecules to mevalonate (Figure 4). The phosphate group attached to the hydroxyl group on C-3 of mevalonate in the intermediate 3-phospho-5-pyrophosphomevalonate is a good leaving group. This phosphate group and the adjacent carboxyl group are released, generating a double bond in the five-carbon product, δ^3-isopentenyl pyrophosphate. This is the first activated isoprene unit, required for cholesterol synthesis. Isomerization of δ^3-isopentenyl pyrophosphate generates a second activated isoprene unit, dimethylallyl pyrophosphate [1].

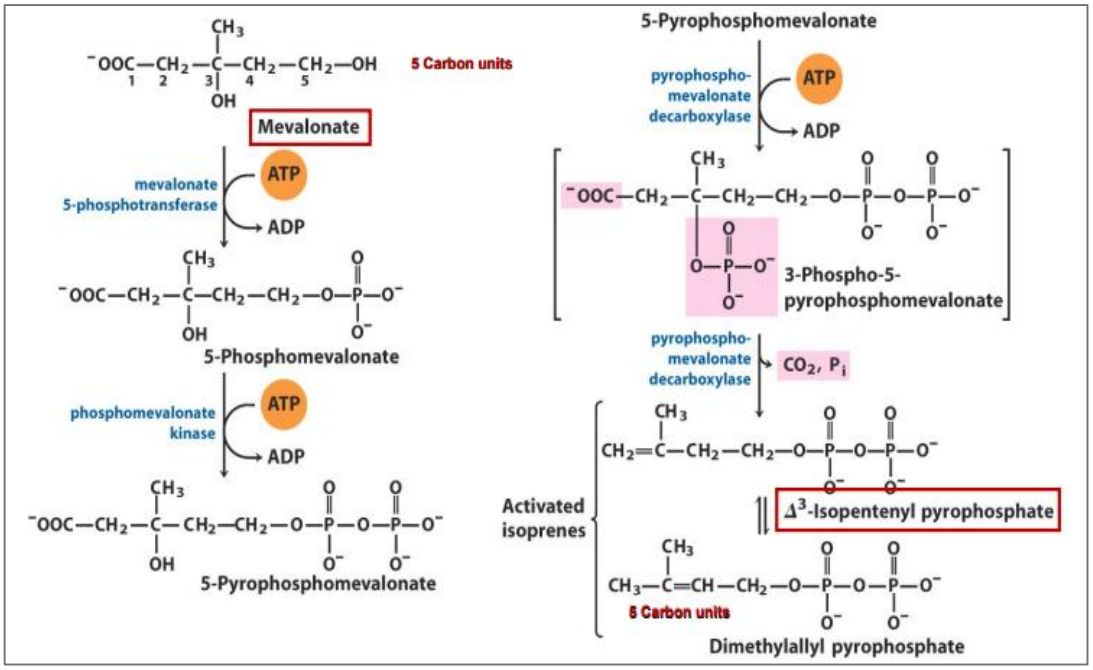

Figure 4 The Mevalonate Pathway: Enzymatic Steps Leading to Activated Isoprenes

STEP 3

Isopentenyl pyrophosphate and dimethylallyl pyrophosphate now undergo a head-to-tail condensation reaction, in which, while a pyrophosphate group is released, a 10-carbon chain, geranyl pyrophosphate, is formed (Figure 5). (The "head" refers to the end of the carbon chain to which the pyrophosphate group is attached). Geranyl pyrophosphate undergoes another head-to-tail condensation reaction, producing a 15-carbon intermediate, farnesyl pyrophosphate. Finally, two molecules of farnesyl pyrophosphate join, this time head-to-head, with the elimination of both pyrophosphate groups, to form squalene [1].

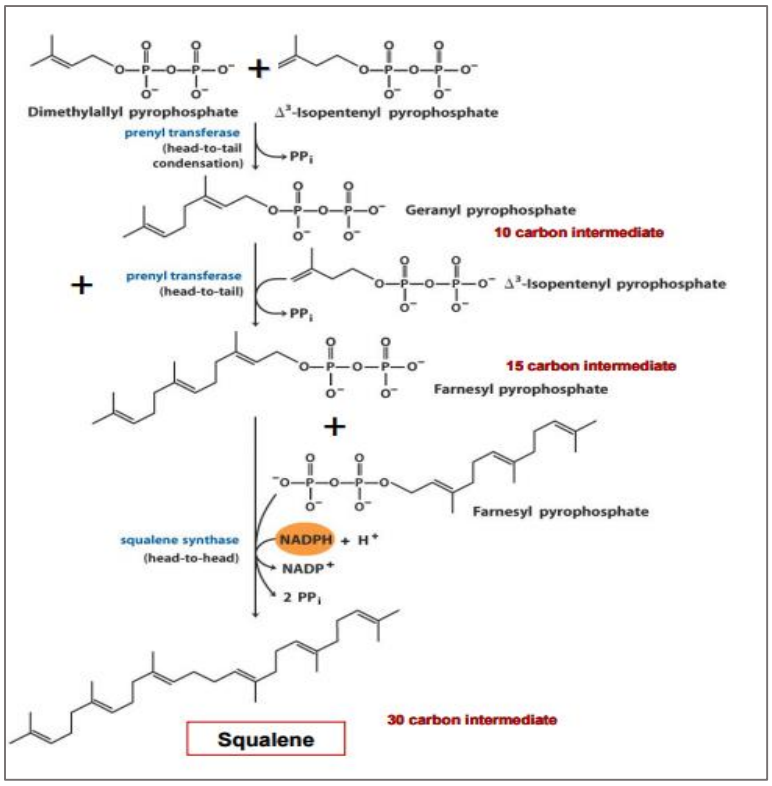

Figure 5 Formation of Squalene from Activated Isoprene. Sequential Condensation Reactions

STEP 4

Sterols are all alcohols, with a hydroxyl group on the C-3 carbon atom, and have four fused rings (steroid nucleus). This is the reason they are called "sterols". To form an epoxide, monooxygenated squalene adds an oxygen atom to the end of the squalene molecule, taking it from the oxygen. This enzyme is another mixed-function oxidase. NADPH converts the other oxygen atom into water. The double bonds of the product of this reaction, squalene 2,3-epoxide, are positioned such that in a coordinated reaction, the linear structure of the squalene epoxide is transformed into a cyclic structure. In animal cells, cyclization produces lanosterol, which has the distinctive four rings of the steroid nucleus. Then, through a series of about twenty reactions, lanosterol is converted to cholesterol (Figure 6). This conversion involves the displacement of some methyl groups and the removal of others. Konrad Bloch, Feodor Lynen, John Cornforth, and George Popjak identified and defined this metabolic pathway in the late 1950s, which was one of the most complex ever studied [1].

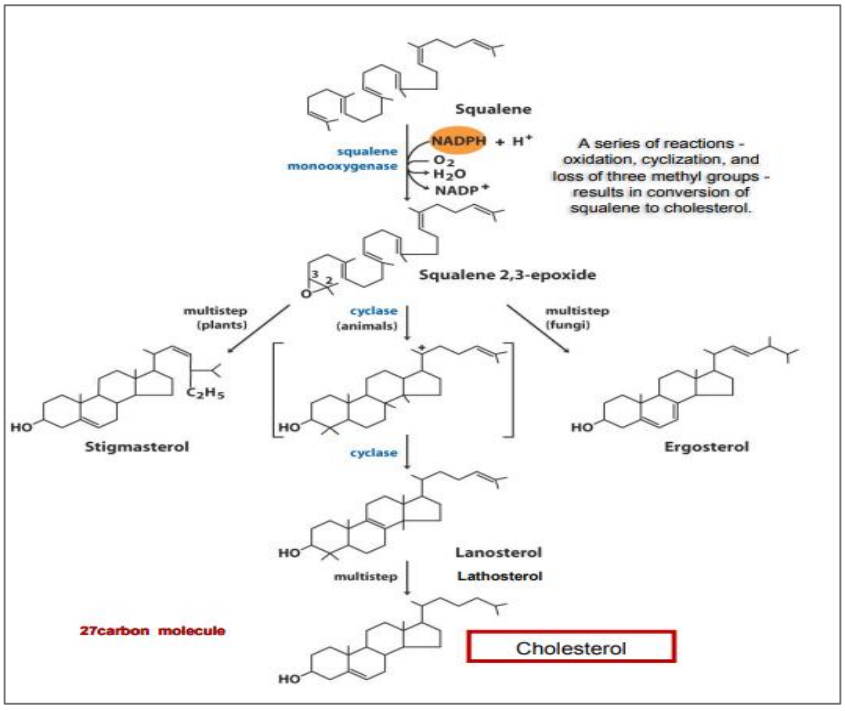

Figure 6 Cyclization of Squalene and Formation of Sterols

CHOLESTEROL TRANSPORT

Most cholesterol is produced in the liver. A small amount is incorporated into hepatocyte membranes, while the majority is exported in one of three possible forms: bile acids, bile cholesterol, or cholesterol esters.

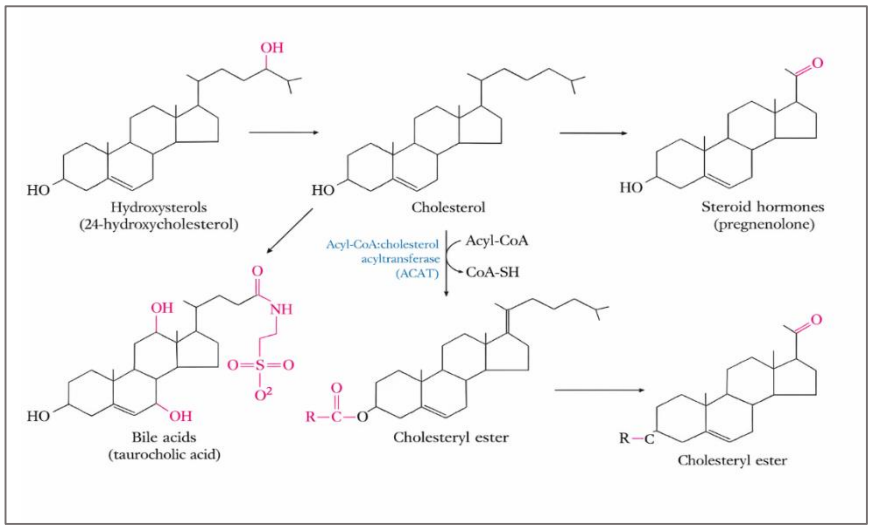

Figure 7 Cholesterol Metabolism

In other tissues, cholesterol is converted into steroid hormones (in the adrenal cortex and gonads, figure 8) or into the hormone vitamin D (in the liver and kidney, figure 9).

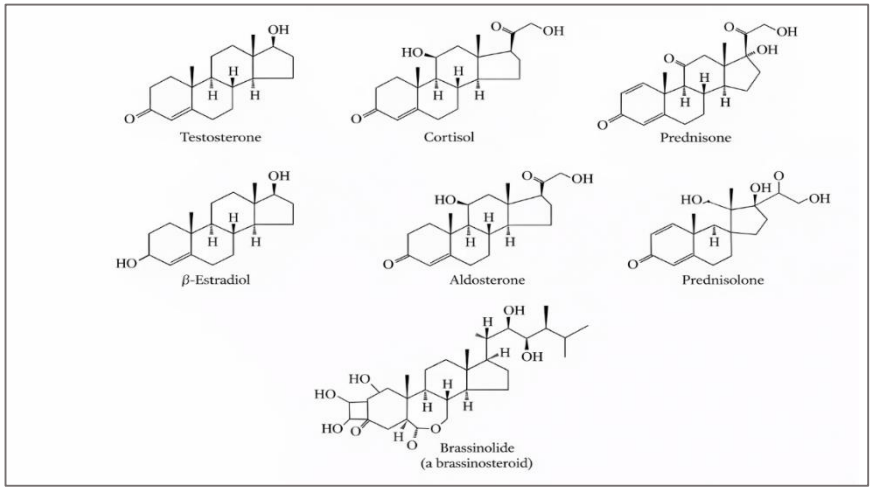

Figure 8 Cholesterol-derived steroids

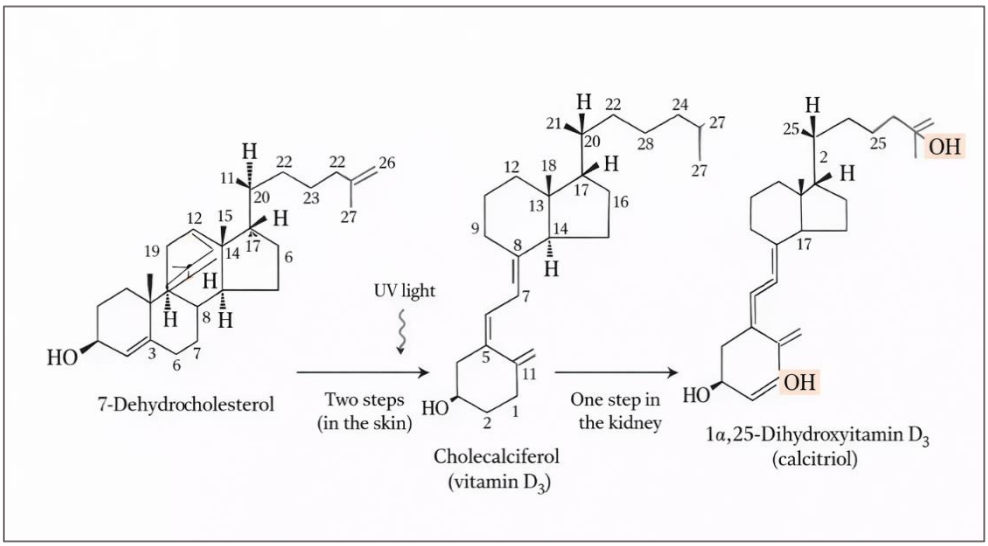

Figure 9 Vitamin D_3 Production

Cholesterol and its esters are insoluble in water. Therefore, they are transported in the blood as plasma lipoproteins, macromolecular aggregates composed of specific carrier proteins, called apolipoproteins, associated in varying proportions with phospholipids, cholesterol, cholesterol esters, and triacylglycerols. Lipoproteins are spherical structures composed of an inner core (containing lipids) and apolipoproteins (associated with phospholipids and cholesterol) (Table 1).

Table 1 Comparative Apolipoprotein Distribution Among Major Lipoprotein Classes

Lipoproteins	**CHYLOMICRONS**	**VLDL**	**LDL**	**HDL**
Apolipoproteins	APOB48 APOE APOCII	ABOB100 APOE APOC1 -C2-C3	APOB100	APOA1-A2 APOE APOC2-C3

Lipoproteins are classified in several ways:

A. Based on **DENSITY**, they include (from lowest to highest density):

   I. CHYLOMICRONS: They have the lowest density, are the largest, and contain a high amount of triglycerides. They are synthesized in the enterocyte ER from dietary fats.

   II. VLDL (Very Low Density Lipoprotein)

   III. LDL (Low Density Lipoprotein)

   IV. HDL (High Density Lipoprotein)

B. Based on **ATHEROGENETIC POWER**:

   I. *HIGHLY ATHEROGENETIC POWER*, lipoproteins that contain a lot of cholesterol:

      1. Chylomicron remnants

      2. VLDL remnants (called IDL, intermediate density lipoproteins)

      3. LDL

   II. *NON-ATHEROGENETIC:*

      1. Chylomicrons

      2. VLDL

   III. *ANTI-ATHEROGENETIC:*

      1. HDL

Lipids are then transported in the bloodstream in the form of lipoproteins, which differ in their functionality, protein and lipid composition, and therefore density. There are two

pathways for cholesterol transport: the exogenous pathway (which transports dietary fats) and the endogenous pathway.

EXOGENOUS PATHWAY

A.1 CHYLOMICRONS

Enterocytes produce chylomicrons, which travel through the lymphatic system and then enter the bloodstream via the left subclavian vein. Chylomicrons release a large portion of the triglycerides to adipose tissue, heart, skeletal muscle, and the mammary gland during lactation. These tissues contain lipoprotein lipase in their capillaries, which interacts with the apo C-II of chylomicrons. Specifically, lipoprotein lipase hydrolyzes chylomicron triglycerides into free fatty acids and glycerol. Free fatty acids (FFA), also called NEFA (Non-Esterified Fatty Acids) can both be absorbed by tissue cells, where they are used to produce energy (Beta-oxidation), and travel in the blood associated with albumins (they cannot travel freely because they are hydrophobic). Glycerol reaches the liver because it is soluble. The chylomicrons thus transform into REMAINS (containing cholesterol, APO-E, and APO-B48).

A.2 CHYLOMICRON REMAINS

They reach the LIVER through the blood, where they are absorbed by endocytosis by the hepatocytes, through the interaction: APO-E + RECEPTORS.

ENDOGENOUS PATHWAY

B.1 VLDL

The liver synthesizes triglycerides and cholesterol esters (ENDOGENOUS LIPIDS) from:

- Fatty acids and cholesterol consumed excessively through the diet

- Carbohydrates
- Fatty acids derived from adipose tissue between meals.

This is how VLDL (very low density lipoproteins) are formed, which reach adipose tissue and skeletal muscle via the blood. In the capillaries of these tissues, lipoprotein lipase and apo-C2 interact, releasing FFAs. These are used by adipocytes to resynthesize triglycerides in the form of intracellular lipid droplets, which myocytes oxidize to obtain energy. After losing their triglycerides, VLDL can be converted into IDL, which in turn can be reabsorbed by hepatocytes thanks to APO-E or APO-B100. Alternatively, IDL further loses their triglycerides and is converted into LDL.

B.2 LDL

LDLs are very rich in cholesterol and cholesterol esters. They are characterized by APO-B100. They transport cholesterol to various tissues (adrenals, gonads, muscle, adipose tissue) equipped with receptors that recognize APO-B100, so LDL entry into cells occurs via receptor-mediated endocytosis. LDL also transports cholesterol to macrophages, which become foam cells. Unused LDL returns to the liver where it is absorbed via LDL receptors on hepatocytes. Cholesterol within hepatocytes can be:

- INCORPORATED INTO MEMBRANES
- CONVERTED INTO BILE ACIDS
- RE-ESTERIFIED BY ACYL-COA-COLESTEROL ACYL TRANSFERASE (ACAT), FORMING CYTOSOLIC LIPID DROPLETS.

B.3 HDL

They originate in the liver and small intestine. Each HDL particle is composed of:

- CHOLESTEROL
- ENZYME Lectin-Cholesterol Acyl-Transferase (LCAT)
- APOLIPOPROTEINS (including APO-A1 and APO-A2)

The LCAT enzyme catalyzes the reaction of cholesterol + lecithin (also called phosphatidylcholine) to cholesterol esters + lysolecithin. HDL can also incorporate cholesterol from extrahepatic cells, macrophages, and foam cells. Mature HDL returns to the liver where it disposes cholesterol. This occurs via the SRBI receptor. This receptor promotes the transfer of cholesterol from HDL into the cell. This mechanism does not involve endocytosis. The now emptied HDL passes into the blood to extract other lipids from chylomicron remnants, VLDL, and cholesterol-rich cells. The cholesterol released by HDL into the liver is mostly converted into bile salts, which are released into the gallbladder, then excreted into the small intestine and finally into the feces.

REGULATION OF CHOLESTEROL SYNTHESIS

Cholesterol synthesis is a process that requires a lot of energy and constant monitoring. Unlike other lipids, cholesterol cannot be used as metabolic fuel, so excess cholesterol must be eliminated. This requirement makes it essential to regulate its production based on both nutrition and the amount already present in cells. The cell evaluates whether to maintain cholesterol synthesis based on three signals: the amount of cholesterol within it, the amount of available ATP, and hormonal signals: insulin and glucagon. The most important checkpoint in the entire biosynthetic pathway is the reaction that converts HMG-CoA to mevalonate, catalyzed by HMG-CoA reductase. Therefore, the cell is able to regulate the enzyme through two pathways: a short-term and a long-term pathway.

SHORT-TERM REGULATION:

HMG-CoA reductase can be rapidly activated or deactivated through reversible phosphorylation. When ATP decreases and AMP increases, AMPK (AMP-activated protein kinase) is activated. AMP kinase phosphorylates HMG-CoA reductase and inactivates it. This slows cholesterol production and stimulates the catabolic pathways that lead to ATP production. Hormonal signals also act similarly, particularly glucagon, which is typical during times of fasting or energy stress, and promotes phosphorylation and therefore inactivation of reductase. Insulin, on the other hand, promotes dephosphorylation and activation of the enzyme, stimulating cholesterol synthesis when energy resources are abundant.

LONG-TERM REGULATION:

The most significant quantitative regulation of the enzyme occurs at the gene level through a transcription control system for the gene encoding HMG-CoA reductase. This highly precise system is based on the SREBP (Sterol Regulatory Element Binding Proteins). SREBPs are attached to the membrane of the endoplasmic reticulum (ER) and, to activate the reductase gene, must undergo a double proteolytic cleavage, releasing a fragment capable of entering the nucleus and activating the genes required for sterol and fatty acid synthesis. Two other proteins play an important role in this system: SCAP (SREBP Cleavage Activator Protein) and INSIGN (Insulin-Induced Gene Protein). These two proteins function as sterol sensors. When sterol levels are abundant and there is no need to produce additional cholesterol, SCAP and INSIGN trap SREBP in the endoplasmic reticulum. When sterol levels decrease, INSIGN detaches from SCAP, allowing SCAP to transport SREBP to the Golgi apparatus, where it is cleaved and activated. The resulting active fragment enters the nucleus and activates the transcription of its target genes, including those of HMG-CoA reductase, LDL receptor protein, and several other proteins necessary for lipid synthesis. In addition to this long-term negative feedback system, the cell can directly eliminate HMG-CoA reductase. Elevated cellular cholesterol levels are

sensed by Insig, which promotes the attachment of ubiquitin molecules to HMG-CoA reductase, leading to its degradation by proteasomes. Furthermore, under conditions of high levels of oxysterols (oxidized cholesterol derivatives), the nuclear receptor (LXR) is activated. This detects excess cholesterol and promotes its elimination or reuse. Once activated, it forms a heterodimer with the retinoid X receptor (RXR). This dimer activates the transcription of a series of genes that facilitate the efflux of cholesterol to HDL and activate bile acid metabolism. Cholesterol homeostasis represents one of the most complex systems in the cell. The SREBP and LXR systems work together to finely regulate this process. The former is activated when cholesterol is low and stimulates its synthesis; the latter is activated when there is excess cholesterol and reduces the need for new synthesis.

OXYSTEROLS

Oxysterols were first identified by Lifshultz in 1913 as products of cholesterol oxidation. About 30 years later, Kandutsch and others developed the "Oxysterol Hypothesis of Cholesterol Homeostasis" theory, which has been the subject of countless revisions over the years [5]. Oxysterols are oxidized products of cholesterol that play diverse roles in various pathophysiological processes, including the control of lipid metabolism, immunological processes, and cytotoxicity [6]. Oxysterols are a category of cholesterol derivatives produced by the addition of a hydroxyl group to the backbone of the cholesterol molecule. They are 27-carbon compounds that vary based on the position, number, and/or oxidation state of the hydroxyl group(s) [7]. The most frequent sites of cholesterol oxidation are those shown in figure 10.

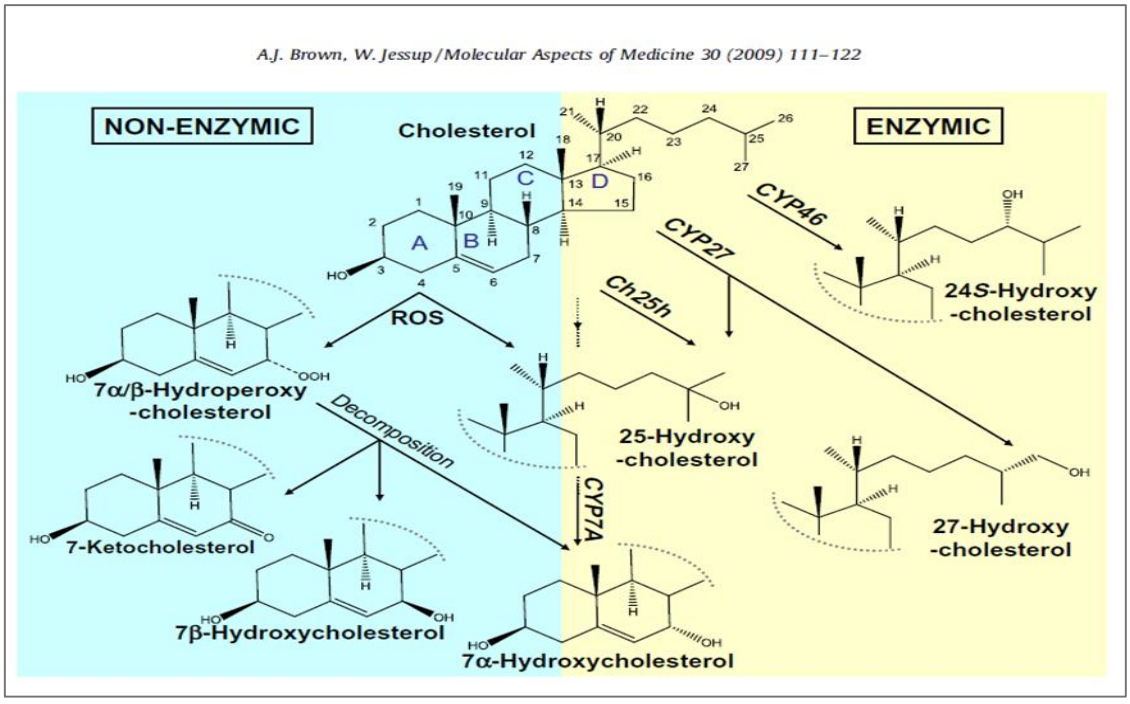

Figure 10. Enzymatic and non-enzymatic pathways of oxysterol formation from cholesterol.
The figure illustrates the main pathways involved in the formation of oxysterols from cholesterol. It shows the complete structure of cholesterol (rings A–D and carbon numbering) and, in simplified form, the structures of oxysterols, highlighting the addition of oxygenated groups. Both enzymatic and non-enzymatic pathways mediated by reactive oxygen species (ROS) are distinguished.

Cholesterol, being a monounsaturated lipid, therefore a molecule with an unsaturated bond or double bond on carbon 5, is susceptible to oxidation in the presence of oxygen, light, heat, radiation, free radicals, metal ions and other factors [8]. Oxysterols are groups of sterols with a structure similar to cholesterol but have a sterol core with a hydroxyl, ketone, or epoxide group and/or a hydroxyl chain in their side molecules [8,9]. They are produced in vivo non-enzymatically after the oxidation of lipids in biological membranes and lipoproteins or enzymatically during cholesterol catabolism. In general, biological oxysterols are divided into two main categories:

- Those oxygenated on the sterol ring, mainly in position 7 (for example 7α/β-hydroperoxycholesterol (7OOHC), 7-ketocholesterol (7KC) and 7α/β-hydroxycholesterol (7HC));
- Those oxygenated on the side chain, such as 24S-hydroxycholesterol (24HC), 25-hydroxycholesterol (25HC) and 27-hydroxycholesterol (27HC).

Ring-oxygenated sterols are typically formed by non-enzymatic pathways, while side-chain oxygenated sterols are typically formed by enzymatic pathways. However, there are some exceptions to this rule: For example, 25HC and 7αHC can be formed by both enzymatic and non-enzymatic pathways.[9]

NON-ENZYMATIC PATHWAY

Nonenzymatic oxidation (or autoxidation), a process involving reactive oxygen species (ROS) and reactive nitrogen species (RNS), produces some oxysterols. Hydroxyl radical (OH), hydrogen peroxide (H2O2), singlet oxygen, and ozone (O3) are ROS that contribute to the nonenzymatic oxidation of cholesterol [7, 10]. Cholesterol is more vulnerable to free radicals because it has two bonds. Oxygen-induced free radicals (ROS), particularly hydroxyl radicals, attach to cholesterol, specifically carbon 7, stripping a hydrogen atom, creating the cholesterol radical, a highly reactive molecule. This cholesterol radical combines with oxygen to produce a peroxyl radical, which in turn combines with another lipid to produce cholesterol hydroperoxides (7a-OOHC and 7b-OOHC). These are the first effects of non-enzymatic oxidation of cholesterol. They do not remain in tissues for long because they are reduced by cellular enzymes or further oxidized. 7-OOHC can transform into 7-α-hydroxycholesterol, 7-β-hydroxycholesterol, and 7-ketocholesterol if metals such as Fe and Cu are present [7].

ENZYMATIC PATHWAY

Enzymes can modify cholesterol by adding a hydroxyl group (-OH) to the side chain forming different oxysterols, each formed by a different enzyme:

- 27-Hydroxycholesterol (27HC) is formed through the action of the enzyme sterol 27-hydroxylase (CYP27A1). The reaction occurs in the mitochondria, especially in the liver and macrophages, and the enzyme adds an OH to cholesterol. The absence of this enzyme is the cause of cerebrotendinous xanthomatosis, a rare genetic disease.
- 24-hydroxycholesterol (24HC) is formed by the action of the enzyme 24-hydroxylase (CYP46A1). Synthesis occurs in the endoplasmic reticulum in neuronal cells and the

- 25-hydroxycholesterol (25HC) is produced through the action of cholesterol 25 hydroxylase, mainly in the endoplasmic reticulum and Golgi. 25HC regulates cholesterol synthesis by inhibiting the SREBP pathway retina. The brain produces most of the 24-hydroxycholesterol that circulates in the blood.

Table 2 Enzymatic Oxysterols

Oxysterol	**Responsible Enzyme**	**Where it is located/ main tissues**	**Main function**
24-hydroxycholesterol (24HC)	**CYP46A1** (cholesterol 24-hydroxylase, P450)	Endoplasmic reticulum; brain and retina	Disposal of cerebral cholesterol; major source of circulating 24HC
25-hydroxycholesterol (25HC)	**Cholesterol 25-**hydroxylase (non-heme iron-containing protein)	Endoplasmic reticulum and Golgi membranes; expressed at low levels in many tissues	Regulator of the SREBP pathway, reduces cholesterol synthesis when there is abundance
27-hydroxycholesterol (27HC)	**CYP27A1** (sterol 27-hydroxylase, P450)	Mitochondria; liver, macrophages, other tissues	First step in the alternative pathway for bile acid synthesis; can further oxidize to a carboxylic acid derivative

CYTOTOXICITY OF OXYSTEROLS

Oxysterols may be involved in the pathogenic processes of atherosclerosis, neurodegeneration, diabetes, renal failure, and ethanol intoxication [11, 12]. Furthermore, they may contribute to the development of age-related macular degeneration [13], cataracts [14] and osteoporosis and have been associated with various types of cancer [15,16]. Oxysterols are important not only in some aspects of physiological processes, but also in pathological conditions, primarily those related to chronic inflammation. Enzymatically formed oxysterols primarily regulate cholesterol homeostasis. In contrast, oxysterols of autooxidative origin appear to have cytotoxic effects. Oxysterols have been observed to be present in amounts above physiological levels in many human diseases associated with cell death and inflammation. The ability of oxysterols to induce cell death is the main reason for their cytotoxicity. Several in vitro studies have described the pro-apoptotic effects of oxysterols on a variety of cell lines (macrophages, fibroblasts, smooth muscle cells, endothelial, monocytic, and enterocytic cells) [17]. The mechanism through which oxysterols activate the apoptotic process is not yet completely clear. Oxysterols can regulate enzymes of the NADPH oxidase (NOx) family and increase the production of reactive oxygen species. Oxidation-reduction reactions, which involve the transfer of electrons between chemical species, play a very important physiological role in health and disease. Cholesterol oxidation is a physiological reaction that plays an important role in cholesterol homeostasis and is an important signaling mechanism for metabolic changes and environmental stresses and changes that occur in all living systems. Changes in this complex communication network can cause acute and chronic pathological conditions, which constitute an important area of investigation in the field of health. Their involvement in aging is inevitable because the metabolism of cholesterol and oxysterols supports key metabolic pathways (figure 11) [18]. Observational studies provide information on the levels of oxysterols present in biological fluids or tissue samples collected from humans. The oxysterols most commonly reported in association with human disease include: 25-hydroxycholesterol (25-OHC), 24-hydroxycholesterol (24-OHC), 5,6-epoxycholesterol isomers (5,6-EC), cholestane-

3β,5α,6β-triol (α-triol), 27-hydroxycholesterol (27-OHC), 7-ketocholesterol (7-KC), 7-α-hydroxycholesterol (7α-OHC), and 7-β-hydroxycholesterol (7β-OHC) [19]. Concentrations of 7-oxycholesterols (7-KC and 7β-OHC) have been found to be increased in many diseases, such as diabetes [20], dyslipidemia [21], hypercholesterolemia [22], and neurodegenerative diseases [18, 23].

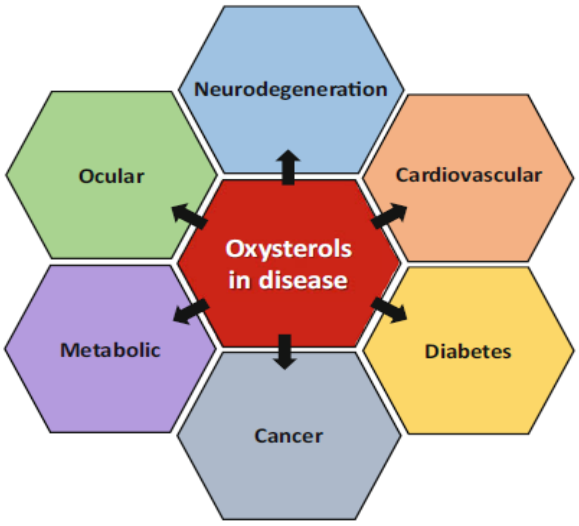

Figure 11 Oxysterols in disease

Many factors, including environmental, genetic, and epigenetic factors, as well as lifestyle factors, contribute to aging, which is a gradual and irreversible process. Together, these factors alter the body's homeostatic mechanisms. The involvement of oxysterols in the aging process is well documented and is linked to many age-related diseases, such as diabetes, cardiovascular disease, neurodegenerative diseases, cancer, and macular degeneration [24, 25,26]. This link has been mainly explained by the contribution of oxysterols to imbalances in RedOX homeostasis [27,28] cholesterol metabolism [29,30], and chronic inflammation [31,32] It is now well known that many oxysterols, including 7-KC, 27-OHC, and 7β-OHC, have cytotoxic effects that lead to cellular dysfunction in numerous tissues of the body [33].

METABOLISM OF VERY LONG CHAIN FATTY ACIDS (FROM C22 TO C26)

Fatty acids are aliphatic monocarboxylic acids. Fatty acids with a carbon number greater than 20 (C>20) are called very-long-chain fatty acids (VLCFA). VLCFAs are found as constituents of cellular lipids, such as sphingolipids and glycerophospholipids, and as precursors of lipid mediators. Long-chain fatty acids (LCFAs) have chain lengths between C11 and C20. Very-long-chain fatty acids are less abundant than LCFAs. VLCFAs with a carbon chain of ≥26 carbons or more are often subclassified as ultra-long-chain fatty acids (ULCFAs) and are found in specific tissues, including the skin, retina, testes, and brain. Another classification of fatty acids is based on the number of double bonds, classifying them into saturated (SFA; no double bonds), monounsaturated (MUFA; one double bond), and polyunsaturated (PUFA; two or more double bonds). PUFAs are further subclassified into n-3 (or ω3) and n-6 (or ω6) series depending on the position of the terminal double bond, i.e., the double bond furthest from the carboxyl group [43]. The classification of very long chain fatty acids is shown in Table 3.

Table 3 Classification of very long chain fatty acids

CLASSIFICATION	FATTY ACID
SATURATED VERY LONG CHAIN FATTY ACIDS	**Behenic acid (22:0)**
	Lignoceric acid (24:0)
	Cerotic acid (26:0)
	Montanic acid (28:0)
	Melissic acid (30:0)
	Laceronic acid (32:0)
MONOUNSATURATED VERY LONG CHAIN FATTY ACIDS	**Cetoleic acid (22:1)**
	Erucic acid (22:1)
	Nervonic acid (24:1)
VERY LONG CHAIN POLYUNSATURATED FATTY ACIDS	**Clupanodonic acid (22:5)**
	Cervonic acid (22:6)

VERY LONG CHAIN FATTY ACID BIOSYNTHESIS PATHWAY

Fatty acids are elongated by enzymes embedded in the endoplasmic reticulum membrane. Fatty acid elongation occurs through a four-step process (condensation, reduction, dehydration, and reduction), shown in figure 12. In the first, rate-limiting step, acyl-CoA is condensed with malonyl-CoA to produce 3-ketoacyl-CoA. This reaction is catalyzed by an FA elongase. Mammals possess seven FA elongases (ELOVL1-7), each of which has a characteristic substrate specificity. In the second step, 3-ketoacyl-CoA is reduced to 3-hydroxyacyl-CoA by a 3-ketoacyl-CoA reductase. Nicotinamide adenine dinucleotide phosphate (NADPH) is used as the reducing agent in this reaction. The 3-hydroxyacyl-CoA is then dehydrated by 3-hydroxyacyl-CoA dehydratase, generating 2,3-trans-enoyl-CoA. Finally, the 3-hydroxyacyl-CoA is reduced to an acyl-CoA with two more carbon chains than the original acyl-CoA. A 2,3-trans-enoyl-CoA reductase catalyzes this reaction using NADPH as a reductant. The enzymes responsible for the fatty acid elongation cycle can interact with each other and form elongase complexes [34]. In particular, the synthesis of VLCFA (C24:0 and C26:0) requires two of the ELOVL enzymes. First the elongation complex with ELOVL6 elongates from C16:0 to C20:0/C22:0 and then ELOVL1 further elongates these fatty acids to C24:0 and C26:0 [35].

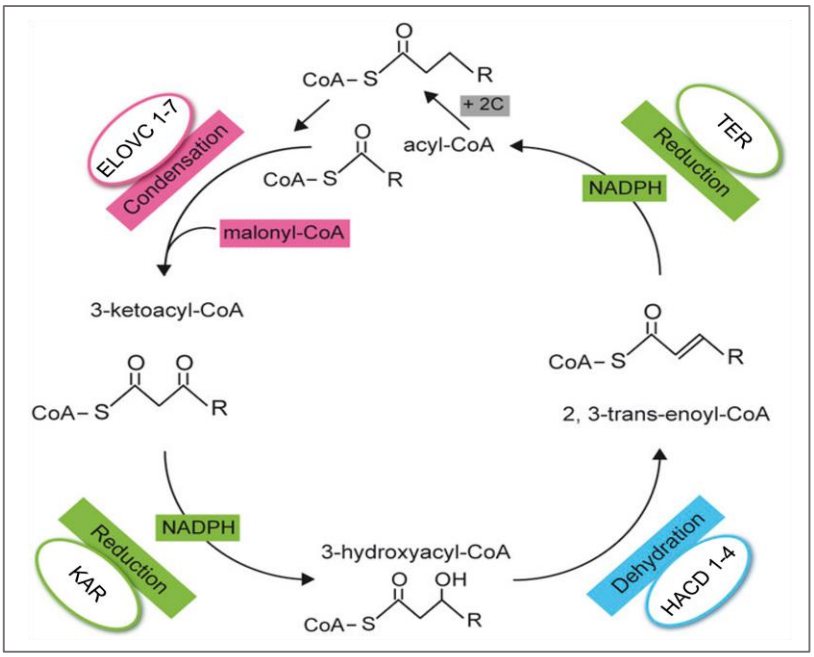

Figura 12 Elongation Cycle of Very Long-Chain Fatty Acids (ELOVL Pathway)

Fatty acid oxidation produces energy, particularly in the heart and skeletal muscle, and is the main source of energy during fasting. Both mitochondria and peroxisomes are capable of degrading saturated fatty acids through oxidation. Short-, medium-, and long-chain saturated fatty acids are degraded predominantly by mitochondria, whereas very-long-chain fatty acids are oxidized exclusively in peroxisomes [36]. Mitochondria lack very long-chain acyl-CoA synthetases, whereas the peroxisomal membrane possesses at least two acyl-CoA synthetases, a long-chain acyl-CoA synthetase and a very long-chain acyl-CoA synthetase, which can activate long-chain fatty acids and VLCFAs for oxidation. While long-chain fatty acids can be oxidized in both mitochondria and peroxisomes, the presence of very-long-chain acyl-CoA synthase on the peroxisomal membrane explains the exclusive oxidation of VLCFAs within peroxisomes. In peroxisomes, oxidation is not coupled to ATP synthesis because the peroxisome lacks any oxidative phosphorylation pathway. Two distinct but partly interchangeable oxidation systems have been identified in peroxisomes, shown in figure 13. One system is responsible for the oxidation of straight-chain fatty acids, while the second oxidation system is designed for the oxidation of branched-chain fatty acids [37].

1. The first system, which is inducible, consists of three enzymes: acyl-CoA oxidase 1 (ACOX1), the rate-limiting enzyme for peroxisomal straight-chain fatty acid oxidation, which produces 2-enoyl-CoA. The second enzyme, known as multifunctional protein 1 (MFP-1) or peroxisomal L-bifunctional enzyme (L-PBE), contains 2-enoyl-CoA hydratase, dienoyl-CoA isomerase, and L-3-hydroxyacyl-CoA dehydrogenase activities. This second step in peroxisomal oxidation produces ketoacyl-CoA, which serves as a substrate for 3-ketoacyl-CoA thiolase (PTL), the third enzyme in the peroxisomal oxidation system. The enzymatic conversion of ketoacyl-CoA results in an acyl-CoA that is two carbon atoms shorter than the original molecule. All three genes of this classic peroxisomal-inducible oxidation system are tightly regulated by PPAR.

2. The second peroxisomal oxidation system performs its function with the help of three enzymes: the first enzyme, 2-methylacyl-CoA-specific oxidases, processes 2-methyl branched-chain fatty acids; the second multifunctional protein (designated MFP-2) contains both hydratase and dehydrogenase activities; while the third enzyme, a 58-kDa sterol carrier protein (SCP-2), contains thiolase activity. MFP-1 hydrates 2-trans-enoyl-CoA to L-3-hydroxyacyl-CoA and dehydrates the L-isomers. MFP-2, on the other hand, converts 2-trans-enoyl-CoA to D-3-hydroxyacyl-CoA and dehydrates the D-isomers.
3. In addition to mitochondrial and peroxisomal oxidation processes, very long-chain fatty acids are also oxidized to dicarboxylic acids by microsomal β-oxidation carried out by cytochrome P450 CYP4A enzymes, regulated by PPARα. Both peroxisomal β-oxidation and microsomal β-oxidation lead to the generation of H2O2.

Unlike the mitochondrial system, peroxisomal β-oxidation is not completed. In the peroxisome, the 2-carbon shortened acyl-CoA re-enters the β-oxidation cycle, and this process repeats for approximately five cycles, resulting in the removal of ten carbon atoms. The chain-shortened acyl-CoAs generated by peroxisomal oxidation are transported into the mitochondria as carnitine esters and/or free fatty acids for completion of oxidation. Peroxisomes contain carnitine acetyltransferase and carnitine octanoylated transferase for the conjugation and transfer of short- and medium-chain acyl-CoAs, respectively [37].

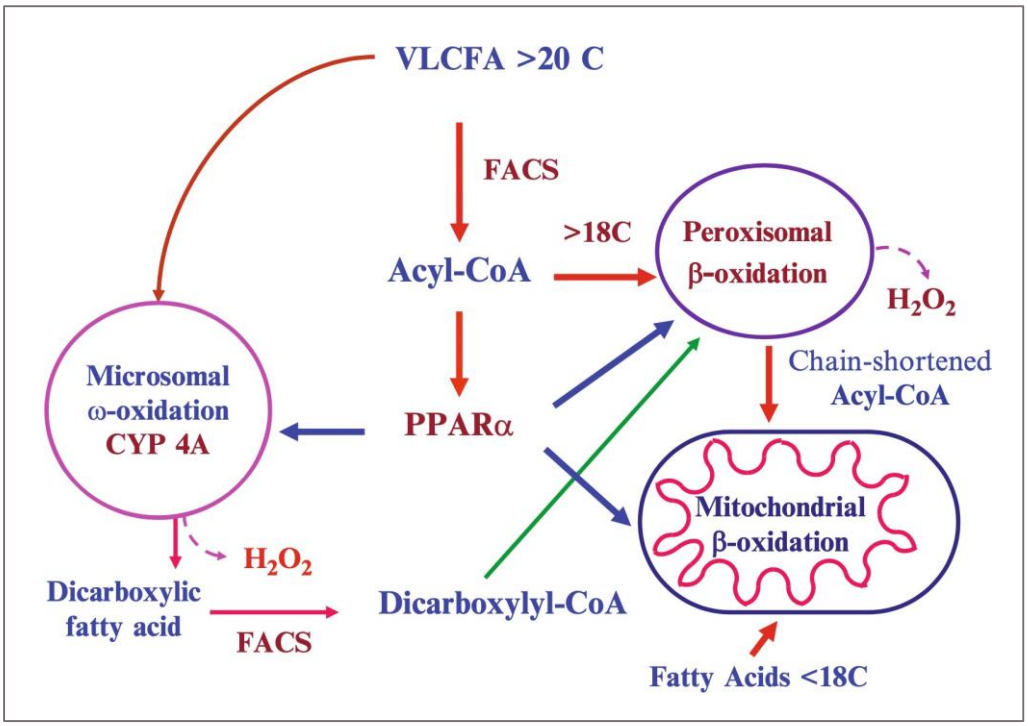

Figure 13 Peroxisomal β-oxidation is responsible for very long-chain fatty acids (>C18), while the mitochondrial β-oxidation system metabolizes medium- and short-chain fatty acids. The microsomal ω-oxidation system metabolizes very long-chain fatty acids, producing a dicarboxylic acid that is a ligand for PPARα and thus regulates PPARα activity.

BRAIN CHOLESTEROL METABOLISM

Cholesterol participates in fundamental processes such as synaptogenesis, neurotransmitter release, signal transduction, and other activities essential for the proper functioning of the central nervous system (CNS). The brain is the organ with the highest cholesterol content, containing ten times more than any other organ, accounting for approximately 25% of total cholesterol. Brain cholesterol is distributed as follows: myelin contains approximately 70% of brain cholesterol, where it acts as an electrical insulator and facilitates the conduction of nerve impulses along axons; 20% is contained in the membranes of neurons and glial cells, particularly microglia and astrocytes; and the remaining 10% is contained in neurons [38]. The blood-brain barrier (BBB) limits the transport of cholesterol from extracerebral tissues to the brain, so almost all cholesterol is synthesized de novo in this organ [38,39]. Most, if not all, of the cholesterol present in

the human brain is synthesized in situ by nerve cells [40]. Biosynthesis levels are highest during the early stages of postnatal development and tend to decline in adulthood. Because cholesterol is a key regulator of neuronal activity, metabolic dysfunction has serious consequences for brain function. A dynamic balance between de novo synthesis, transport, storage, and removal maintains cholesterol homeostasis. Depending on their needs, different brain cells can utilize two different biochemical pathways for cholesterol production: the Kandutsch–Russell pathway and the Bloch pathway. The choice of synthesis pathway therefore depends on the cell type, the availability of enzymes, and metabolic needs:

Table 4 Cell-Specific Cholesterol Biosynthesis Pathways in the Central Nervous System

CELL	FUNCTIONS	FINAL PRECURSOR	PATHWAY
Neurons	limited production of cholesterol for internal functions.	7-DEHYDROCHOLESTEROL	Kandutsch–Russell
Astrocytes	massive production of cholesterol to supply to neurons.	DESMOSTEROL	Bloch
Oligodendrocytes	high synthesis for myelin formation.	DESMOSTEROL	Bloch

The **Kandutsch-Russell pathway** involves converting lanosterol to 7-dehydrocholesterol, which is then reduced to cholesterol. Axon growth, the formation of a small number of synapses, and cell survival are all essential neuronal functions that require limited amounts of cholesterol produced through this pathway. Due to the low levels of key enzymes in neurons, synthesis can stop at the lanosterol stage. This limits the ability of neurons to produce large amounts of cholesterol [41].

The **Bloch pathway**, used by astrocytes and oligodendrocytes, requires lanosterol to be converted, through a series of enzymatic reactions, into desmosterol, the final precursor before the formation of cholesterol. In oligodendrocytes, cholesterol formation is essential for the production of myelin, the sheath that allows nerve signals to be conducted along axons. In astrocytes, cholesterol production is partly stored for internal use, while another portion is transported, via apolipoprotein E (ApoE), to neurons for proper synaptogenesis and neuronal plasticity [41].

CHOLESTEROL TRANSPORT IN THE NERVOUS SYSTEM

Neurons, as mentioned, reduce de novo cholesterol production at the end of the main developmental processes of the nervous system, namely myelination and synaptogenesis. This does not occur due to a functional deficiency, but rather due to a metabolic strategy; in fact, the synthesis process requires a lot of energy, approximately 100 ATP molecules per mole of cholesterol [42]. Neurons require a constant supply of cholesterol to maintain an adequate membrane concentration. This acquisition occurs exclusively through two pathways:

- De novo synthesis by the enzyme HMGCoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase). This pathway is active and appears to be the main one at the neuronal level only in the embryonic phase and, to a minimal extent, in mature neurons.
- Incorporation from the extracellular environment of cholesterol synthesized in astrocytes and transported in lipoproteins containing Apo E. This cholesterol is incorporated into the neuron by means of two main receptors: the LDL receptor (LDLR) and the LDLR Related Protein (LRP). Both of these receptors recognize Apo-E, which therefore constitutes a fundamental apolipoprotein at the brain level (in the absence of ApoB100, which is present only in plasma).

In the adult brain, the "outsourcing hypothesis" has emerged, according to which neurons are no longer self-sufficient and internalize the cholesterol produced by astrocytes [43]. Astrocytes synthesize cholesterol and release it in the form of HDL-like lipoproteins containing apolipoprotein E (ApoE). ApoE is a key protein in cholesterol transport in the brain. Its main function is to bind cholesterol to other lipids, forming soluble particles that can move through the extracellular space and reach neurons. The transported cholesterol can derive either from de novo synthesis in astrocytes or from the recycling of cholesterol from degenerating axons. Neurons have receptors on their surface, LDL-R and LRP1, capable of recognizing ApoE and internalizing it through endocytosis. Internalized cholesterol enters early endosomes. These mature into late endosomes through progressive acidification and the replacement of membrane proteins from RAB5 to RAB7, with structural and functional changes. Late endosomes finally fuse with lysosomes, where lysosomal enzymes hydrolyze cholesterol esters and, thanks to NPC1/NPC2, free cholesterol is transferred into the cytoplasm for distribution to membranes, synapses, and the endoplasmic reticulum. The NPC1/NPC2 system involves NPC2 binding cholesterol in the lysosome and transposing it to NPC1 on the lysosome membrane, thereby expelling the cholesterol into the cytoplasm, where it can follow various functional pathways. CNS cells are able to utilize cholesterol through three main pathways:

- ESTERIFIED: Cholesterol can be metabolized by Acyl-CoA cholesterol acyltransferase (ACAT), forming esterified cholesterol.

- OXIDIZED: Alternatively, it can be oxidized at positions 24, 25, or 27, forming oxysterols, which, being more hydrolyzed than cholesterol, can pass the blood-brain barrier [41,44]

- USE AS A PRECURSOR FOR OTHER STEROIDS: Cholesterol can be used in the synthesis of other steroids.

In the adult central nervous system, most cholesterol synthesis is regulated by the formation of a hydroxylated metabolite, 24-hydroxycholesterol (24-OHC). Indeed, it has been demonstrated that the most important regulatory pathway for cholesterol homeostasis in the CNS is its oxidation to 24-OHC, a process that occurs primarily in neurons. 24-OHC is also able to cross the blood-brain barrier and enter the systemic circulation, so virtually all 24-OHC present in plasma is of brain origin [39].

NEURODEGENERATIVE DISEASES AND CHOLESTEROL

Cholesterol contributes to the structure and function of membranes in the central nervous system. The blood-brain barrier effectively prevents the absorption of cholesterol from the circulation. Consequently, almost all cholesterol in the central nervous system is synthesized de novo [39]. The neuronal-specific enzyme cholesterol 24-hydroxylase (CYP46A1) converts excess cholesterol into 24OHC. 24OHC circulates primarily in the brain. The number of metabolically active neurons in the brain and, consequently, the volume of gray matter structures are reflected by plasma 24OHC levels. Plasma 24OHC is reduced in proportion to the degree of brain atrophy in neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and Huntington's disease [39]. Less than 1% of total 24OHC excretion occurs through the cerebrospinal fluid (CSF). This small fraction appears to reflect neuronal damage and the rate of neuronal loss rather than the total number of metabolically active neuronal cells. Increased 24OHC levels have been found in CSF samples from patients with neurodegenerative diseases [39]. Neurodegeneration, associated with neuronal loss, would lead to a reduction in 24OHC levels and its efflux from the brain into the circulation. Plasma 24OHC levels can therefore be considered a surrogate marker for the number of metabolically active neurons, located primarily in the cerebral gray matter. Cholesterol metabolism may play a key role in amyloidogenesis in the brain. Experimental studies in animals and cell cultures suggest that excess cholesterol in the brain increases amyloid production. The importance of cholesterol homeostasis in the CNS for neuronal function,

brain plasticity, synaptogenesis, and the response to brain damage is highlighted by the pathogenesis of several neurodegenerative diseases. (Table 5)

Table 5 Cholesterol-Related Genetic Disorders

NEURODEGENERATIVE DISEASE	GENES	COGNITIVE IMPAIRMENT/DEMENTIA
Alzheimer's disease	ApoE4, APP	YES
Niemann-Pick disease type C1	NCP1, NCP2	YES
Cerebrotendinous xanthomatosis	CYP27A1	YES
Tangier disease	ABCA1	NO

The presence of a spectrum of degenerative diseases, all associated with alterations in cholesterol homeostasis in the CNS or peripheral nervous system, leads to the clear need for a more detailed understanding of the various pathways involved in cholesterol metabolism. This requirement, in addition to being of significant scientific interest, is also essential for therapeutic purposes.

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a gradually debilitating neurodegenerative disease characterized by progressive memory loss, cognitive decline, and personality changes, accompanied by specific structural abnormalities in the brain. AD is the most common dementia worldwide and, along with other dementia-causing diseases, is considered the seventh leading cause of death worldwide. Approximately 55 million people are affected, and the number is estimated to rise to approximately 130 million by 2050, making it a global public health priority. There are many different risk factors, and the aging population nearly entirely determines the disease's epidemiology. Despite tremendous advancements in research over the last 20 years, the disease's causes remain a mystery. Amyloid beta (Aβ) extracellular deposits in the form of senile plaques and intracellular inclusions of hyperphosphorylated tau protein in the form of neurofibrillary tangles in the structures of the medial temporal lobe and cortical sections of the brain are the primary histological characteristics of AD.

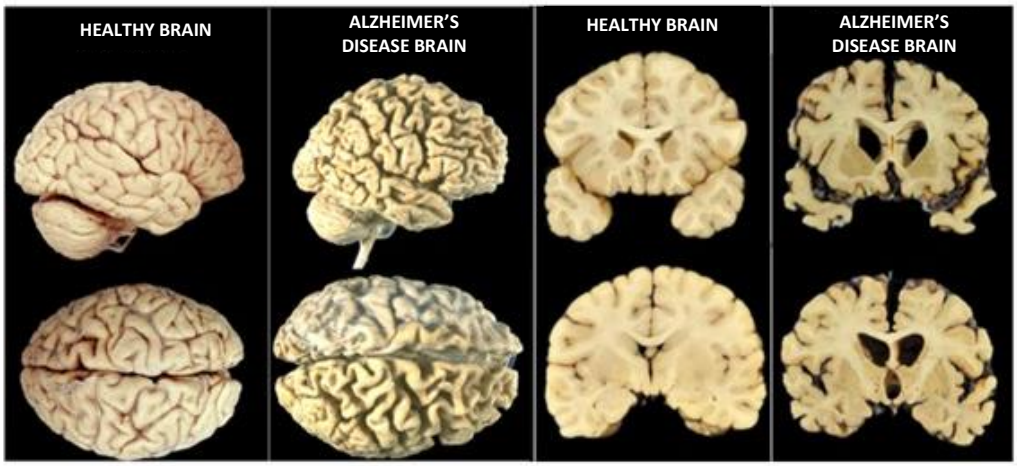

Figure 14 External (left) and internal (right) comparison between healthy and AD-affected brain tissue, observed from lateral, superior and frontal viewpoints

The involvement of particular genes in the onset of the disease has been emphasized by numerous genetic investigations. There are currently four genes that have been conclusively linked to the development of Alzheimer's disease:

a) the gene, located on chromosome 21, encoding the amyloid precursor protein (APP);

b) the two genes, located on chromosomes 14 and 1, respectively, encoding the proteins called 'presenilins' (PS1 and PS2);

c) the gene encoding apolipoprotein E (ApoE) on chromosome 19.

Extremely rare mutations in the first three genes are directly responsible for the early-onset familial form. The gene encoding ApoE, however, represents a susceptibility factor for the risk of developing the disease. ApoE, characterized by polymorphism, is present in the population in three different forms: E2, E3, and E4. Each individual can possess two of the three alternative forms (alleles) of the gene. The E4 allele is the most frequently present in individuals with Alzheimer's disease. In this sense, it can be argued that having the E4 allele in one's genetic makeup represents a greater risk of developing the disease. It would appear that the disease is based on a multifactorial component, including oxidative stress, altered cholesterol metabolism, and inflammation. Oxidative stress is associated with neuroinflammation and neurodegeneration. In fact, ROS activate glial cells, resulting in the release of inflammatory mediators, which in turn increase oxidative stress and amplify neurodegeneration. Inflammation is believed to play a key role in the neuropathological changes in AD. In recent years, it has become increasingly clear that altered cholesterol metabolism plays a crucial role in the development of Alzheimer's disease. Elevated total cholesterol has been linked to an increased risk of Alzheimer's disease, according to longitudinal research. Intracellular cholesterol levels and beta-amyloid formation have been linked in a number of studies. In particular, a decrease in intracellular cholesterol has been demonstrated to result in a decrease in beta-amyloid

synthesis. The role of oxysterols in the pathogenesis of AD is not fully understood. It has been observed that CYP46, the enzyme that catalyzes the oxidation of cholesterol to 24-OHC in the brain, is expressed differently between healthy and diseased subjects. Specifically, in healthy elderly subjects, it is expressed in neurons and astrocytes, while in patients with the disease, it is expressed predominantly in degenerated neurons. 24-OHC is a very interesting factor, both for the pathogenesis of AD and for its use as an early marker of disease onset.

NIEMANN-PICK DISEASE TYPE C1

Niemann-Pick disease (NPD) is a group of autosomal recessive lysosomal disorders characterized by lipid accumulation in the cells of the reticuloendothelial system in the spleen, liver, lungs, bone marrow, and central nervous system (CNS). Based on genetics and symptomatology, four subtypes with different clinical and pathogenetic characteristics: types A, B, C, and D, in addition to type E, which has an adult-onset. Niemann-Pick disease type C (NCP) is caused by a defect in one of the two proteins responsible for intralysosomal cholesterol transport: NPC1 (95% of cases), locus 18q11-q12, and NPC2 (5% of cases), locus 14q24.3 [45]. As a result, non-esterified cholesterol and other lipids build up in lysosomes, causing cellular damage in target organs such the liver and spleen, but primarily progressive neurodegeneration at the level of the central nervous system. A pathogenic mutation on both alleles of the NPC1 or NPC2 gene is necessary for the afflicted person to exhibit the condition since it is inherited as an autosomal recessive characteristic. It can be classified as newborn hepatitis, severe infantile hepatitis, late-onset infantile hepatitis, or juvenile hepatitis, depending on when it first appears. Clinical manifestations include severe liver disease, respiratory distress, neuropsychomotor retardation, seizures, dystonia, motor incoordination, feeding problems, and the inability to perform vertical eye movements [45]. The classic diagnostic process for the disease involves: 1) clinical suspicion; 2) skin biopsy to perform the Filipin test on cultured fibroblasts, which, in the case of disease, highlights the cellular

accumulation of non-esterified cholesterol; 3) confirmation of the disease through molecular analysis of the NPC1 and NPC2 genes. Given the variety of disease presentations, a score index has been developed to facilitate diagnostic suspicion. Based on the overall score achieved, the probability of having a patient with NPC is defined as low, moderate, or high. A defect in one of these proteins results in a deficit in the processing and utilization of exogenous cholesterol (LDL). This causes the liver and spleen to lysosomally accumulate excess unesterified cholesterol, sphingomyelin, phospholipids, and glycolipids, and the central nervous system (the disease's main target organ) to accumulate glycolipids. These alterations trigger a cascade of pathogenic events, including defective oxysterol synthesis, delayed beginning of cholesterol homeostasis reactions, aberrant sphingosine metabolism, neuroinflammation, and apoptosis activation. Cells with mutated NPC1 also lack expression of LXR-regulated genes, and the production of 24-OHC and 27-OH is decreased. Exogenous administration of these oxysterols can eliminate excess cholesterol in these cells, thus correcting the phenotype of mutated NPC1. Regarding therapy, there are currently no specific treatments capable of modifying the progression of the disease. Numerous studies are underway to test the effectiveness of a drug, miglustat, which inhibits sphingolipid synthesis. A family with apparent autosomal dominant Alzheimer's disease has been described for the first time. They did not have any known AD-associated mutations, but they did have a heterozygous mutation in the NPC1 gene. The subjects described in the study presented a clinical, biochemical, and neuroimaging picture compatible with a late-onset form of Alzheimer's disease, without typical signs of Niemann-Pick disease. The study also showed an increase in serum oxysterols, suggesting an alteration in lipid metabolism [45,46].

HYPERTENSION

Aging is a gradual and irreversible process caused by environmental, genetic, and epigenetic factors, as well as lifestyle factors, which together lead to progressive alterations in an organism's homeostatic mechanisms. The vascular endothelium plays a crucial role in maintaining the body's health, adapting its functions and structures to various insults. Specifically, the endothelium plays a key role in blood homeostasis, adequate organ perfusion, activation of the immune response, and regulation of peripheral resistance [47,48]. The endothelium's compensatory mechanisms are progressively compromised during aging, causing distinctive changes in vascular cell morphology and vessel structure. Aging blood vessels are characterized by increased arterial stiffness due to increased intima-media thickness, reduced elasticity resulting from collagen accumulation and decreased elastin content, as well as vascular calcification [49]. A healthy endothelium maintains antithrombotic, vasodilatory, anti-inflammatory, and antioxidant properties, tightly regulated to meet the body's needs. According to the most recent data (2017-2018) from the NHANES (National Health and Nutrition Examination Survey) [50], the prevalence of hypertension in the general population of the United States is 45.4%, with wide fluctuations across age groups. In fact, while it is 22% in the young population (18-39 years), it reaches 74.5% in the population over the age of 60. Genetic, behavioral, and environmental factors can influence the pathogenesis of hypertension. In particular, the aging population, physical inactivity, and obesity are the factors that have favored the increase in the problem in the Western population and, more recently, also in developing countries. Arterial hypertension, defined as resting blood pressure values equal to or greater than 140/90 mmHg [51], is a widely represented condition in the general population, with an age-related prevalence. The concept of "hypertension" defines blood pressure values for which treatment certainly provides benefits in terms of preventing cardiovascular disease. The threshold values used to define a hypertensive subject are effectively arbitrary, as blood pressure values are a normally distributed variable in the general population; hypertension can therefore be defined as the blood pressure level at which the benefits of treatment, both

in terms of lifestyle modification and pharmacological treatment, unequivocally outweigh the risks [52]. However, there is no unanimous consensus in defining the threshold blood pressure values with which to consider a subject as hypertensive or normotensive. In fact, if we take into consideration the guidelines of the European Society of Cardiology and European Society of Hypertension (ESC/ESH), a patient is defined as hypertensive if he/she has systolic blood pressure values ≥ 140 mmHg or diastolic blood pressure values ≥ 90 mmHg [51], while the American College of Cardiology/American Heart Association (ACC/AHA) defines a subject as hypertensive if he/she has systolic blood pressure values ≥ 130 mmHg or diastolic blood pressure values ≥ 80 mmHg [51,53]. (Table 6-7).

Table 6 Classification of blood pressure according to ESC/ESH guidelines. Systolic and diastolic blood pressure values are expressed in mmHg [53].

GRADE	**SYSTOLIC BLOOD PRESSURE (mmHg)**		**DIASTOLIC BLOOD PRESSURE (mmHg)**
Optimal	<120	and	<80
Normal	120–129	and/or	80–84
High normal	130–139	and/or	85–89
Grade 1 hypertension	140–159	and/or	90–99
Grade 2 hypertension	160–179	and/or	100–109
Grade 3 hypertension	≥180	and/or	≥110
Isolated systolic hypertension	≥140	and	<90

Table 7 Classification of blood pressure according to ACC/AHA guidelines. Systolic and diastolic blood pressure values are expressed in mmHg [54].

GRADE	**SYSTOLIC BLOOD PRESSURE (mmHg)**		**DIASTOLIC BLOOD PRESSURE (mmHg)**
Normal	<120	and	<80
Elevated	120–129	and	<80
Stage 1 hypertension	130–139	or	80–89
Stage 2 hypertension	140–159	and/or	90–99

Blood pressure levels depend on two fundamental hemodynamic variables: cardiac output and peripheral resistance [53,54], according to the formula:

Arterial Pressure = Cardiac Output × Peripheral Resistance

Therefore, factors capable of influencing cardiac output and/or peripheral resistance can produce either increases or decreases in arterial blood pressure values. The physiological mechanisms that ensure the maintenance of adequate blood pressure levels and pressure variability necessary for the body are numerous and closely interconnected. These include the arterial baroreceptor system, the sympathetic nervous system (SNS), the renin–angiotensin–aldosterone system (RAAS), endothelial vasodilatory and vasoconstrictive factors, and the role of the kidney in providing appropriate natriuresis in response to different blood pressure levels. Dysfunction of one or more of these processes may promote the development of hypertension through an increase in cardiac output, an increase in peripheral resistance, or both. In approximately 95% of hypertension cases, it is not possible to identify a single causative factor; therefore, the terms essential or primary hypertension have been adopted. In about 5% of cases, an identifiable cause can be demonstrated (secondary hypertension), such as endocrine disorders (primary hyperaldosteronism, mineralocorticoid excess, pheochromocytoma, Cushing's disease), renal artery stenosis, chronic kidney disease, or obstructive sleep apnea. The traditional "candidate gene" approach can explain only the rare Mendelian forms of hypertension, such as Liddle syndrome, glucocorticoid-remediable hyperaldosteronism, or Gitelman syndrome, in which genetic mutations alter renal water and electrolyte transport, promoting the development of hypertension due to increased plasma volume. Activation of these receptors induces inflammation, production of oxidative substances, and cellular proliferation, all of which contribute to vascular remodeling. Moreover, through the expression of adhesion molecules in vascular smooth muscle cells, the inflammatory and pro-fibrotic response involved in the pathogenesis of atherosclerosis is enhanced.

Persistent pressure overload over time leads to structural damage to so-called “target organs,” manifesting as left ventricular hypertrophy, retinopathy, renal damage, vascular dementia, and hypertensive arteriopathy [54]. The identification of subclinical organ damage in the individual hypertensive patient helps guide and support the need for pharmacological treatment of hypertension. In this context, all patients should undergo routine blood testing to assess electrolytes, blood glucose, renal function, and lipid profile. Hypertension and hypercholesterolemia are frequently associated in population and interventional studies. Both factors are involved in the development and progression of atherosclerosis and promote the occurrence of major cardiovascular events. Pathophysiological studies have shown that hypertension and hypercholesterolemia induce alterations in the vascular wall, leading to endothelial dysfunction, reduced nitric oxide availability, increased oxidative stress, and activation of inflammatory pathways [55]. Despite advances in diagnosis and treatment, arterial hypertension remains a major public health issue. It is one of the leading global risk factors for premature death [53,54]. Furthermore, the majority of deaths related to arterial hypertension are due to ischemic heart disease, hemorrhagic stroke, and ischemic stroke [52]. Studies have also documented that increased blood pressure is associated with a higher risk of developing cognitive impairment and dementia. The strong focus on the study and treatment of hypertension derives from epidemiological and interventional studies demonstrating that this condition represents one of the main cardiovascular risk factors. The WHO Technical Report defines arterial hypertension as “the most common cardiovascular disorder,” affecting approximately 20% of the adult population in many countries [54,55,56], with prevalence and incidence increasing with age.

ATHEROSCLEROSIS

Atherosclerosis is an inflammatory disease of arteries exposed to high pressure that leads to the formation of fibro-fatty plaques, with possible stenosis or occlusion of the vascular lumen. The “risk factors” associated with a more rapid and/or pronounced development of atherosclerosis and its complications can be divided into “modifiable” and “non-modifiable” factors. Both categories contribute to increasing the likelihood of complications; however, only the “modifiable” ones can be targeted through preventive medical interventions. The main risk factors are:

Table 8 Non-modificable and modificable risk factors

NON-MODIFIABLE RISK FACTORS	***MODIFIABLE RISK FACTORS***
Age	Hypercholesterolemia
Sex	Smoking
Family history of premature ischemic heart disease	Arterial hypertension
Ethnicity	Lifestyle habits (obesity, atherogenic diet, physical inactivity)

Atherosclerotic lesions can occur as a result of all the variables mentioned, but two key circumstances are necessary for their development: high blood pressure and noticeably higher blood cholesterol levels. A growing body of evidence suggests that oxysterols, oxidation products of cholesterol contained in oxidized low-density lipoproteins (oxLDL), significantly contribute to the vascular remodeling observed in atherosclerosis, as they are involved in several key steps of this age-related disease. Oxysterols are also implicated in endothelial dysfunction: they promote platelet aggregation and impair arterial relaxation by reducing nitric oxide (NO) bioavailability. Furthermore, they promote vascular smooth muscle cell (VSMC) migration and proliferation, which thickens the intima, deposits collagen, and causes calcification, all of which enhance arterial stiffness.

Lastly, because oxysterols can cause oxidative stress, inflammation, and apoptosis, they may be important in vascular aging (Figure 15).

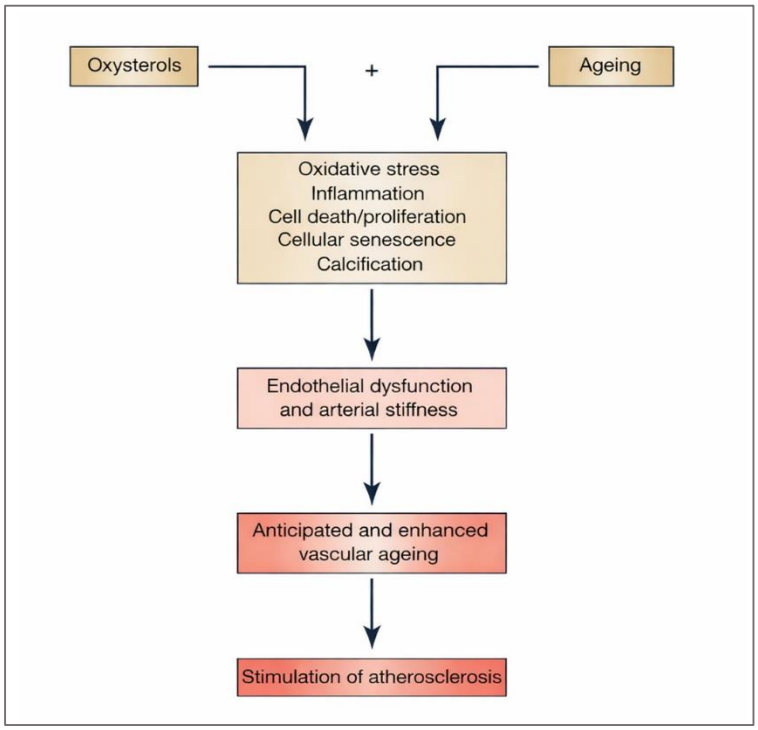

Figure 15 Effects of oxysterols on VSMCs and vascular aging. Oxysterols promote VSMC migration and proliferation, intimal thickening, calcification, and oxidative stress, contributing to vascular aging. Reproduced from Gargiulo et al., 2016.

Atherosclerosis is a multifactorial degenerative disease affecting large- and medium-sized arteries and is characterized by chronic inflammation of the vascular wall. Monocytes are continuously recruited and migrate from the circulation into the subendothelial region as a result of this inflammatory condition. Growth factors, chemokines, cytokines, and endothelial adhesion molecules are all upregulated during the process. After entering the subintimal space, monocytes undergo macrophage differentiation and internalize oxidized LDL (oxLDL) through scavenger receptors such SR-A and CD36. Unlike LDL receptors, scavenger receptors are not regulated by a negative feedback mechanism; consequently, macrophages avidly accumulate oxidized lipids, transforming into foam

cells while releasing a wide array of pro-inflammatory cytokines. The development of a chronic inflammatory state and the advancement of the atherosclerotic lesion are encouraged by the persistence of this process. It is now widely known that oxidized LDL (oxLDL) causes atherosclerosis. These particles cause inflammation, apoptosis, vascular smooth muscle cell (VSMC) migration and proliferation, and endothelial activation, all of which contribute to the development, progression, and instability of fibrotic plaque [57]. It is therefore plausible that oxLDL also significantly contribute to vascular aging. Although the involvement of oxidized lipoproteins in atherogenesis and aging is unquestionable, the distinct roles of the various components present in oxLDL have not yet been fully clarified. Many reactive compounds, including as peroxides, hydroxides, aldehydes, oxidized phospholipids, and cholesterol oxidation products, are produced when the lipid fraction of LDL is oxidized [58,59]. Research groups have focused a lot of emphasis on the latter class of chemicals, which are quantitatively relevant in oxLDL. Oxysterols in particular are of great interest since they are thought to play a key role in the pathophysiology of atherosclerosis. There is now little doubt that pathophysiologically relevant oxysterols play a central role in the different stages of atheroma formation, from endothelial dysfunction to plaque fibrosis and rupture, through macrophage infiltration and VSMC differentiation. In this context, an excess of oxysterols has been detected in human atherosclerotic plaques. Atherosclerotic plaques have been reported to contain oxysterols that are frequently seen in the plasma of hypercholesterolemic patients, and there is a substantial link between the total oxysterol content and the total cholesterol levels in these plaques. The idea that cholesterol oxidation is a significant, if not essential, step in the arterial remodeling linked to atherosclerosis is further supported by this finding. Vascular reactive oxygen species (ROS) are mainly derived from the activity of NOX enzymes, multimeric complexes present in endothelial cells (ECs) and smooth muscle cells (SMCs), which catalyze the reduction of oxygen to superoxide anion ($O_2 \cdot^-$). Several NOX isoenzymes are constitutively expressed in the vascular system, particularly NOX1, NOX4, and NOX5, whereas NOX2—typically expressed in cells of the macrophage lineage—may become involved when ECs acquire an inflammatory phenotype. All these

enzymes release $O_2\cdot^-$, which functions as a signaling molecule in numerous pathophysiological processes, including cell growth, migration, fibrosis, apoptosis, and inflammation. Oxysterols may further disrupt these already damaged organelles, hence encouraging the beginning or maintenance of oxidative imbalance in the endothelium redox state. Reduced mitochondrial activity in aged endothelial cells is a reasonable expectation.

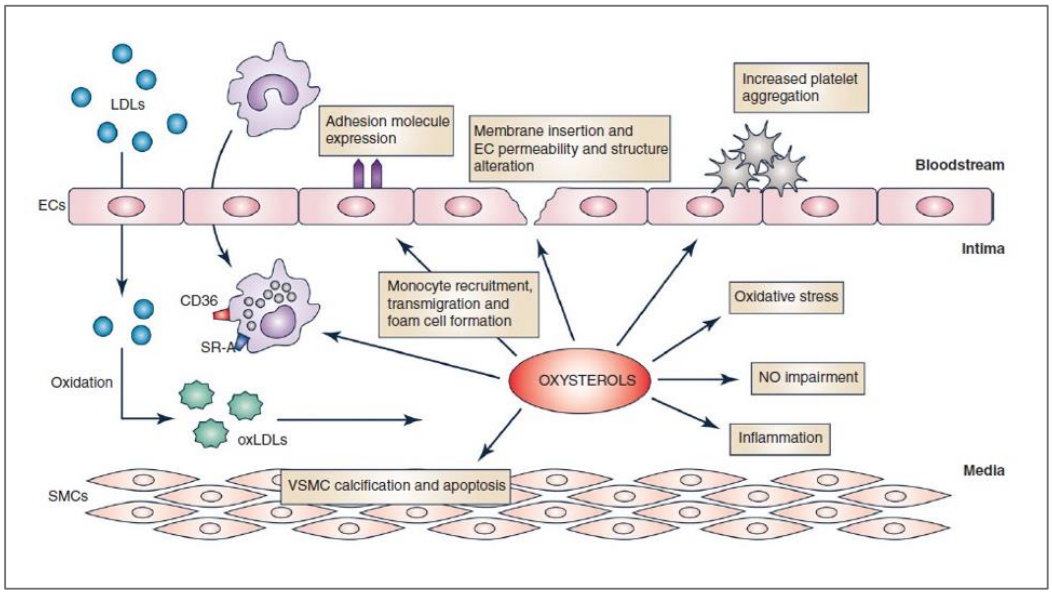

Figure 16 Involvement of oxysterols in endothelial dysfunction

Atherosclerosis, which can be regarded as a form of accelerated aging affecting specific segments of the arterial system, is characterized by chronic inflammation. This process starts off as a defense mechanism but turns destructive when it is repeatedly and uncontrollably triggered inside the artery wall. Atherosclerosis's pathogenesis is maintained throughout all stages, including the rupture of unstable plaques, by inflammation, which is essential in the disease's early stages. Oxidized low-density lipoprotein (oxLDL) is the primary trigger for endothelial cells' (ECs) production of adhesion molecules and chemokines, which in turn attracts circulating monocytes in the atherosclerotic process. Inflammation-dependent increased permeability of the

endothelial layer allows inflammatory cells to migrate into the subendothelial space, where they differentiate into macrophages and begin secreting chemokines and cytokines, thereby attracting additional inflammatory cells and activating vascular cells, namely ECs and vascular smooth muscle cells (VSMCs). Additionally, inflammation facilitates VSMCs' transition from a contractile to a fibroblast-like phenotype, which permits their migration and proliferation from the media to the intima. The arterial wall's structure is significantly and permanently changed as a result. All of these combine to cause arterial stiffness and endothelial dysfunction. Inflammation also contributes to vascular remodeling associated with aging by progressively disrupting extracellular matrix (ECM) homeostasis, both by stimulating fibrogenesis (the inflammatory cytokine TGF-β1 is strongly profibrogenic) and by inducing marked upregulation of matrix metalloproteinases (MMPs). In this context, oxysterols have been shown to induce TGF-β1 expression and synthesis in human promonocytic U937 cells [60] and in murine Raw264.7 macrophages [61]. In all investigations, a physiologically acceptable blend of oxysterols was employed, with 7-ketocholesterol (7-K) predominating. Non-oxidized cholesterol, on the other hand, had no effect on TGF-β1 production or expression in U937 cells [60]. The loss of vascular wall homeostasis associated with aging would undoubtedly be exacerbated by irregular and unchecked ECM breakdown. This process is primarily caused by MMPs and their particular inhibitors, TIMPs, whose expression is influenced by inflammation. In this regard, oxysterols are among the active molecules contributing to arterial ECM disruption: MMP-2 and MMP-9 have been shown to increase in VSMCs in response to 7-K and α-epoxide (α-EPOX) via activation of extracellular signal-regulated kinase (ERK) [62], a signaling kinase involved in nuclear factor κB (NF-κB) activation and subsequent overexpression of inflammatory genes. Notably, 7-K has been shown to drive human peripheral blood monocyte-derived macrophages toward a proinflammatory phenotype by inducing numerous inflammatory mediators, including MMP-9 [63]. Furthermore, one of the most abundant oxysterols in human blood, 27-hydroxycholesterol (27-OH), significantly increases MMP-9 expression and synthesis in human promonocytic U937 cells through activation of Toll-like receptor 4 (TLR4) and/or

induction of CD14, a TLR4 coreceptor [51]. Regarding TIMPs, 27-OH has been reported not to modulate their levels in promonocytic cells [51]. The arteries of ApoE−/−mice given oxysterols exhibited rapid plaque disintegration and rupture, which was of special interest. This was linked to increased monocyte infiltration and increased MCP-1 and MMP activity. Ezetimibe treatment inhibited intestinal oxysterol absorption, preventing all these effects in the same animal model, indicating a role for oxysterols in plaque vulnerability in high-risk patients [64]. Moreover, MCP-1 and β1-integrin were found to be upregulated in U937 cells in response to an oxysterol mixture reproducing that detectable in the plasma of hypercholesterolemic subjects [51,65]. The same oxysterol mixture was also able to induce foam cell formation through upregulation of the scavenger receptor CD36 [60,65]. Unlike the oxysterol mixture, non-oxidized cholesterol did not modulate MCP-1, β1-integrin, or CD36 expression and synthesis [51,65,66]. Many oxysterols seem to be able to cause and maintain vascular inflammation by overexpressing different adhesion molecules, cytokines, and chemokines. In human vascular endothelial cells, 7β-hydroxycholesterol (7β-OH) and 7-K both cause the production of E-selectin, ICAM-1, and VCAM-1 in addition to interleukin-1β (IL-1β). Additionally, 27-OH has been shown to increase IL-1β, IL-8, TNF-α, and MMP-9 expression in U937 cells [51]. Additionally, human macrophage cell lines exhibit substantial induction of MCP-1, TNF-α, IL-8, and macrophage inflammatory protein-1β (MIP-1β) by 7β-OH, 7-K, and 25-hydroxycholesterol (25-OH). The ERK/activator protein-1 (AP-1) pathway in THP-1 cells causes 7β-OH and 25-OH to upregulate IL-8 while the TLR4/NF-κB route in U937 cells causes 27-OH to do the same [51,65,66]. In terms of oxysterol-induced adhesion molecule expression, it has been demonstrated that 25-OH improves monocyte adherence to ECs and increases VCAM-1 expression in human aortic endothelial cells [67].

STUDY AIMS

The aim of the research project is to evaluate the use of oxysterols as potential biomarkers for the diagnosis and/or monitoring of the progression of degenerative diseases and aging.

FIRST STUDY

Study of oxysterols of pathophysiological relevance in the cerebrospinal fluid (CSF) of patients with Alzheimer's disease (AD), non-Alzheimer's disease (NAD), and non-degenerative disorders (controls). The aim is to observe how the metabolomic/lipidomic profile changes.

SECOND STUDY

Study of the effects of treatment with miglustat on the clinical, neuropsychological, and biochemical status of patients with Alzheimer's disease (NCP mutation c.3034G>T; p.Gly1012Cys)

THIRD STUDY

Study of the metabolomic/lipidomic profile in normotensive subjects and hypertensive patients, focusing specifically on fatty acids, sterols, phytosterols, and oxysterols. The study assessed whether individual biomarkers are independently associated with the presence of hypertension. The study also investigated the relationship between biomarkers and cardiovascular organ damage quantified by measuring Pulse Wave Velocity (PWV), carotid intima-media thickness (IMT), and Left Ventricular Mass Index (LVMI).

MATERIALS AND METHODS

The study included 189 subjects, divided into three main groups:

- First Study: 41 subjects diagnosed with Alzheimer's disease (11 subjects) and non-Alzheimer's degenerative dementia (22 subjects), and controls with non-degenerative disorders (8 subjects).
- Second Study: 48 subjects, 3 with late-onset autosomal dominant Alzheimer's disease (AD) caused by a heterozygous NPC1 c.3034G>T (p.Gly1012Cys) mutation in the NPC1 gene (NM_000271.5, rs1555632941), and 45 controls.
- Third Study: 204 subjects, 150 normotensive and 54 hypertensive.

FIRST STUDY

STUDY POPULATION

A total of 41 subjects were enrolled and divided into three subgroups: 11 patients with AD, 22 patients with NAD, and 8 patients with non-neurodegenerative disorders serving as internal controls (C). Each patient underwent a multidisciplinary evaluation including neurological and neuropsychological assessment, brain magnetic resonance imaging (MRI), routine laboratory testing, and lumbar puncture for the determination of cerebrospinal fluid (CSF) biomarkers. All enrolled patients also underwent a comprehensive sleep evaluation including clinical assessment and polysomnography (PSG). The diagnosis of dementia was established according to standard diagnostic criteria, namely the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5), and the NIA-AA criteri [68,69].

LABORATORY ANALYSES

According to international biomarker guidelines, samples of cerebrospinal fluid (CSF) were centrifuged at room temperature for 10 minutes at 2,000 g, aliquoted, and kept at -80 °C until analysis [70]. Commercially available sandwich ELISA kits (Innotest, Innogenetics/Fujirebio, Belgium) were used to quantify the amounts of total tau (t-Tau), phosphorylated tau (p-Tau181), and beta-amyloid 1–42 (Aβ42) in CSF independently and in triplicate. Sterols and oxysterols were quantified by isotope dilution gas chromatography–mass spectrometry (GC–MS). Briefly, 500 µL of CSF were added to a screw-capped vial sealed with a Teflon septum, together with structurally homologous internal standards (see table 9), 50 µL of K3-EDTA (10 g/L, Sigma-Merck) to prevent auto-oxidation, ethanol, and 1 M KOH. Each vial was then flushed with argon for 10 minutes to remove air.

Table 9 Structurally homologous internal standards

Compound (Supplier)	Amount
epicoprostanol (Sigma-Aldrich, Canada)	10 µg
lathosterol-d7 (d7-latho)	250 ng
lanosterol-d6 (d6-lano)	20 ng
7α-hydroxycholesterol-d7 (d7-7αOHC)	20 ng
7β-hydroxycholesterol-d7 (d7-7βOHC)	20 ng
7-ketocholesterol-d7 (d7-7KC)	20 ng
α-epoxycholesterol-d7 (d7-α-epoxyC)	20 ng
5β,6β-epoxycholesterol-d7 (d7-β-epoxyC)	20 ng
cholestane-3β,5α,6β-triol-d7 (d7-triol)	20 ng
24(R/S)-hydroxycholesterol-d7 (d7-24OHC)	20 ng
27-hydroxycholesterol-d6 (d6-27OHC) (Avanti Polar Lipids Inc., USA)	20 ng
butylated hydroxytoluene (5 g/L, Sigma-Aldrich, Canada)	50 µL

Standardized Protocol for Sample Preparation and Oxysterol Analysis

The standardized protocol for sample preparation and oxysterol analysis consists of several steps:

- **Ethanolic saponification with KOH** to hydrolyze sterol, oxysterol, and fatty acid esters.
- **Addition of the antioxidant K3-EDTA** (tripotassium ethylenediaminetetraacetic acid) to prevent metal ions present in air from binding to molecules in the sample during laboratory handling, thereby limiting artificial oxidation.
- **Use of deuterated internal standards** for sterols and oxysterols. These are structural analogs of the analyzed metabolites in which some hydrogen atoms are replaced by deuterium. Deuterium is a stable isotope with mass number 2, sharing the same atomic number as hydrogen but containing one proton and one neutron in its nucleus. Its incorporation allows accurate isotope dilution quantification and improves analytical precision.
- **Epicoprostanol (3α-hydroxy-5β-cholestanol)**, a structural analog of oxysterols containing a trimethylsilyl (TMS) group instead of hydroxyl hydrogens, is used as an internal standard for cholesterol quantification.
- **Derivatization of sterols and oxysterols with TMS groups**, in which hydroxyl hydrogens are replaced by trimethylsilyl (TMS) moieties to protect against oxidation by external agents such as oxygen, sulfur, and iron.
- **Selection of both deuterated and non-deuterated internal standards** as structural analogs of the molecules of interest quantified by mass spectrometry.
- **Extraction of the lipid fraction** using the organic solvent n-hexane.

- **Evaporation under nitrogen (N_2) flow** to remove the organic solvent (hexane), leaving the dried lipid residue.
- **Resuspension in butylated hydroxytoluene (BHT)**, a stabilizer used to prevent peroxide (O_2) formation, dissolved in diethyl ether (Et_2O).
- **Evaporation to dryness** of the fraction of interest.
- **Trimethylsilylation using BSTFA**, a chemical derivatizing agent that renders sterols sufficiently volatile for gas chromatography analysis. Because sterols have high boiling points, they are otherwise poorly amenable to GC analysis.
- **Second-order nucleophilic substitution (SN2)** replaces the alcoholic hydrogen of sterically hindered hydroxyl groups in oxysterols with a bistrimethylsilyl (TMS) group, increasing volatility. This widely validated method for side-chain oxysterol preparation enables selective isolation of lipids of interest while removing more apolar lipids present in high concentrations due to greater nucleophilic reactivity.
- **Final evaporation and resuspension.**
- **Analysis by GC–MS.**

Quantification Method: GC–MS

Gas chromatography–mass spectrometry (GC–MS) is an analytical technique that combines a gas chromatograph, which separates the compounds present in a sample, with a mass spectrometer, which acts as a detector by converting vaporized analytes into electrical signals. GC–MS is ideal for the identification and quantification of numerous metabolic molecules, including acids, alcohols, amino acids, sugars, sterols, catecholamines, toxins, and drugs. The instrument integrates both the retention time, defined as the time required for an analyte to travel through the chromatographic

column, and the mass spectrum, enabling precise, reliable, and accurate analysis selective for molecules with specific mass-to-charge (m/z) ratios of interest.

The derivatizing agent BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) is added to enhance detectability and volatility in the gas chromatograph by converting analytes into volatile, thermodynamically stable, non-ionic derivatives. Quantification is performed using isotopically labeled, stable deuterated internal standards (OS-deuterated) to ensure analytical accuracy and reproducibility. Peak integration was performed manually, and oxysterols were quantified by selected ion monitoring (SIM) analysis through comparison with internal standards, using calibration curves constructed for the listed sterols.

Chromatogram acquisition was carried out in total ion current (TIC) mode (Total Ion Current versus time), which displays signal intensity as the sum of mass-to-charge (m/z) ratios of ion concentrations and internal standards. In this representation, time is plotted on the x-axis and signal intensity (m/z) on the y-axis. The area of chromatographic peaks was determined through full scanning of all fragment ions, while focusing on specific ions of interest for trace analysis. Mass spectrometry data were acquired in selected ion monitoring (SIM) mode at specific m/z values corresponding to the internal standards, in order to increase sensitivity, enhance mass scan speed, and optimize dwell time (the time spent analyzing each selected mass).

STATISTICAL ANALYSIS

Statistical analyses were performed using Jamovi software. Data normality was assessed with the Shapiro–Wilk test. Differences between groups were analyzed using one-way ANOVA with Tukey's post hoc test. Correlations between variables were evaluated using Pearson's correlation test. The level of statistical significance was set at $p = 0.05$.

SECOND STUDY

STUDY POPULATION

A total of 48 subjects were included: 45 controls (mean age 75.5 ± 3.1 years; 22 males, 23 females) and 3 patients (1 male, 2 females) with late-onset Alzheimer's disease (AD), all carrying the heterozygous NPC1 mutation c.3034G>T (p.Gly1012Cys) (NM_000271.5, rs1555632941). All subjects met the diagnostic criteria for AD according to revised guidelines for staging and diagnosis, as well as the ATN classification system, based on CSF sampling and amyloid-PET imaging. However, according to the NPC suspicion index, they did not meet the criteria for Niemann–Pick type C (NPC) disease. Besides the heterozygous p.Gly1012Cys mutation in NPC1, whole-exome sequencing revealed no other pathogenic or likely pathogenic variants associated with the patients' phenotypes. Miglustat (1,5-(butylimino)-1,5-dideoxy-D-glucitol) is a synthetic derivative of polyhydroxylated alkaloids or iminosugars that inhibits glucosylceramide synthase, a key enzyme in glycosphingolipid biosynthesis. It is FDA-approved for Gaucher disease and authorized in the EU for NPC patients. The recommended adult dose for NPC is 200 mg three times daily. Therapeutic use of miglustat in our patients was approved by the local ethics committee (Protocol No. 23, 314/2023), and informed consent was obtained from patients or their legal representatives. Neuropsychological assessments were conducted at baseline (T0), 6 months (T6), and 12 months (T12). Brain imaging with amyloid-PET was performed at T0 and T12, while serum oxysterol concentrations were measured at T0, T3, T9, and T12. CSF neurodegeneration biomarkers and oxysterol levels were analyzed at T12. Additionally, cognitive tests, PET imaging, and biochemical analyses were compared to data obtained 12 months prior to treatment (T-12) to assess longitudinal trends.

LABORATORY ANALYSIS

The following oxysterols were quantified: lathosterol, desmosterol, 7β-hydroxycholesterol (7βOHC), 7-ketocholesterol (7-KC), 5β,6β-epoxycholesterol, 5α,6α-epoxycholesterol, and cholestane-3β,5α,6β-triol (C-triol). CSF biomarkers of AD pathology (Aβ40, Aβ42, total tau [tTau], and phosphorylated tau [pTau181]) were measured using Lumipulse assays. All patients underwent PET-CT scans with [^{18}F]flutemetamol, an amyloid-β radioligand, using a fully automated multiparametric acquisition protocol (Flow Motion Multiparametric PET, Siemens Biograph Vision 600 PET/CT, Siemens Healthineers, Knoxville, USA) with an axial field of view of 26.2 cm.

STATISTICAL ANALYSIS

This study employed a descriptive longitudinal approach. Due to the small sample size, inferential statistics were not applied. Intra-individual changes over time were evaluated for each subject. Results are reported as longitudinal trends and clinically relevant variations.

THIRD STUDY

STUDY POPULATION

A total of 204 subjects were included and stratified according to hypertensive status in *normotensive group* (n=150 subjects; 35 males, 115 females; mean age 42 ± 10 years) and *hypertensive group* (n=54 subjects; 30 males; mean age 55 ± 11 years). All participants underwent evaluation of the following hematochemical parameters: glucose, total cholesterol, LDL, HDL, triglycerides, creatinine, and eGFR. Additionally, lipidomic and metabolomic profiles were assessed. For a subset of subjects, cardiovascular risk and target organ damage were evaluated through Pulse Wave Velocity (PWV), Carotid Intima-Media Thickness (IMT), Left Ventricular Mass Index (LVMI).

LABORATORY ANALYSIS

Blood Glucose

Blood glucose was measured on the Cobas c503 (Roche Diagnostics GmbH, Mannheim, Germany) using an enzymatic method based on hexokinase (EC number 2.7.1.1), which phosphorylates glucose to glucose-6-phosphate. This is subsequently oxidized in the presence of nicotinamide adenine dinucleotide phosphate (NADP) to gluconate-6-phosphate, with the concomitant production of NADPH in an amount directly proportional to the glucose concentration in the sample.

The assay, standardized against the primary reference method based on isotope dilution mass spectrometry (IDMS), monitors NADPH production spectrophotometrically at a wavelength of 340 nm.

Total Cholesterol Measurement

Total cholesterol was measured in lithium-heparin plasma samples using an enzymatic colorimetric assay. Cholesterol esters are hydrolyzed by cholesterol esterase (EC 3.1.1.13) to yield free cholesterol and fatty acids. Subsequently, cholesterol oxidase (EC 1.1.3.6) catalyzes the oxidation of free cholesterol to cholest-4-en-3-one, with the concomitant formation of hydrogen peroxide (H_2O_2). In the presence of peroxidase (EC 1.11.1.7), the hydrogen peroxide generated promotes the oxidative coupling of phenol and 4-aminoantipyrine (4-AAP), resulting in the formation of a red quinoneimine dye. The intensity of the colored complex is directly proportional to the total cholesterol concentration in the sample and is determined by measuring the increase in absorbance at approximately 500 nm (typically 500–520 nm, depending on reagent specifications). The assay exhibits a linearity range of 3.86–800 mg/dL (0.10–20.7 mmol/L), according to the manufacturer's specifications.

HDL Cholesterol Measurement

HDL cholesterol was measured using a homogeneous enzymatic colorimetric assay. In this method, non-HDL lipoproteins (including LDL, VLDL, and chylomicrons) are selectively blocked by specific detergents and polyanions, preventing their participation in the enzymatic reaction. HDL particles remain accessible to cholesterol esterase (CHER; EC 3.1.1.13) and cholesterol oxidase (CHOD; EC 1.1.3.6). Cholesterol esters are hydrolyzed to free cholesterol, which is subsequently oxidized to cholest-4-en-3-one with the generation of hydrogen peroxide (H_2O_2). In the presence of peroxidase (EC 1.11.1.7), H_2O_2 reacts with a chromogenic system to produce a colored compound. The absorbance of the resulting dye, measured photometrically (typically at 580–600 nm, method-dependent), is directly proportional to the HDL cholesterol concentration.

LDL Cholesterol Measurement

LDL cholesterol was measured enzymatically in plasma using a selective homogeneous assay. Specific surfactants solubilize LDL particles while inhibiting the activity of cholesterol esterase (CHER) and cholesterol oxidase (CHOD) toward non-LDL lipoproteins. Following selective solubilization, LDL cholesterol undergoes enzymatic hydrolysis and oxidation, generating hydrogen peroxide, which reacts with a chromogen in the presence of peroxidase to produce a colored compound measured photometrically. Analyses were performed on the Cobas c503 (Roche Diagnostics). The assay exhibited a linear measurement range of 0.10–14.2 mmol/L.

Triglycerides Measurement

Triglycerides were determined using an enzymatic colorimetric method. Triglycerides are hydrolyzed by lipoprotein lipase (EC 3.1.1.34) to glycerol and free fatty acids. Glycerol is subsequently phosphorylated by glycerol kinase (EC 2.7.1.30) to glycerol-3-phosphate, which is oxidized by glycerol-3-phosphate oxidase (EC 1.1.3.21) to dihydroxyacetone phosphate with the production of hydrogen peroxide (H_2O_2). In the presence of peroxidase (EC 1.11.1.7), H_2O_2 reacts with 4-aminophenazone (4-AAP) and 4-chlorophenol to form a red quinoneimine dye. The intensity of the color, measured photometrically, is directly proportional to the triglyceride concentration. Analyses were performed on the Cobas c503 (Roche Diagnostics). The assay demonstrated a linear range of 0.1–10.0 mmol/L.

Serum Creatinine Measurement

Serum creatinine was measured enzymatically in plasma using a multi-step enzymatic assay involving creatininase (EC 3.5.2.10), creatinase (EC 3.5.3.3), and sarcosine oxidase (EC 1.5.3.1). Creatinine is sequentially converted to creatine, sarcosine, and finally glycine, formaldehyde, and hydrogen peroxide (H_2O_2). The generated H_2O_2 reacts with a

chromogenic substrate in the presence of peroxidase to form a colored compound, quantified colorimetrically at 546 nm. The assay was standardized against isotope dilution mass spectrometry (IDMS) reference methods. The measurement range was 0.17–25 mg/dL.

$$eGFR = 141 \times (\min(\frac{\text{Scr}}{\kappa}, 1))^{\alpha} \times (\max(\frac{\text{Scr}}{\kappa}, 1))^{-1.209} \times (0.993)^{\text{età}} \times F$$

- **Scr** = serum creatinine concentration (mg/dL)
- **κ (kappa)** = 0.7 for women, 0.9 for men
- **α (alpha)** = -0.329 for women, -0.411 for men
- **min(Scr/κ, 1)** = the minimum value between Scr/κ and 1
- **max(Scr/κ, 1)** = the maximum value between Scr/κ and 1
- **(0.993)^age** = age adjustment factor (years)
- **F** = sex- and ethnicity-specific adjustment factor:
    - 1.018 for women
    - 1.159 for African American individuals

Evaluation of Organ Damage

Hypertension-Mediated Organ Damage (HMOD) refers to subclinical structural or functional alterations in the arteries, heart, and large vessels that precede major clinical events such as stroke and myocardial infarction. In this study, organ damage was assessed using three main markers:

Arterial Stiffness

Arterial stiffness was measured using Pulse Wave Velocity (PWV), which evaluates the speed at which the pressure wave propagates along the arteries. PWV < 10 m/s indicates elastic arteries, while PWV > 10 m/s indicates stiff arteries. PWV is considered the gold standard for assessing arterial stiffness.

Carotid Vascular Damage

Carotid intima-media thickness (IMT) was measured via ultrasound as an early indicator of atherosclerosis and cardiovascular risk. Values between 0.5 and 0.9 mm are considered normal, values between 1 and 1.5 mm indicate wall thickening, and values greater than 1.5 mm are considered pathological.

Cardiac Remodeling

The Left Ventricular Mass Index (LVMI) indicates left ventricular hypertrophy and cardiac remodeling induced by pressure overload. Normal values are 49–115 g/m² for men and 43–95 g/m² for women. LVMI was calculated using 2D/M-mode transthoracic echocardiography, taking into account interventricular septal thickness, posterior wall thickness, and left ventricular end-diastolic diameter. Left ventricular mass was calculated using the Devereux formula:

$$LV\ mass = 0.8 \times \{1.04[(IVSd + LVIDd + PWd)^3 - (LVIDd)^3]\} + 0.6$$

Quantification Method: GC–MS

The analytical method used for serum is similar to that employed for cerebrospinal fluid, with some differences. To a glass vial with a Teflon septum, 100 µL of serum was added together with a mixture of internal standards (see table 9) and 2 mL of 1 M ethanolic KOH solution. Hydrolysis and saponification were carried out at room temperature under constant agitation for 60 minutes. The liquid–liquid extraction involved a first extraction of neutral lipids with 5 mL of n-hexane, followed by a second extraction with n-hexane after acidification with HCl. The two organic phases were combined, evaporated under a stream of nitrogen, and derivatized as previously described. GC-MS analyses were performed using an Agilent 6890N system with an HP7687 autosampler and 7683 injector

coupled to an HP5975B Inert MSD mass spectrometer (Agilent Technologies). The column used was an ELITE-HP5MS (30 m × 0.32 mm i.d. × 0.25 μm film, 5%-phenyl-methylpolysiloxane phase; Agilent Technologies, Santa Clara, CA, USA) with helium as carrier gas at a flow rate of 1 mL/min. Injection was splitless at 300°C. The temperature program started at 200°C for 1 min, followed by a linear ramp of 10°C/min up to 300°C, held for 7 min. Mass spectrometer parameters were: transfer line at 290°C, filament at 150°C, and quadrupole at 250°C. GC-MS analysis was performed by a 6890N Network GC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a HP 7687 series autosampler and a HP 7683 series injector (Agilent Technologies), and coupled to a quadruple mass selective detector HP5975B Inert MSD (Agilent Technologies). For GC separation was used an ELITE- HP5MS column (30 m × 0.32 mm id × 0.25 mm film, (5%-phenyl)-methylpolysiloxane phase; Agilent Technologies). Helium was used as carrier gas at a flow rate of 1 ml/min. Injection of the sample (1 μl) was carried out in splitless mode, at 250°C with a flow rate of 20 ml/min. The temperature program was as follows: initial temperature 200 °C held for 1 min, followed by a linear ramp of 10 °C/min to 300 °C, and then held for 7 min. The MS temperature parameters were 290°C for the transfer line, 150°C for the filament, and 250°C for the quadrupole, according with the manufacturer indication. TMS-ethers mass spectrometric data were acquired in selected ion-monitoring mode. Peak integration was performed manually, and analytes were quantified against internal standards using standard curves for the listed ion monitoring mode (SIM): m/z = 355 for nonadecanoic (C19), m/z = 383 for heneicosanoic (C21), m/z 411 for tricosanoic (C23), m/z = 361 for arachidonic (C20:4), m/z = 359 for eicosapentaenoic (C20:5), m/z = 363 for meadic (C20:3), m/z = 365 for eicosadienoic (C20:2), m/z = 365 for eicosenoic (C20:1), m/z = 367 for eicosanoic (C20), m/z = 215 for decosohexenoic (c22:6), m/z = 395 for brassidic and eurcic acid (C22:1), m/z = 397 for behenic (C22), m/z = 423 for nervonic (C24:1), m/z = 425 for lignoceric (C24), m/z = 451 for C26:1 and m/z = 453 for cerotic (C26) acid, at m/z = 463 for 7α-hydroxycholesterol-d7, m/z = 456 for 7α-hydroxycholesterol, at m/z = 463 for 7β-hydroxycholesterol- d7, m/z = 456 for 7β-hydroxycholesterol, m/z = 479 for 7-ketocholesterol- d7 and m/z = 472 for 7-

ketocholesterol, m/z = 131 for 25-hydroxycholesterol and m/z 137 for 25-hydroxycholesterol-d6, m/z 413 for D7-24Shydroxycholesterol and 24S-hydroxycholesteorl, m/z = 462 for 27-hydroxycholesterol—d6, m/z = 456 for 27-hydroxycholesterol at m/z = 370 for epicoprostanol, and m/z = 368 for cholesterol. GC.MS quantitative analysis of medium and long chain fatty acids, and cholesterol was performed by a 6890N Network GC system (Agilent Technologies) equipped with a HP 7687 series autosampler and a HP 7683 series injector (Agilent Technologies), and coupled to a quadruple mass selective detector HP5975B Inert MSD (Agilent Technologies). For GC separation was used an ELITE- HP5MS column (30 m × 0.32 mm id × 0.25 mm film, (5%-phenyl)-methylpolysiloxane phase; Agilent Technologies). Helium was used as carrier gas at a flow rate of 1 ml/min. Injection of the sample (1 μl) was carried out in split 1:10 mode, at 250°C with a flow rate of 20 ml/min. The temperature program was as follows: initial temperature 170 °C held for 2 min, followed by a linear ramp of 10 °C/min to 300 °C, and then held for 7 min. The MS temperature parameters were 290°C for the transfer line, 150°C for the filament, and 220°C for the quadrupole, according with the manufacturer indication. TMS-ethers mass spectrometric data were acquired in selected ion-monitoring mode. Peak integration was performed manually, and analytes were quantified against internal standards using standard curves for the listed ion monitoring mode (SIM): pentadecanoic acid (C15, IS) M/z = 299 and heptadecanoic acid (C17, IS), m/z = 327), lauric acid (C12) m/z = 257, myristoleic acid (C14:1ω6), m/z 283, myristic acid (C14), m/z = 285, palmitic acid (C16) m/z = 313, palmitoleic acid (C16:1(9) m/z = 311, stearic acid (C18), m/z 341, oleic acid /C18:1(9) m/z = 339, vaccenic acid (c18:1t11), m/z = 339, linoleic acid (C18:2(9,12) = m/z 337, α-linolenic-acid (C18:3(9,12,15) = m/z 335. The cell number of each sample was evaluated to normalize the corresponding lipid amounts.

RESULTS

FIRST STUDY

The table summarizes the findings of biochemical analyses of cerebrospinal fluid (CSF) from the 41 study participants. The data are presented as mean ± standard deviation, along with the related P values. Beta-amyloid 1-42 (Aβ42), phosphorylated tau (p-Tau181), and total tau (t-Tau) were the CSF dementia biomarkers that were assessed in these patients with Alzheimer's disease (AD), non-Alzheimer degenerative dementia (NAD), and non-degenerative diseases.

Table 10 Mean values ± SD of the clinical pathology parameters measured in the three groups of patients: AD, NAD and C.

Parameters	AD	NAD	C	P values		
				AD vs C	NAD vs C	AD vs NAD
Patients (n)	11	22	8			
t-Tau (pg/mL)	446.0 ± 233.0	361.1 ± 188.7	182.7 ± 131.4	<0.05	NS	NS
p-Tau$_{181}$ (pg/mL)	69.1 ± 31.7	65.3 ± 47.6	30.0 ± 21.8	NS	NS	NS
Aβ$_{42}$ (pg/mL)	484.8 ± 80.8	908.4 ± 290.3	952.9 ± 297.4	<0.001	NS	<0.001
MDA (µg/L)	8.5 ± 1.0	7.8 ± 1.7	7.6 ± 1.9	NS	NS	NS
Lathosterol (ng/mL)	1846.8 ± 423.4	1649.5 ± 342.9	1143.2 ± 324.7	<0.001	<0.01	NS
Desmosterol (ng/mL)	1234.0 ± 192.9	978.2 ± 205.4	699.6 ± 107.4	<0.001	<0.01	<0.01
Lanosterol (ng/mL)	178.6 ± 45.7	148.0 ± 62.0	73.7 ± 17.9	<0.001	<0.01	NS
Cholesterol (mg/L)	5676 ± 1024	4063 ± 960	2880 ± 661	<0.001	<0.01	<0.001
7αOHC (ng/mL)	3.7 ± 0.6	2.6 ± 0.6	1.5 ± 0.3	<0.001	<0.001	<0.001
7βOHC (ng/mL)	4.0 ± 0.8	2.5 ± 0.6	1.6 ± 0.3	<0.001	<0.01	<0.001
7KC (ng/mL)	7.4 ± 1.0	5.2 ± 1.6	2.4 ± 0.4	<0.001	<0.001	<0.001
α-epoxyC (ng/mL)	1.6 ± 0.4	1.2 ± 0.3	0.6 ± 0.1	<0.001	<0.001	<0.001
β-epoxyC (ng/mL)	1.2 ± 0.3	1.0 ± 0.2	0.5 ± 0.1	<0.001	<0.01	NS
Triol (ng/mL)	1.4 ± 0.2	1.1 ± 0.2	0.8 ± 0.1	<0.001	<0.01	<0.001
24OHC (ng/mL)	3.4 ± 0.3	3.1 ± 0.7	2.2 ± 0.2	<0.001	<0.001	NS
27OHC (ng/mL)	1.3 ± 0.3	1.3 ± 0.3	0.9 ± 0.1	<0.01	<0.01	NS

The CSF obtained from patients with AD and NAD had higher levels of a number of oxysterols than the control group. CSF levels for non-enzymatic oxysterols 7α-OHC, 7β-OHC, 7KC, α-epoxyC, β-epoxyC, and triol were 1.5–3.0 times more than those found in controls (figure 17).

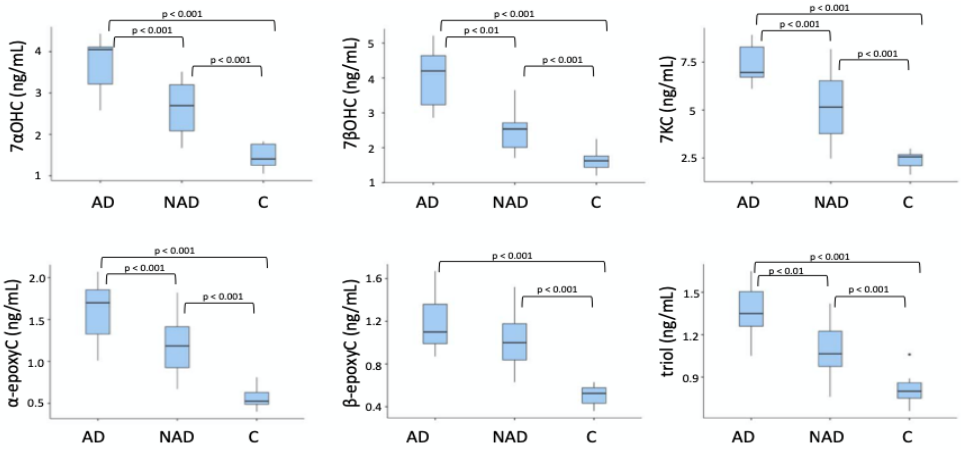

Figure 17 Oxysterols of nonenzymatic origin quantified in the cerebrospinal fluid (CSF) of patients with diagnosed Alzheimer's dementia (AD), non-Alzheimer's degenerative dementia (NAD) and nondegenerative disorders (C).

The two enzymatic oxysterols, 24OHC and 27OHC, were also significantly increased, by 1.5- and 1.4-fold respectively, in the AD and NAD groups compared to the control group (figure 18).

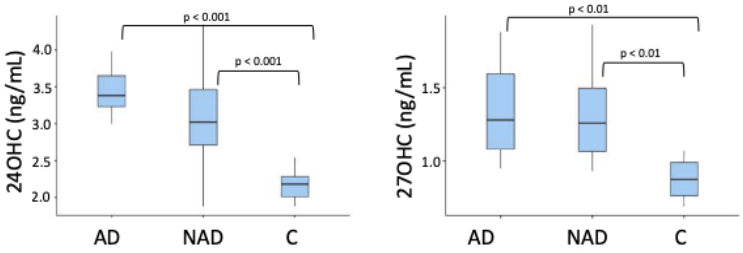

Figure 18 Oxysterols of enzymatic origin quantified in the cerebrospinal fluid (CSF) of patients with diagnosed Alzheimer's dementia (AD), non-Alzheimer's degenerative dementia (NAD) and nondegenerative disorders (C).

Cholesterol and its precursors, lanosterol, lathosterol, and desmosterol—markers of cholesterol biosynthesis—were also measured in order to further understand the observed differences. As shown in figures 21, all four sterols were significantly increased in the CSF of patients with AD and NAD compared to the CSF of patients without neurodegenerative disease, used as internal controls. Furthermore, cholesterol content in the CSF of AD patients was significantly higher than that observed in NAD patients, mainly due to the increase in desmosterol (figures 19).

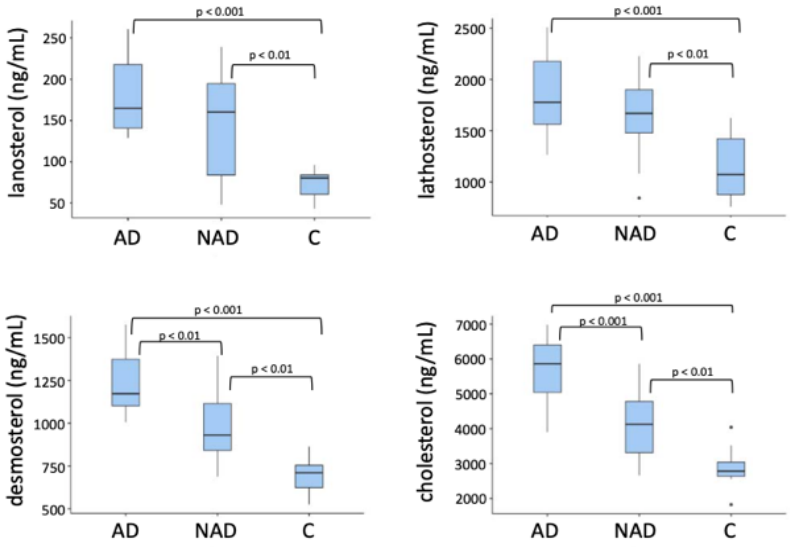

Figure 19 CSF content of cholesterol and its precursors lanosterol, lathosterol and desmosterol in AD, NAD and C groups of patients.

SECOND STUDY

Patients regularly took miglustat, which was generally well tolerated. However, during the first two months of therapy, dose escalation had to be slowed due to diarrhea, particularly in patient 1. Another side effect was a mild weight loss, likely related to gastrointestinal disturbances. Consequently, a personalized diet and a probiotic (Saccharomyces boulardii) were recommended. No clinically significant alterations were observed during routine laboratory tests. Biochemical analyses were performed on both serum and cerebrospinal fluid (CSF), and values were compared with age- and sex-matched healthy controls. Unfortunately, patient 1 declined consent for a repeat lumbar puncture, so only the pre-treatment CSF sample was available for this subject. Serum oxysterol assessment at T0 showed very high levels of 7-KC and C-triol, similar to those observed one year earlier. During miglustat treatment, plasma levels of 7-KC and C-triol showed a persistent reduction. In parallel, CSF levels of 7-KC and C-triol decreased at T12 compared to values measured one year before the start of therapy (T-12).

Table 11 Summary of CSF and plasma findings.

Months	***Triol plasma µg/L (n.v. 15.46±8.88)***	***7KC plasma µg/L (n.v. 31.03±8.43)***	***Triol CSF µg/L (n.v. 0.7±0.1)***	***7KC CSF µg/L (n.v. 0.86±0.25)***	***Aβ42 CSF pg/mL (normal >450)***	***Aβ42/40 CSF (normal >0.060)***	***Tau CSF pg/mL (normal <275)***	***pTau CSF pg/mL (normal <55)***
P1								
T-12	63.6	105.36	1.19	1.38	471	0.051	747	94
T0	59.08	110.08	/	/	/	/	/	/
T3	42.86	89.22	/	/	/	/	/	/
T9	30.84	62.58	/	/	/	/	/	/
T12	26.6	63.81	/	/	/	/	/	/
P2								
T-12	58.16	135.32	1.28	1.18	271	0.027	1520	329
T0	47.16	128.9	/	/	/	/	/	/
T3	42.81	102.5	/	/	/	/	/	/
T9	44.47	98.54	/	/	/	/	/	/
T12	40.43	99.68	0.84	0.95	776	0.036	1705	300
P3								
T-12	32.16	119.12	0.94	2.12	428	0.053	486	58
T0	39.06	120.38	/	/	/	/	/	/
T3	32.07	81.88	/	/	/	/	/	/
T9	26.81	68.12	/	/	/	/	/	/
T12	24.15	60.08	0.67	1.19	961	0.057	623	90

Neuropsychological tests showed a substantial stabilization of cognitive performance from T0 to T12 in patient 2. In patients 1 and 3, cognitive abilities initially continued to decline, as observed at the T6 assessment. However, during the following six months, clinical stabilization was achieved, confirmed by the T12 evaluation, which showed unchanged cognitive functions. Scores from the neuropsychological assessments, conducted on each occasion by the same clinical neuropsychologist (B.P.).

Table 12 Neuropsycological test score

Months	*MMSE (normal ≥23.8)*	*MODA (normal >85.5)*	*CDT (normal >6)*	*15 words Rey immediate recall (normal ≥28.53)*	*15 words Rey delayed recall (normal ≥4.69)*
P1					
T-12	14	65.6	/	/	/
T0	11	47.5	2	/	/
T6	9	/	0	/	/
T12	9	/	0	/	/
P2					
T-12	23.4	84.4	9	/	/
T0	17	74.9	9	/	/
T6	17	76.4	7	/	/
T12	17	74.9	9	/	/
P3					
T-12	28.05	/	9	/	/
T0	29.15	/	8	38	6.24
T6	26.15	/	6	28.22	5.48
T12	26.15	/	7	28.22	5.48

Amyloid PET at T0 showed that amyloid deposition had increased further in all patients, but it was most noticeable in patient 2 and in the temporal, frontal, and precuneus/posterior cingulate cortices. Surprisingly, at T12, cerebral amyloid load decreased in all three patients. In patients 2 and 3, the decrease in neocortical Aβ binding was particularly noticeable, especially in the frontal and precuneus/posterior cingulate

cortices. The cerebral amyloid PET data for our three patients are shown in figure 20, along with how they have changed over time.

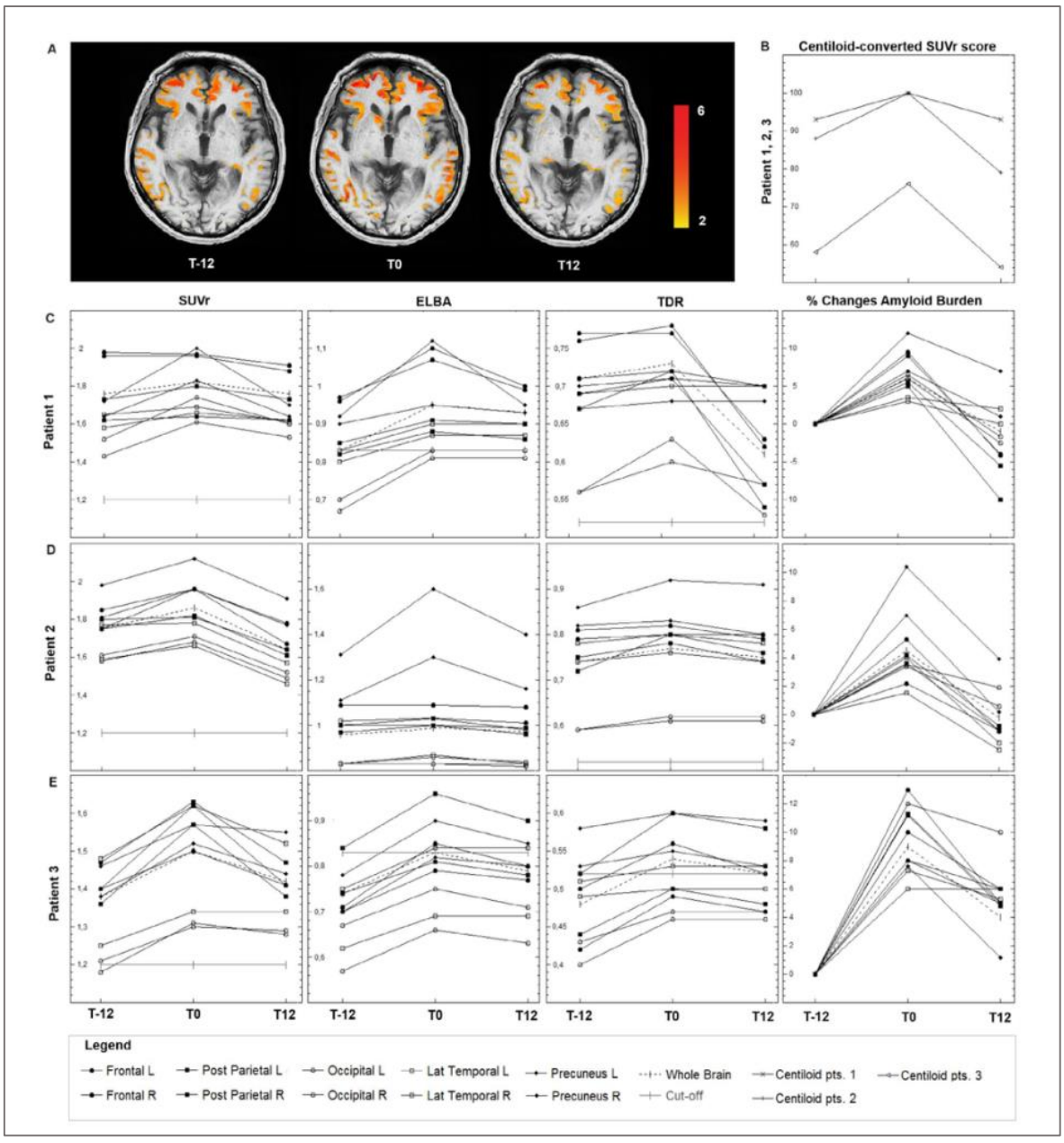

Figure 20 Longitudinal assessment of cortical amyloid burden: MRI images with amyloid overlay (T-12, T0, T12)

THIRD STUDY

Table 13 describes the baseline characteristics of the study population, stratified according to hypertensive status.

Table 13 Baseline demographic and clinical characteristics of the study population

VARIABLE	*NORMOTENSIVE*	*HYPERTENSIVE*	*P-VALUE*
DEMOGRAPHY			
Number	150	54	-
Age, years	42.1 ± 10.3	55.7 ± 11.2	<0.001
Male sex, n (%)	35 (23.3%)	30 (55.6%)	<0.001
CARDIOVASCULAR RISK FACTORS			
BMI, kg/m²	25.1 ± 3.2	26.5 ± 4.0	0.006
Current smoking, n (%)	81 (54.0%)	25 (46.3%)	0.32
SBP, mmHg	121.6 ± 11.1	136.7 ± 18.3	<0.001
DBP, mmHg	76.4 ± 8.4	83.0 ± 15.2	<0.001
DM, n (%)	11 (7.3%)	3 (5.6%)	0.67
BIOCHEMICAL VARIABLES			
Fasting glucose, mg/dL	89.3 ± 12.2	89.8 ± 20.0	0.30
Total Cholesterol, mg/dL	191.0 ± 31.3	203.0 ± 38.5	0.048
HDL cholesterol, mg/dL	54.7 ± 13.6	57.0 ± 14.4	0.37
Triglycerides, mg/dL	95.2 ± 52.9	107.0 ± 46.6	0.048
LDL cholesterol, mg/dL	121.5 ± 27.8	123.5 ± 36.4	0.81
Creatinine, mg/dL	0.82 ± 0.2	0.88 ± 0.2	0.028
eGFR, mL/min/1.73 m²	98.9 ± 18.8	88.4 ± 15.1	<0.001
CARDIOVASCULAR DRUGS			
Statin therapy, n (%)	0 (0.0%)	5 (9.3%)	0.003
RAAS inhibitors, n (%)	0 (0.0%)	17 (31.5%)	<0.001
HYPERTENSION MEDIATED ORGAN DAMAGE			
PWV, m/s	7.40 ± 1.25	8.51 ± 1.93	<0.001
IMT, mm	0.58 ± 0.19	0.75 ± 0.16	<0.001
LVMI, g/m²	52.2 ± 12.6	55.6 ± 16.1	0.06

Table 14 shows the distribution of metabolomic and lipidomic biomarkers in hypertensive and normotensive subjects. Values are expressed as mean ± standard deviation. Comparisons between hypertensive and normotensive subjects were performed using the independent-samples t-test. p-values refer to differences between the groups. Metabolomic and lipidomic biomarkers are reported according to fatty acid class and sterol/oxysterol profile.

Table 14 distribution of metabolomic and lipidomic biomarkers in hypertensive and normotensive subjects

BIOMARKER	**NORMOTENSIVE**		**HYPERTENSIVE**		
	MEDIA	*DS*	*MEDIA*	*DS*	*p-value*
C12	7,87	1,93	5,49	1,74	**<0.001**
C14:0	12,85	4,53	10,23	3,80	**<0.001**
C16	542,09	114,43	516,78	135,47	0.186
C16:1A	0,50	0,18	0,62	0,27	**0.001**
C16:1B	4,65	2,00	5,24	2,30	0.072
C18:0	163,62	31,60	159,59	34,98	0.436
C18:1	259,96	50,10	228,14	64,72	**<0.001**
C18:2	204,54	35,01	182,86	49,32	**0.001**
ALA	11,53	2,25	9,15	2,66	0.867
AA	130,90	27,20	92,28	32,30	**<0.001**
EPA	25,83	10,38	13,63	8,57	**<0.001**
C20:3B	6351,78	1869,65	4190,25	2004,03	**<0.001**
C20:3A	204,33	55,84	138,52	41,91	**<0.001**
C20:2C	1932,44	476,14	1241,02	456,61	**<0.001**
C20.2B	453,96	120,51	307,87	134,49	**<0.001**
C20.2A	347,22	95,44	187,86	77,68	**<0.001**
C20:1	2790,90	762,73	1983,56	814,46	**<0.001**
C20	3414,66	693,18	2285,28	729,22	**<0.001**

C22:5	11,61	1,99	6,47	2,91	**<0.001**
DHA	85,40	24,64	56,20	24,61	**<0.001**
C22:4	23,11	6,40	13,74	7,18	**<0.001**
C22:1	411,49	240,64	1102,19	375,35	0.028
C22	433,25	74,90	298,39	74,79	0.007
C24	361,67	66,09	254,87	59,70	**<0.001**
C24:1	132,56	23,06	125,45	29,80	0.025
C26	78,21	23,33	57,80	19,48	0.043
C26:1a	8,12	2,20	7,08	3,13	**<0.001**
C26:1b	5,28	1,60	4,69	1,28	0.080
lathosterol	3145,69	1083,68	2723,31	1195,52	**<0.001**
desmosterol	1592,35	425,69	1441,14	475,66	**<0.001**
lanosterol	90,35	27,75	83,39	28,41	0.156
7bOHC	13,49	2,47	20,90	3,98	**<0.001**
7KC	38,57	12,01	42,74	15,69	**<0.001**
sitosterol	1942,04	456,91	1480,44	368,54	0.858
campesterol	1687,74	468,49	1369,21	476,64	**<0.001**
stigmasterol	70,68	34,18	64,05	27,26	0.200
sitostanol	69,12	30,76	39,32	15,92	**<0.001**
24OHC	25,79	10,10	18,80	9,07	0.827
27OHC	146,52	32,08	147,81	49,31	**<0.001**

Table 15 shows the results of multivariate logistic regression analyzing the association between metabolomic and lipidomic biomarkers and arterial hypertension. Logistic regression models were constructed with arterial hypertension as the dependent variable. Each metabolomic and lipidomic biomarker was analyzed individually. Models were adjusted for age, sex, smoking status, body mass index, systolic blood pressure, estimated glomerular filtration rate, total cholesterol, triglycerides, fasting glucose, statin

therapy, microalbuminuria, and antihypertensive treatment. Results are reported as odds ratios (OR) with 95% confidence intervals (CI) per unit increase. Biomarkers significantly associated with hypertension are reported in the table; nonsignificant biomarkers are not shown.

Table 15 Significant biomarkers

Biomarker	OR	95% CI lower	95% CI upper	p-value
C12	0.485	0.332	0.708	<0.001
C14:0	0.711	0.575	0.881	0.002
C18:1	0.981	0.969	0.993	0.002
C18:2	0.979	0.963	0.994	0.008
ALA	0.523	0.369	0.741	<0.001
AA	0.929	0.896	0.963	<0.001
EPA	0.764	0.662	0.881	<0.001
DHA	0.917	0.879	0.958	<0.001
C20:3B	0.999	0.999	1.000	<0.001
C20:3A	0.937	0.908	0.967	<0.001
C20:2C	0.994	0.991	0.997	<0.001
C20:2B	0.985	0.977	0.992	<0.001
C20:2A	0.954	0.927	0.982	0.001
C20:1	0.994	0.991	0.997	<0.001
C20	0.994	0.991	0.997	<0.001
C22:5	0.147	0.046	0.470	0.001
C22:4	0.917	0.879	0.958	<0.001
C22:1	1.007	1.004	1.011	<0.001
C22	0.996	0.994	0.998	<0.001
C24	0.966	0.949	0.982	<0.001
C26	0.938	0.904	0.974	0.001
C26:1a	0.669	0.511	0.875	0.003
Lathosterol	0.999	0.998	1.000	0.003
Desmosterol	0.998	0.997	1.000	0.027
7β-OHC	1.984	1.386	2.840	<0.001
Sitosterol	0.996	0.994	0.998	<0.001
Sitostanol	0.917	0.879	0.958	<0.001
24-OHC	0.937	0.908	0.967	<0.001
27-OHC	0.994	0.991	0.997	<0.001

The biomarkers not significantly associated with hypertension are reported in Table 16.

Table 16 Non-significant biomarkers

Biomarker	OR	95% CI lower	95% CI upper	p-value
C16:1A	1.680	0.099	28.614	0.720
C24:1	1.027	0.963	1.095	0.420
C26:1b	0.723	0.495	1.057	0.094
Lanosterol	0.564	0.116	2.734	0.477
7-KC	1.017	0.998	1.036	0.074
Campesterol	0.973	0.942	1.005	0.093
Stigmasterol	1.002	0.985	1.018	0.844

A multivariate linear regression was performed in both normotensive and hypertensive subjects. The outcomes selected were:

- PWV (arterial stiffness)
- IMT (carotid intima-media thickness)
- LVMI (left ventricular mass index)

All models were adjusted for age, sex, smoking, BMI, systolic blood pressure, eGFR, LDL, and triglycerides. The following tables show the β values. A negative β value indicates that higher metabolite concentrations are associated with lower organ damage. A positive β value indicates that higher metabolite concentrations are associated with greater organ damage. Many long-chain fatty acids are correlated with lower PWV, IMT, and LVMI, whereas 7β-hydroxycholesterol shows opposite correlations.

Multivariable linear regression models were used to evaluate the association between individual metabolomic variables and hypertension-mediated organ damage. Each metabolomic variable was analyzed separately. Models were adjusted for age, sex, smoking status, body mass index, systolic blood pressure, estimated glomerular filtration rate, LDL cholesterol, and triglycerides.

Table 17 Multivariable linear regression models for continuous hypertension-mediated organ damage variables in normotensive subjects

	PWV				IMT				LVMI			
	β	95% CI		p-value	β	95% CI		p-value	β	95% CI		p-value
		Lower limit	Upper limit			Lower limit	Upper limit			Lower limit	Upper limit	
C18.2	−0.00052	−0.00089	−0.00015	0.006	−0.00031	−0.00055	−0.00007	0.011	−0.061	−0.112	−0.010	0.019
C20:3A	−0.00001	−0.00002	−0.000002	0.016	−0.000008	−0.000015	−0.000001	0.026	−0.0016	−0.0030	−0.0002	0.025
C20:2C	−0.0006	−0.0010	−0.0002	0.004	−0.0003	−0.0005	−0.0001	0.008	−0.047	−0.078	−0.016	0.003
C20:2B	−0.0004	−0.0007	−0.0001	0.006	−0.0002	−0.0004	−0.00005	0.011	−0.026	−0.041	−0.011	0.001
C20:2A	−0.0005	−0.0009	−0.0001	0.009	−0.0004	−0.0007	−0.0001	0.010	−0.049	−0.082	−0.016	0.004
C20:1	−0.0003	−0.0006	−0.00003	0.029	−0.0002	−0.0004	−0.00001	0.041	−0.036	−0.059	−0.013	0.002
C20	−0.0002	−0.0004	−0.00001	0.038	−0.0001	−0.0003	−0.00001	0.047	−0.024	−0.045	−0.003	0.025
C22:5	−0.062	−0.114	−0.010	0.020	−0.028	−0.049	−0.007	0.010	−2.41	−4.38	−0.44	0.017
C22:4	−0.018	−0.031	−0.005	0.007	−0.008	−0.014	−0.002	0.009	−0.86	−1.64	−0.08	0.031
C22:1	0.004	0.001	0.007	0.011	0.0018	−0.0002	0.0038	0.08	0.22	−0.07	0.51	0.14
C22	−0.011	−0.019	−0.003	0.006	−0.005	−0.009	−0.001	0.012	−0.69	−1.24	−0.14	0.014
C24	−0.0003	−0.0005	−0.0001	0.002	−0.00015	−0.00026	−0.00004	0.008	−0.049	−0.081	−0.017	0.003
C24:1	−0.0002	−0.0004	−0.00003	0.018	−0.00011	−0.00021	−0.00002	0.017	−0.037	−0.066	−0.008	0.012
C26	−0.006	−0.011	−0.001	0.017	−0.002	−0.004	−0.0004	0.014	−0.31	−0.58	−0.04	0.024
C26:1a	−0.024	−0.041	−0.007	0.006	−0.010	−0.017	−0.003	0.006	−1.12	−1.97	−0.27	0.010
EPA	−0.003	−0.005	−0.001	0.001	−0.0016	−0.0028	−0.0004	0.009	−0.21	−0.33	−0.09	<0.001
DHA	−0.002	−0.004	−0.0004	0.012	−0.0014	−0.0026	−0.0002	0.022	−0.19	−0.32	−0.06	0.004
C20:3B	−0.0009	−0.0015	−0.0003	0.003	−0.0004	−0.0007	−0.0001	0.006	−0.058	−0.104	−0.012	0.014
7β-OHC	0.013	0.004	0.022	0.004	0.007	0.002	0.012	0.006	0.94	0.41	1.47	<0.001
Sitostanol	−0.007	−0.012	−0.002	0.006	−0.003	−0.006	−0.001	0.008	−0.79	−1.27	−0.31	0.001
Lathosterol	−0.0005	−0.0009	−0.0001	0.012	−0.00025	−0.00047	−0.00003	0.026	−0.018	−0.033	−0.003	0.020
Desmosterol	−0.0003	−0.0006	−0.00003	0.028	−0.00018	−0.00035	−0.00001	0.038	−0.014	−0.027	−0.001	0.036
Sitosterol	−0.0010	−0.0016	−0.0004	0.001	−0.0005	−0.0009	−0.0001	0.009	−0.062	−0.101	−0.023	0.002
24-OHC	−0.003	−0.006	−0.0004	0.021	−0.0015	−0.0029	−0.0001	0.034	−0.29	−0.55	−0.03	0.028
27-OHC	−0.0008	−0.0014	−0.0002	0.008	−0.0005	−0.0009	−0.0001	0.015	−0.09	−0.16	−0.02	0.014

Multivariable linear regression models evaluating the association between individual metabolomic variables and hypertension-mediated organ damage. Each metabolomic variable was analyzed separately. Models were adjusted for age, sex, smoking status, body mass index, systolic blood pressure, estimated glomerular filtration rate, LDL cholesterol, triglycerides, diabetes mellitus, and pharmacological therapies, including renin–angiotensin–aldosterone system inhibitors and statins. (Table 18)

Table 18 Multivariable linear regression models for continuous hypertension-mediated organ damage variables in hypertensive subjects

	PWV				IMT				LVMI			
	β	95% CI		p-value	β	95% CI		p-value	β	95% CI		p-value
		Lower limit	Upper limit			Lower limit	Upper limit			Lower limit	Upper limit	
C18.2					−0.00028	−0.00049	−0.00007	0.009	−0.074	−0.129	−0.019	0.009
ALA	−0.021	−0.041	−0.001	0.039	−0.006	−0.013	0.001	0.08	−0.98	−1.89	−0.07	0.035
AA	−0.014	−0.028	−0.001	0.036	−0.005	−0.010	−0.001	0.021	−0.84	−1.52	−0.16	0.016
EPA	−0.004	−0.006	−0.002	<0.001	−0.002	−0.003	−0.001	<0.001	−0.29	−0.41	−0.17	<0.001
DHA	−0.003	−0.005	−0.001	0.002	−0.002	−0.003	−0.001	0.002	−0.25	−0.39	−0.11	<0.001
C20	−0.0003	−0.0005	−0.0001	0.001	−0.0002	−0.0004	−0.00005	0.01	−0.029	−0.051	−0.007	0.011
C20:3B	−0.0012	−0.0018	−0.0006	<0.001	−0.0005	−0.0009	−0.0002	0.002	−0.071	−0.118	−0.024	0.003
C20:3A	−0.00002	−0.00003	−0.00001	<0.001	−0.00001	−0.00002	−0.000004	0.004	−0.0021	−0.0036	−0.0006	0.006
C20:2C	−0.0009	−0.0013	−0.0005	<0.001	−0.0004	−0.0006	−0.0002	<0.001	−0.054	−0.086	−0.022	0.001
C20:2B	−0.0004	−0.0006	−0.0002	<0.001	−0.0003	−0.0004	−0.0001	0.001	−0.031	−0.048	−0.014	<0.001
C22:5	−0.089	−0.141	−0.037	0.001	−0.031	−0.054	−0.008	0.008	−3.21	−5.46	−0.96	0.005
C24	−0.0004	−0.0006	−0.0002	<0.001	−0.0002	−0.0003	−0.0001	<0.001	−0.061	−0.093	−0.029	<0.001
C24:1	−0.0003	−0.0005	−0.0001	0.003	−0.0001	−0.0002	−0.00004	0.002	−0.048	−0.078	−0.018	0.002
C26	−0.007	−0.012	−0.002	0.006	−0.003	−0.005	−0.001	0.006	−0.39	−0.69	−0.09	0.010
7β-OHC	0.021	0.011	0.031	<0.001	0.009	0.004	0.014	<0.001	1.18	0.61	1.75	<0.001
Lathosterol	−0.0006	−0.0010	−0.0002	0.003	−0.0003	−0.0005	−0.0001	0.004	−0.021	−0.036	−0.006	0.006
Desmosterol	−0.0004	−0.0007	−0.0001	0.008	−0.0002	−0.0004	−0.00004	0.014	−0.017	−0.030	−0.004	0.010
Sitosterol	−0.0012	−0.0018	−0.0006	<0.001	−0.0006	−0.0009	−0.0003	<0.001	−0.071	−0.113	−0.029	0.001
Sitostanol	−0.009	−0.014	−0.004	<0.001	−0.004	−0.007	−0.002	<0.001	−0.91	−1.42	−0.40	<0.001
24-OHC	−0.004	−0.007	−0.001	0.004	−0.002	−0.004	−0.0004	0.012	−0.36	−0.63	−0.09	0.009
27-OHC	−0.0011	−0.0016	−0.0006	<0.001	−0.0006	−0.0010	−0.0002	0.003	−0.11	−0.19	−0.03	0.006

DISCUSSION

In recent years, there has been heightened focus on the possible translational applicability of various biochemical and biological discoveries related to molecules implicated in pathophysiological processes. This is also pertinent to cholesterol oxidation products, redox-active molecules that have been thoroughly examined for their involvement in human pathology.

DISCUSSION FIRST STUDY

Changes in cholesterol metabolism and hypercholesterolemia, which are often associated with both AD and NAD have rarely been studied in relation to oxidative stress, which is well known in Alzheimer's disease (AD) [71,72] and other neurodegenerative diseases (NAD) [73]. In this pilot investigation, we examined a small group of patients with AD and NAD as well as individuals without neurodegenerative or inflammatory disease to determine the relationship between oxidative stress measurement and changes in cholesterol metabolism. For a long time, it has been demonstrated that excessive free cholesterol accumulation causes neuronal death [74]. Strong pro-oxidant, pro-apoptotic, and pro-inflammatory characteristics are displayed by oxysterols, especially those of non-enzymatic origin; these effects are more noticeable than those of free cholesterol [75,76]. Oxysterols' biochemical characteristics enable them to play a major role in the development of cerebral degenerative processes, particularly in their progression. Interestingly, there were no noticeable changes in CSF oxysterol levels or cholesterol metabolism in the brains of control subjects. We found that the CSF of patients with degenerative dementia (AD and NAD) was significantly higher than that of those with non-degenerative diseases for all quantifiable oxysterols, both enzymatic and non-enzymatic. With the exception of β-epoxyC, the rise was especially noticeable for auto-oxidation-derived oxysterols, which are indicators of oxidative stress, in AD patients as opposed to NAD (figures 19 and table 10). On the other hand, there were no statistically significant differences in the CSF concentrations of 24OHC and 27OHC between the AD and NAD

groups (figures 20). Therefore, there are reliable candidates as oxidative stress biomarkers among auto-oxidation-derived oxysterols that may be measured in CSF. Furthermore, because oxysterols are byproducts of cholesterol oxidation, their pathological increase in CSF naturally reflects changes in cholesterol metabolism. This was also confirmed in patients with AD and NAD by concurrent increases in lanosterol, cholesterol, and intermediates of the Bloch (desmosterol) and Kandutsch–Russell (lathosterol) biosynthesis pathways (figures 21 and Table 10). Further evidence that oxidative stress and altered cholesterol metabolism are linked in degenerative dementias comes from the finding that metabolites of cholesterol auto-oxidation exacerbate the redox imbalance that is a constant in neurodegenerative processes. This study should be regarded as a feasibility pilot study due to its primary limitation, which is the small number of enrolled patients, especially those without neurological disorders. CSF samples from individuals without neurodegenerative disorders cannot be obtained due to ethical issues. Another drawback is that patients without degenerative disorders tended to be somewhat younger on average than those with AD and NAD. Although inherently difficult, comparing age-matched subgroups is nevertheless an objective [77,78].

DISCUSSION SECOND STUDY

There has long been discussion about the connection between Alzheimer's disease (AD) and cholesterol metabolism. Alois Alzheimer first reported intracellular lipid deposits in 1906, in addition to amyloid plaques and neurofibrillary tangles [79]. The wealth of information on this subject has recently given rise to new, intricate pathogenetic models that highlight the critical role that altered cerebral cholesterol metabolism plays in AD, which has been identified as a possible lipid metabolic illness. NPC and AD have been shown to have bidirectional relationships in the past. There are notable neuropathological similarities between these disorders, such as altered cholesterol metabolism, dysregulation of Aβ metabolism, and neurofibrillary tangles. With a common hereditary component to cholesterol metabolism, lipid imbalance may be the cause of

neurodegenerative processes in both. The first family with autosomal dominant AD caused by a heterozygous NPC1 mutation was previously reported by our group [80,81,82]. The idea of a potential new AD entity is supported by clinical, biochemical, genetic, and neuroimaging evidence from our family as well as earlier research on heterozygous NPC1 mutations. Although lower than in NPC patients, plasma levels of 7-KC and other oxysterols were considerably higher in carriers of the NPC1 mutation than in healthy controls [82]. Our patients were 68–78 years old, which suggests that more prominent biochemical phenotypes may be linked to advanced age, in contrast to earlier research that used carriers who were about 40 years old. The investigation of treatment implications was spurred by this possible new ADAD entity as well as growing evidence of lipid-based AD pathogenesis [82]. Through the reduction of cellular lipid levels, NPC treatments can halt the progression of the disease. Miglustat lowers the GM2 and GM3 ganglioside load in NPC by blocking glucosylceramide synthase. According to several studies, AD brains have higher levels of GM2 and GM3, and some gangliosides may promote the synthesis of Aβ or its aggregation into neurotoxic complexes. In human AD cellular models, miglustat has shown anti-amyloidogenic effects by promoting the non-amyloidogenic route and modifying APP processing. Here, we describe for the first time how three individuals with heterozygous NPC1 mutations responded to therapeutic-dose miglustat in AD. Cerebral amyloid PET after one year of treatment (T12) showed marked reduction in amyloid load in all patients, which was increased at baseline (T0) compared to one year prior (T-12). A time-dependent effect of miglustat was suggested by the cognitive tests, which revealed stabilization in one patient from the start of treatment and beginning decrease in the other two throughout the course of six months before stabilizing in the subsequent six months [83]. Decreased beta-amyloid formation through manipulation of cholesterol homeostasis may be the cause of the reduction in CSF amyloid and PET amyloid load, which could improve endogenous clearance. Nevertheless, this study had several drawbacks, including being uncontrolled, only involving three patients from the same family, and having highly variable biomarker measurements. However, the data suggest a potential anti-amyloidogenic effect of miglustat in AD

patients with heterozygous NPC1 mutations, warranting future controlled trials. This cohort may represent a unique AD model particularly responsive to miglustat. Potential effects may also extend to sporadic AD independent of NPC1 mutation [83].

DISCUSSION THIRD STUDY

The study population included 204 subjects: 54 hypertensive and 150 normotensive. Hypertensive subjects were older (55.7 years) and predominantly male (56%). As expected, blood pressure was higher in hypertensives. Normotensive subjects were younger (42.1 years) and predominantly female (76.7%). Within the normotensive group, there are also individuals classified as normotensive who present systolic blood pressure in the range of 120–139 mmHg and/or diastolic blood pressure range of 70–89 mmHg, although not yet meeting criteria for overt hypertension.

According to the guidelines of the European Society of Cardiology / European Society of Hypertension (ESC/ESH) and the American College of Cardiology / American Heart Association (ACC/AHA), these individuals may fall within the category consistent with “elevated” or borderline blood pressure and potentially associated with increased long-term cardiovascular risk.

Total cholesterol and triglycerides were slightly but significantly higher in hypertensives. Renal function was reduced, with higher creatinine and lower eGFR. Only hypertensives were on RAAS inhibitors (31%) or statins (9%). Hypertensives also exhibited increased PWV, carotid IMT, and LVMI, consistent with subclinical vascular damage. Hypertensives showed altered lipid profiles, with significant reductions in polyunsaturated fatty acids (PUFAs) and very long-chain fatty acids (VLCFAs). Protective omega-3 and omega-6 fatty acids, including EPA, DHA, AA, and linoleic acid (C18:2), were significantly lower, reducing anti-inflammatory and vasoprotective effects. Reduced long-chain fatty acids may decrease endothelial protection and nitric oxide production, contributing to increased vascular elasticity. These reductions may result from low dietary intake, oxidative stress consumption, or altered lipid metabolism. Multivariate models showed that higher EPA

and DHA levels were associated with lower PWV, IMT, and LVMI, suggesting that chronic hypertension–related arterial and myocardial remodeling may be linked to their deficiency. VLCFA production depends on peroxisomal and mitochondrial activity. Peroxisomal dysfunction can reduce structural membrane lipids and increase oxidized lipids. Reduced VLCFAs were consistently associated with higher arterial stiffness, carotid thickness, and LVMI, indicating that altered peroxisomal metabolism may contribute to vascular and cardiac remodeling in hypertensives. Mean 7β-OHC levels were higher in hypertensives (20.90 ng/L) than normotensives (13.49 ng/L, $p<0.001$). 7-KC showed a trend ($p=0.074$), while 24OHC and 27OHC were variably associated. Adjusted multivariate analysis revealed that each increment in 7β-OHC was significantly associated with higher hypertension risk (OR 1.98; $p<0.001$), highlighting its potential as a biomarker. Derived from non-enzymatic cholesterol oxidation under oxidative stress, 7β-OHC promotes oxidative stress, endothelial apoptosis, and atherogenesis by acting as a cytotoxic and pro-inflammatory agent on vascular and endothelial cells. Reduced protective PUFAs may predispose membranes and LDL to oxidation, increasing oxysterols like 7β-OHC. Oxidized LDL (oxLDL) causally contribute to atherosclerosis initiation, progression, and plaque destabilization, inducing inflammation, apoptosis, vascular smooth muscle cell proliferation/migration, and endothelial activation, and likely also vascular aging. [84,85] Elevated 7β-OHC in hypertensives suggests its role both as an oxidative stress biomarker and a cardiovascular risk indicator. Our data show consistent associations between lipidomic alterations and oxysterol levels in hypertensives, particularly reduced EPA, DHA, AA, and increased 7β-OHC, 7-KC, and 24OH. These results support a pathogenic hypothesis: decreased protective omega-3 and omega-6 leads to increased oxidative stress, elevated 7β-OHC, endothelial dysfunction, and hypertension. Combined monitoring of oxysterols and lipid profiles may aid cardiovascular risk stratification and guide targeted nutritional interventions [52]. Limitations include a small sample size and cross-sectional biomarker measurements. Multivariate linear regression showed that higher long-chain fatty acid concentrations were associated with PWV, IMT, and LVMI both in normotensives and hypertensives. Associations in both groups suggest that the

metabolic–vascular axis operates even before overt hypertension [48]. Among all analytes, 7β-OHC was most strongly associated with organ damage, showing positive correlations with PWV, IMT, and LVMI, with stronger and more numerous associations in hypertensives [48,55].

FINAL DISCUSSION AND CONCLUSIONS

The doctoral training program was structured around a combination of theoretical and practical activities aimed at developing critical reasoning skills. The goal of the training path was to improve the scientific and methodological skills required for in-depth critical instruction. The interdisciplinary approach of the PhD in Environmental and Health Sciences and Technologies offered a wide range of educational and professional experiences, enriching the doctoral candidate's cultural and professional background from a multidisciplinary perspective. To increase the transversal knowledge required for a comprehensive education, other research projects were conducted in addition to the primary PhD study. By the end of the program, six papers had been published in international journals, along with contributions in national journals and scientific posters. The doctoral project, titled *Metabolomic and Lipidomic Studies in Degenerative and Aging Diseases*, aimed to identify serum biomarkers using metabolomic and lipidomic approaches. The goal was to enable early diagnosis, predict disease progression, and/or anticipate therapeutic response. Over the three years, multiple lines of research were pursued, focusing on lipid profiles and, in particular, oxysterols. Cholesterol is one of the most important chemical compounds in the human body. It is present in all cells, mainly as a structural element of lipid bilayers where it regulates membrane fluidity. Cholesterol is also a key substrate for bile acid synthesis, steroid hormones, vitamin D, and oxysterols. Oxysterols share almost the same structure as cholesterol and its precursor, 7-dehydrocholesterol, with an additional hydroxyl, epoxy, or ketone group. Oxidation occurs via enzymatic or non-enzymatic mechanisms. Enzymatically derived oxysterols include 27-hydroxycholesterol (27-OHC), 24(S)-hydroxycholesterol (24-OHC), 25-hydroxycholesterol (25-OHC), and 7α-hydroxycholesterol (7α-OHC). Non-enzymatically formed oxysterols include mainly 7α-OHC, 7β-hydroxycholesterol (7β-OHC), and 7-ketocholesterol (7-KC). The broad spectrum of oxysterols in the human body varies in origin and function. Those present in peripheral tissues and the central nervous system are essential for cholesterol metabolism and may participate in both physiological and pathological processes. Because disease can disrupt the oxysterol concentration balance

in body fluids, they are helpful diagnostic tools. Elevated oxysterol levels have shown promise for assessing coronary disease risk or diagnosis. Proper oxysterols may provide a rapid, simple, and non-invasive diagnostic method, even in early stages when conventional tests cannot detect pathology. Current data suggest that oxysterols are particularly useful for diagnosis and/or monitoring of neurodegenerative disease progression. During the doctoral program, one of our publications, *"The Diagnostic Use of the Plasma Quantification of 24S-Hydroxycholesterol and Other Oxysterols in Neurodegenerative Disease"* (Advances in Experimental Medicine and Biology, vol. 1440, 2024), explored the use of 24-OHC in neurodegenerative diseases. In approximately 40 patients with Alzheimer's disease (AD), non-Alzheimer degenerative dementia (NAD), and non-degenerative disorders, dementia biomarkers were measured in cerebrospinal fluid (CSF): total tau (t-Tau), phosphorylated tau (p-Tau181), and beta-amyloid 1-42 (Aβ42). Cut-offs were applied as follows: t-Tau >300 ng/L (ages 21–50), t-Tau >450 ng/L (ages 51–70), t-Tau >500 ng/L (ages >71), Aβ42 >500 pg/mL p-Tau181 <61 pg/mL. Cholesterol, its precursors, and oxysterols were quantified in CSF using gas chromatography and isotope-dilution mass spectrometry. Comparisons between AD and control groups revealed elevated concentrations of several oxysterols in AD and NAD patients. Non-enzymatic oxysterols (7α-OHC, 7β-OHC, 7-KC, α-epoxyC, β-epoxyC, triol) were 1.5–3.0 times higher than in controls (Figure 1). Enzymatic oxysterols (24OHC, 27OHC) were significantly increased by 1.5 and 1.4 times, respectively, in AD and NAD versus controls (Figure 20). 7α-OHC, 7β-OHC, 7-KC, α-epoxyC, and triol levels were significantly higher in AD compared to NAD (Figure 19). Cholesterol and its precursors (lanosterol, lathosterol, desmosterol) were also elevated in AD and NAD versus controls, with higher desmosterol contributing to increased cholesterol in AD CSF (Figure 21). These findings were published (Pradotto et al., Redox Experimental Medicine, 2024) and following the validation of the mass spectrometry techniques, future plans call for increasing the cohort and include investigation of the CSF phospholipid and fatty acid profiles.

Studies have shown increased dementia risk and amyloid deposition in heterozygous NPC1 mutation carriers. Recent work identified the first late-onset autosomal dominant

AD family caused by heterozygous NPC1 mutation. No effective treatments exist currently, but miglustat, a drug affecting oxysterol metabolism, has shown anti-amyloidogenic effects in human AD cell models. In an exploratory uncontrolled study, three family members were treated with miglustat for 12 months, and were undergoing monthly clinical evaluations, routine blood tests, neuropsychological assessments, amyloid PET imaging, and biochemical analysis of plasma and CSF. All patients achieved clinical stability, reduced serum oxysterol levels, and markedly decreased cerebral amyloid burden (Lopergolo et al., European Journal of Neurology, 2025). Preliminary observations support further investigation of miglustat in larger cohorts of heterozygous NPC1 AD patients and its potential as a disease-modifying therapy.

A study on 204 subjects (150 normotensive, 54 hypertensive) was also conducted. Literature indicates that both long-chain and very long-chain fatty acids, as well as oxysterols, can alter lipid profiles and contribute to hypertension development. In our cohort, metabolic markers, renal function, and organ damage indices were analyzed. Hypertensives exhibited altered lipid profiles, with significant reductions in polyunsaturated fatty acids (PUFAs) and VLCFAs, and a significant increase in 7β-OHC. Protective omega-3 and omega-6 fatty acids were particularly reduced.

Results confirm a link between metabolomic-lipidomic profiles and arterial hypertension. Lipid profile alterations were associated with both hypertension and signs of cardiovascular organ damage. These findings support a progressive model in which lipidomic and metabolomic changes may contribute to early cardiovascular risk stratification. In summary, these studies agree on the significance of an integrated approach that incorporates lipidomic, metabolomic, and clinical data. The findings highlight how clinical, metabolic, and pharmacological methods must be combined to create precision medicine that can prevent, diagnose, and cure complicated illnesses.

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