Oxysterols: implication in biological processes and diseases.

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OXYSTEROLS: IMPLICATION IN BIOLOGICAL PROCESSES AND DISEASES.

BEATRIZ GARRIDO CARMONA.

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RESUM

OXISTEROLS: IMPLICACIONS EN PROCESSOS BIOLÒGICS I EN MALALTIES.

El colesterol es distribueix per tot el cos i és necessari per la biosíntesis de les hormones esteroides, de sals biliars i pel manteniment de l'estabilitat de les membranes biològiques en cèl·lules animals. La seva regulació és garantida per l'acció conjunta d'uns factors/enzims/receptors específics com són SREBP, HMG-CoA o LXR. LDL i HDL s'encarreguen del transport i de proveir a les cèl·lules per la construcció i manteniment de les membranes, entre d'altres funcions. El colesterol, així com els lípids en general, és més susceptible a l'oxidació en algunes posicions, degut a la seva estructura química, de patir aquest atac oxidatiu; els derivats del colesterol més comuns produïts per aquest atac oxidatiu són de tipus hidroxil, keto, hidroperòxids, epòxids i derivats de carboxils. Aquest derivats del colesterol són coneguts amb el nom d'oxisterols o òxids de colesterol.

Aquesta oxidació és deguda a processos d'autooxidació en els aliments que han estat exposats a tractaments de calor en presència d'oxigen o bé que han estat emmagatzemats durant llargs períodes de temps o exposats a la llum del sol i a l'oxigen. Aquest procés d'autooxidació constitueix el que es coneix com a font exògena d'oxisterols que poden ser ingerits a través de la dieta. Per una altra banda, l'oxidació del colesterol pot ocasionar-se per l'acció d'uns enzims que pertanyen a la família del citocrom P450, constituint, conjuntament amb els processos d'autooxidació que es poden donar dins el propi cos, amb la font d'oxisterols endògena.

Els oxisterols es caracteritzen per posseir una àmplia diversitat d'activitats biològiques, com són els efectes en el metabolisme dels esfingolípids, l'agregació plaquetària, l'apoptosis, la citotoxicitat, la seva unió a transportadors específics, la seva relació amb la ruta Hedgehog, la unió a receptors d'estrògens i la prenilació de proteïnes. L'activitat més destacada dels oxisterols es relaciona amb la regulació del colesterol, que sembla estar controlada, si més no en part, per una sèrie complexa d'interaccions dels oxisterols amb varis receptors com els lligands d'oxisterols, les proteïnes d'unió als àcids nucleics, les proteïnes d'unió als elements reguladors d'esterols, els receptors nuclears orfes LXR i els receptors de lipoproteïnes de baixa densitat.

Es sospita que els oxisterols poden estar implicats en la homeòstasi del colesterol a més de jugar un paper important en la progressió de malalties neurodegeneratives i en la carcinogènesis, especialment per la seva interacció amb mecanismes de mort cel·lular (apoptosis) i degut a la seva relació amb processos inflamatoris.

Degut a les seves propietats biològiques i la seva implicació en processos sistèmics importants, així com en l'etiologia o patofisiologia de certes malalties, aquesta revisió ofereix alguns aclariments del rol que juguen els oxisterols en la homeòstasi del colesterol, i al mateix temps ofereix alguns coneixements del seu rol directe o indirecte en la patofisiologia de l'Alzheimer i el Càncer, així com si seria possible la seva utilització com a dianes terapèutiques.

La investigació bibliogràfica condueix a concloure que, actualment, es sap que els oxisterols no són els únics reguladors en l'homeòstasi del colesterol tot i que sí exerceixen un paper regulador rellevant en el procés. I per una altra banda, s'ha demostrat que estan involucrats en processos relacionats amb l'inici i/o la progressió de malalties neurodegeneratives tals com l'Alzheimer, a més de en la carcinogènesis. En base a tot això, els

oxisterols podrien representar una gran revolució en el camp de la biomedicina com a potencials dianes directes o indirectes per a la seva utilització terapèutica. No obstant, és necessari que es facin més experiments *in vivo* per tal de poder traduir quin efecte real tenen les seves activitats en humans i poder decidir quina estratègia podria aplicar-se per desenvolupar un tractament potencial en contra de certes malalties.

RESUMEN

OXISTEROLES: IMPLICACIÓN EN PROCESOS BIOLÓGICOS Y EN ENFERMEDADES.

El colesterol se distribuye por todo el cuerpo y es necesario para la biosíntesis de las hormonas esteroideas, las sales biliares y para el mantenimiento de la estabilidad de las membranas biológicas en las células animales. Su regulación se ve garantizada por la acción conjunta de unos factores/enzimas/receptores específicos como SREBP, HMG-CoA o LXR. LDL y HDL se encargan del transporte y de proveer a las células para la construcción y mantenimiento de las membranas, entre otras funciones. El colesterol, como los lípidos en general, es susceptible a la oxidación en algunas posiciones en su estructura química de padecer el ataque oxidativo; los derivados del colesterol más comunes producidos por este ataque oxidativo son del tipo hidroxilo, keto, hidroperóxido, epóxido y derivados de carboxilos. Estos derivados del colesterol se conocen como oxisteroles u óxidos de colesterol.

Esta oxidación se debe a procesos de autoxidación en alimentos que han estado expuestos a tratamientos de calor en presencia de oxígeno o bien han sido almacenados por largos períodos de tiempo o han sido expuestos a la luz del Sol junto con el oxígeno. Este proceso de autoxidación constituye lo que se conoce como fuente exógena de oxisteroles, que pueden ser ingeridos a través de la dieta. Por otro lado, la oxidación del colesterol puede estar ocasionada por la acción de unas enzimas que pertenecen a la familia del citocromo P450, constituyendo, de forma conjunta con los propios procesos de autoxidación que se dan dentro del cuerpo, la fuente endógena de oxisteroles.

Los oxisteroles se caracterizan por poseer una amplia diversidad de actividades biológicas, como los efectos en el metabolismo de los esfingolípidos, la agregación plaquetaria, la apoptosis, la citotoxicidad, su unión a transportadores específicos, su relación con la vía Hedgehog, la unión a receptores de estrógenos y la prenilación de proteínas. La actividad más destacada de los oxisteroles se relaciona con la regulación del colesterol, que parece estar controlada, en cierto modo, por una serie compleja de interacciones de los oxisteroles con varios receptores como son sus propios ligandos, las proteías de unión a los ácidos nucleicos, las proteínas de unión a los elementos reguladores de los esteroles, los receptores nucleares huérfanos LXR y los receptores de lipoproteínas de baja densidad.

Se sospecha que los oxisteroles pueden estar implicados en la homeostasis del colesterol, además de jugar un papel importante en la progresión de enfermedades neurodegenerativas y en la carcinogénesis, especialmente por su interacción con mecanismos de muerte celular (apoptosis) y debido a su relación, también, con procesos inflamatorios.

Por las propiedades biológicas que poseen y su implicación en procesos sistémicos de gran importancia, así como en la etiología o patofisiología de algunas enfermedades, esta revisión ofrece algunas aclaraciones del rol que juegan estos oxisteroles en la homeostasis del colesterol y, al mismo tiempo, ofrece algunos conocimientos de su rol directo o indirecto en la patofisiologia del Alzheimer y el Cáncer, además de plantear si sería posible su utilización como dianas terapéuticas.

La investigación bibliográfica nos lleva a concluir que, actualmente, se sabe que los oxisteroles no son los únicos reguladores en la homeostasis del colesterol aunque sí es cierto que ejercen un papel regulador relevante en el proceso. Y, por otra parte, se ha demostrado que están involucrados en procesos relacionados con el inicio y/o la progresión de enfermedades neurodegenerativas como el Alzheimer, además de en la carcinogénesis. En base a todo esto, los oxisteroles podrían representar una gran revolución en el campo de la biomedicina como potenciales dianas directas o indirectas para uso terapéutico. No obstante, son necesarios más experimentos *in vivo* para poder así traducir sus efectos reales en humanos y poder decidir qué estrategia podría aplicarse para desarrollar un tratamiento potencial para ciertas enfermedades.

ABSTRACT

OXYSTEROLS: IMPLICATION IN BIOLOGICAL PROCESSES AND DISEASES.

Cholesterol is ubiquitous and necessary for the biosynthesis of steroidal hormones, bile salts and to maintain the stability of biological membranes in animal cells. Its regulation and homeostasis is ensured by the conjugated action of specific factors/enzymes/receptors such as SREBP, HMG-CoA or LXR. LDL and HDL are responsible to transport cholesterol and provide the cells to build and maintain the membranes, etc. Cholesterol, as lipids in general, is susceptible to oxidation and some positions in the chemical structure are more prone to oxidative attack; the most common cholesterol derivatives produced by oxidative attack are hydroxyl derivatives, keto derivatives, hydroperoxydes, epoxides and carboxyl derivatives. Those oxidized cholesterol derivatives are called Oxysterols or Cholesterol Oxides.

Cholesterol oxidation occurs by autoxidation processes in dietary food exposed to heating treatments in the presence of oxygen or have been stored for long periods and exposed to sunlight and oxygen. This autoxidation process constitutes an exogenous source of oxysterols i.e. they can be ingested from the diet. On the other hand, cholesterol oxidation can be mediated by enzymes belonging to the family of cytochrome P450 of oxygenases and autoxidation processes are also possible inside the body constituting both the endogenous source of oxysterols.

Oxysterols are characterized by a diverse profile of biological activities, including effects on sphingolipid metabolism, platelet aggregation, apoptosis, cytotoxicity, binding to specific transporters, relation with the Hedgehog pathway, binding to the oestrogen receptor and protein prenylation. The most notable oxysterol activity is related to the regulation of cholesterol homeostasis, which appears to be controlled in part by a complex series of interactions of oxysterols with various receptors, such as the oxysterol binding protein, the cellular nucleic acid binding protein, the sterol regulatory element binding protein, the LXR nuclear orphan receptors, and the low-density lipoprotein receptor.

Oxysterols are suspected to be involved in cholesterol homeostasis but also seem to play a role or at least are involved in the progression of neurodegenerative diseases and in carcinogenesis especially due to their interaction with cell death mechanisms (apoptosis) and because of their relationship with inflammatory processes.

Because of their relevant biological properties and their implication in important systemic processes and in the etiology or pathophysiology of certain diseases, this work provides clarifications on the role of Oxysterols in cholesterol homeostasis and provides insights on their direct or indirect role in the pathophysiology of Alzheimer's disease and Cancer as well as if these could represent a direct or indirect target for therapeutic action.

The bibliographical investigation led to conclude that, currently, it is known that oxysterols are not the sole regulators of cholesterol homeostasis, although they are significant players in the regulation of this process. On the other hand, it has been demonstrated that they are involved in processes related to the onset and/or progression of neurodegenerative diseases such as Alzheimer, and with carcinogenesis. Because of this, Oxysterols could represent a big revolution in the field of biomedicine as potential direct or indirect targets for therapeutic action, but there is still the need of more *in vivo* experiments to translate their activities in humans and before deciding which strategy is applicable to develop a potential disease treatment.

1. INTRODUCTION

Oxysterols, or cholesterol oxidation products (COPs), are oxygenated derivatives of cholesterol which are formed endogenously in the first steps of cholesterol metabolism by non-enzymatically processes or by enzymes which belong to the microsomal cytochrome P450. In addition, oxysterols may also be absorbed as esters from the diet and further transported in the plasma by chylomicrons or by lipoproteins. COPs are localized in the membranes and also in lipoproteins at trace levels, though they can exert profound biological effects. However, they are always accompanied by a great excess (as much as 10^6-fold) of cholesterol (Otaegui-Arrazola *et al*. 2010).

Oxysterols play important roles in signalling and cell development through regulation of Hedgehog signalling pathway, which is important in determining embryonic patterning and cell fate in multiple structures of the developing embryo, and oestrogen receptor function. Furthermore, oxysterols are potent bioactive lipids that "regulate" lipid metabolism by controlling a number of transcription factors, immune function and cytotoxicity. These functions are mediated by specific oxysterol sensors, including liver X receptors (LXR) which are responsible of the transcriptional regulation of cholesterol adsorption and cellular efflux, cholesterol and bile acid synthesis, neutral lipid secretion into bile, inflammation and immune response, insulin induced genes (Insigs) which is implicated in regulation of SREBP maturation; cholesterol and fatty acid biosynthesis and LDL receptor expression, Oxysterol-binding proteins (OSBP) and their related proteins called ORP (Shibata & Glass 2010). Most of the oxysterol effects are mediated through their interactions with cytoplasmic ORP family members (Zhou 2013).

Oxysterols levels are increased in plasma of patients with cardiovascular diseases and in atherosclerotic lesions. They are also suspected to be involved in degenerative diseases such as Alzheimer's disease, osteoporosis, and age-related macular degeneration and also implicated with certain types of cancer, such as breast and prostate cancer (Pommier *et.al.* 2013).

2. HYPOTHESIS AND OBJECTIVES

Hypothesis

1st hypothesis: Cholesterol homeostasis is regulated by oxysterols.

Based on largely indirect evidence, and in spite of their low levels in vivo, oxysterols are generally believed to be important physiological mediators of cholesterol-induced effects.

2nd hypothesis: Oxysterols and/or the Binding Proteins mediating their intracellular activity could be identified as potential direct or indirect targets for the treatment of Alzheimer's disease and Cancer.

This idea is supported by the properties of these cholesterol metabolites, including their activity in the inflammatory responses, as immune modulators, and their cytotoxicity attributable to their ability to induce apoptosis.

Objectives

1. To understand the physiological processes related with cholesterol homeostasis and the relationship between oxysterols and cholesterol itself.
2. To relate oxysterols functions or properties with the pathophysiology of Alzheimer's disease and Cancer and explore the hypothesis of oxysterols being a direct or indirect direct for therapeutic treatment.

3. REVIEW OF THE LITERATURE

Lipids are small hydrophobic molecules that carry out a multitude of crucial roles as forming the bilayer barrier of cells and organelles and further contribute to the intrinsic properties of membranes such as thickness, permeability, asymmetry and curvature; they store energy and they function as signaling molecules in cellular response pathways. In mammalian cells, lipids are divided into three major groups: glycerolipids, sphingolipids and sterols (Breslow, 2013/ C.Champe *et.al.* 2007). The absorption and integration of sterols, the group of interest for this review, is shown in the figure 1. Disturbances of lipid homeostasis can provoke a variety of cellular responses, and further trigger life-threatening metabolic diseases. It is hence important to understand lipid signalling, transport and related cellular processes, because there are related to contribute to a variety of diseases such as cancer, atherosclerosis, hypertension, type 2 diabetes and cardiovascular diseases. In addition, increased lipid signalling is suggested to play a role in the development of tumors from benign to malignant stage (Wymann & Schneiter. 2008 / You Zhou 2013).

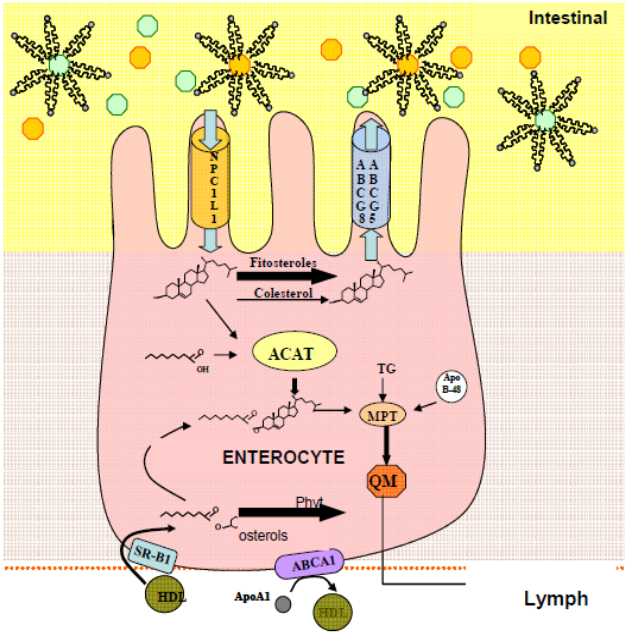


Fig. 1: Sterol and probably oxysterol absorption, esterification and incorporation into chylomicrons. NPC1L1: lipid micelle transporter; ABC: ATP Binding Cassette transporters; ACAT: Acyl-coA Cholesterol Acyl Transferase; TG: Triglycerides; MPT: Microsomal TG transfer protein; QM: Chylomicrons; HDL: High Density Lipoprotein; SR-B1: Scavenger Receptor type B. [Source: Menéndez-Carreño, 2009.]

Cholesterol belongs to the group of sterols, a subgroup of steroids. Based on a planar four-ring structure, different sterols have similar physical properties and intracellular distribution (Christie, W 2014).

3.1 Cholesterol

According to Escurriol and his co-workers, it is healthy to take cholesterol in range of 150-450mg/day; it is found in animal food products such as eggs, milk, cheese, red meat… (Escurriol *et al*., 2010). Systemic healthy cholesterol levels ranges from 180mg/dL - 200 mg/dL. Higher levels of blood cholesterol are associated with a higher risk of cardiovascular diseases (American Heart Association).

3.1.1 Definition and location

Cholesterol is ubiquitous, where much of it is located in the membranes, and necessary for the viability of mammalian cells and maintenance of the permeability barrier function of the plasmatic membrane Furthermore, it is implicated in the generation of steroid hormones and bile acids production. The highest proportion (60-80%) of un-esterified cholesterol occurs in the plasma membrane, while mitochondria and the endoplasmic reticulum have very low cholesterol contents, and the Golgi contains an intermediate amount. Cholesterol is also enriched in early and recycling endosomes, but not in late endosomes. (C.Champe, 2007/ Christie, W.W. 2014).

3.1.2 Regulation – Cholesterol homeostasis

Cellular cholesterol levels are largely maintained through the activity of two transcription factors: the sterol regulatory element binding protein (SREBP1 and SREBP2) and the liver X receptors (α and β).

SREBP is produced in the endoplasmic reticulum (ER) and controls the expression of genes involved in cholesterol synthesis. It is bound to the SREBP-cleavage activating protein (SCAP) which is like a cholesterol sensor; if cholesterol levels are low, SCAP releases SREBP and the gene transcription is stimulated. However, when cholesterol levels are high, Insig protein retains SCAP-SREBP complex in ER and the gene transcription is inhibited. Moreover, cholesterol production may be stopped by the cholesterol binding to SCAP, when cholesterol levels are increased in ER (Cenipalma, & Humana, 2001/ Hui, D. Y., & Howles, P. N. 2005).

There are two isoforms of nuclear receptors called liver X receptors (LXRs) in mammals, LXRα and LXRβ. LXRs form heterodimers with retinoid X receptor (RXR), a common partner for several nuclear receptors such as peroxisome proliferator activated receptors (PPARs) and vitamin D receptor (Zhou 2013). LXRα/β are regulators of several metabolic pathways, including lipid and cholesterol metabolism, as well as attenuating inflammation in immune cells through their ability to directly repress inflammatory gene expression through a process called transrepression. When excess oxysterols (e.g., 22-(R) and 25-hydroxycholesterol) are accumulate in the cell, they can directly bind to and activate LXRs, resulting in transcriptional up-regulation of genes involved in cholesterol efflux (Harris, J. R., 2010/ Hui, D. Y., & Howles, P. N. 2005).

As an important constituent of the plasma membrane, cholesterol is necessary for the cell and can be provided by the diet or by an endogenous cellular synthesis. Exogenous cholesterol is transported to the target tissues by the major cholesterol carrying low-density lipoprotein (LDL) and delivered to the cells via LDL receptor binding and endocytosis. LDL transport cholesterol around the body and when cholesterol levels exceed the healthy range give way for the cholesterol deposition and atheromatous plaques are formed, that can trigger atherosclerosis. This is why is called "bad cholesterol".

Meanwhile, high-density lipoprotein (HDL) transport cholesterol from peripheral tissues to liver, preventing cholesterol deposition in veins and arteries, so it is commonly called "good cholesterol." (American Heart Association). When the cell needs for cholesterol are satisfied, cholesterol inhibits its *de novo* synthesis, so LDL/HDL are another way to regulate cholesterol levels. This homeostasis is achieved through negative feedback regulation of the HMG-CoA-synthase (the first enzyme involved in the cholesterol biosynthetic pathway), the HMG-CoA-reductasa and the LDL receptor synthesis (Castro, J. J. L. (2013). HMG-CoA reductasa, a resident ER enzyme important in controlling the rate of sterol biosynthesis, is the one of the proteins expressed when SREBP reaches the nucleus for gene transcription. It catalyses the conversion of HMG-CoA to mavelonate, one of the steps in cholesterol synthesis.

Cholesterol esters are another form of cholesterol and are part of the regulation process of cholesterol. They are major constituents of the adrenal glands, and they accumulate in the fatty lesions of atherosclerotic plaques. As cholesterol esters accumulate in the core of the lipoprotein, cholesterol is removed from its surface thus promoting the flow of cholesterol from cell membranes into HDL. Subsequently, cholesterol esters are transferred to the other lipoprotein fractions LDL and very low-density lipoprotein (VLDL), a reaction catalysed by cholesteryl ester transfer protein. VLDL transport endogenous lipids (TAG) around the body. This process promotes the efflux of cholesterol from peripheral tissues ('reverse cholesterol transport'), especially from macrophages in the arterial wall, for subsequent delivery to the liver. Lecithin—cholesterol acyltransferase (LCAT) is often stated to be the main driving force behind this process, and it is of great importance for cholesterol homeostasis and a suggested target for therapeutic intervention against cardiovascular disease.

In other animal tissues, a further enzyme acyl-CoA:cholesterol acyltransferase (ACAT) synthesizes cholesterol esters from CoA esters of fatty acids and cholesterol. ACAT exists in two forms: ACAT1 is present in many tissues, but especially in macrophages and adrenal and sebaceous glands, which store cholesterol esters in the form of cytoplasmic droplets. It is also responsible for the synthesis of cholesterol esters in arterial foam cells in human atherosclerotic lesions. ACAT2 is found only in the liver and small intestine, and it is believed to be involved in the supply of cholesterol esters to the nascent lipoproteins (Christie, W.W. 2014/ Pramfalk *et.al.* 2012).

A major part of cholesterol is known to bypass the vesicle transport through the Golgi apparatus. The key regulators involved in vesicular trafficking are Rab GTPases that provide compartmental specificity for membrane trafficking while vesicle fusion is mediated by soluble N-ethylmaleimide sensitive factor attachment receptors (SNAREs) (You Zhou, 2013).

3.1.3 Biological importance

Cholesterol is required to build and maintain membranes, decreasing their fluidity and permeability and promoting the formation of special liquid-ordered microdomains that are known as lipid rafts.

It has vital structural roles in membranes and in lipid metabolism in general. It is a biosynthetic precursor of bile acids, vitamin D and steroid hormones (glucocorticoids, estrogens, progesterones, androgens and aldosterone). In addition, it contributes to the development and working of the central nervous system, and it has major functions in signal transduction and sperm development. It is found

in covalent linkage to specific membrane proteins or proteolipids ('hedgehog' proteins), which have vital functions in embryonic development. (Christie, W.W., 2013).
When increased levels of sterols other than cholesterol are found in plasma, they usually serve as markers for abnormalities in lipid metabolism associated with disease states. (W.W. Christie, 2013). For example, elevated cholesterol and cholesterol ester levels are associated with the pathogenesis of cardiovascular disease (atherosclerotic plaques, myocardial infarctions, and strokes).

3.1.4 Oxidation

Like lipids, cholesterol is prone to oxidation, either through a process called autoxidation or by enzymatic processes. The oxidation of cholesterol produces primary by-products called oxysterols or cholesterol oxides. Oxysterols like cholesterol can be absorbed from the diet (Vaya, *et al*. 2011).

3.2 Cholesterol oxides (Oxysterols)

3.2.1 Chemistry

Cholesterol is an aliphatic molecule (cholest-5-en-3β-ol) and consists of a tetracyclic cyclopental[a]phenanthrene structure (Fig 2) with an *iso-octyl* side-chain at carbon 17. The four rings (A, B, C and D) have *trans* ring junctions, and the side chain and two methyl groups (C-18 and C-19) are at an angle to the rings above the plane with β stereochemistry. There is a double bond between carbons 5 and 6. Due to the presence of this double bound, sterols can undergo oxidative processes (Maraschiello, 1998).

This oxidation results in the formation of oxysterols or cholesterol oxidation products.

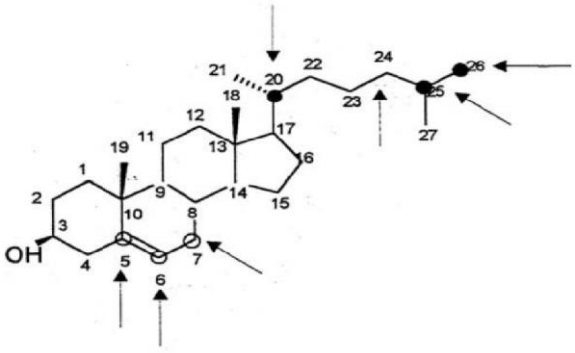


Fig. 2 Susceptible positions for the oxidative attack of the cholesterol molecule. 20C and 25C of the lateral aliphatic chain are the most susceptible positions.

The common modifications of cholesterol occurring in oxysterols are hydroxyl, keto, hydroperoxy, epoxy and carboxyl moieties.

3.2.2 Origins

An extensive study about the world of oxysterols (Otaegui-Arrazola *et. al.* 2010) have clarified the different sources of oxysterols in plasma: endogenous and exogenous. The exogenous one is related with the absorption of oxysterols from the diet. And the other one includes the enzymatic and the non-enzymatic (provoked by radical mechanism) transformation of sterols *in vivo.* The non-enzymatic mainly affect the sterol ring while the enzymatic one react in the side chain of sterol structures (Ryan *et. al*. 2005). But there are exceptions: 25-HC and 7α-HC can be generated by both metabolic pathways. There are some oxysterols structures and their origins represented in the figure 3.

3.2.2.1 Non-enzymatic oxidation

By definition, autoxidation is a chemical reaction between an organic compound and atmospheric oxygen (Maraschiello, 1998) and it includes free radical, lipid peroxide, or divalent cation-induced oxidative processes.

Cholesterol is attacked by reactive oxygen species (ROS) abstracting an allylic hydrogen atom at C-7 of the sterol ring. The radical generated can react with oxygen to form a cholesterol peroxyl radical, which further reacts abstracting a hydrogen and generates the relatively stable cholesterol 7α/β- hydroperoxides. At this point, hydroperoxides may continue oxidizing non-enzymatically or enzymes will reduce them to epoxycholesterols. The non-enzymatic oxidation generates 7 α/β-HC and 7-KC, which are the major non-enzymatic oxysterols present in most tissues (Brown and Jessup, 2009).

All foods containing cholesterol are susceptible to oxidation before entering the organism; especially those which have been exposed to heating treatments in the presence of oxygen or have been stored for long periods subjected to sunlight and oxygen. Cholesterol in dry state is resistant to autoxidation in air when low or moderate temperatures (80ºC) are used, but higher temperatures (180ºC) drastically increase the oxidative degradation of cholesterol to 7-ketocholesterol as a major product (Kim and Nawar, 1993). The fact of resuspend cholesterol in water and heated at 80ºC caused the production of the C7-derivatives and the epoxides (Kim and Nawar, 1993). 7-ketocholesterol, 7β-hydroxycholesterol, β-epoxide and cholestanetriol were unstable at high temperature and β-epoxide was shown to be more labile than its α-isomer (Kim and Nawar, 1993). Cholesterol in the presence of unsaturated fatty acids in models systems oxidized more rapidly and high temperatures favoured the production of ketones. Increasing the heating time raised the production of oxysterols at constant temperature (Maraschiello, 1998).

Finally, acidic pH increased the production of 7-ketocholesterol from the 7-hydroperoxides due to the more favoured dehydration of the hydroperoxides (Kim and Nawar, 1993). B-epoxide was found to be more labile than α-epoxide at acidic pH (Maraschiello, 1998).

Formation of oxysterols in animal products can be minimized by application of low processing temperatures, use of oxygen-proof packaging and a protective atmosphere, by low-temperature and light-free storage, and/or dietary addition of antioxidant to animal feed or antioxidant addition to foods (Otaegui-Arrazola *et. al*. 2010).

3.2.2.2 Enzymatic oxidation

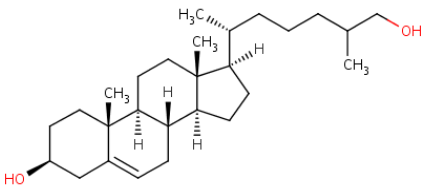
Buege and Aust defined that as microsomal lipid peroxidation observed in the presence of NADPH, ADP and iron mostly used as Fe^{3+} salts (Maraschiello, 1998). The microsomes are a

mixture of vesicles from the endoplasmic reticulum and the plasma membrane. The initiation of lipid peroxidation is due to the production of the superoxide radical O_2^- (one electron reduction of molecular oxygen) by e.g. the microsomal NADPH-cytochrome P450 reductase: O_2^- reduces Fe^{3+} to give Fe^{2+} which can decompose H_2O_2 into the very reactive OH leading to the initiation of lipid peroxidation. Moreover, during the handling of microsome preparation, a few hydroperoxides can be formed. Natural nutrients such as α-tocopherol (Vitamin E), β-carotene and ascorbic acid (Vitamin C) can prevent the formation of lipid hydroperoxides and are called chain-breaking antioxidants (Maraschiello 1998).

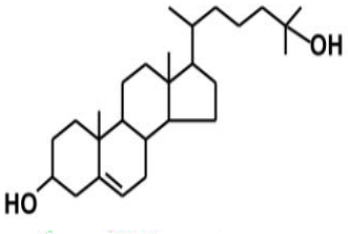
The hepatic microsomal 7α-hydroxylase synthesizes 7α-hydroxycholesterol from cholesterol and serves as a precursor in the bile acid synthesis pathway (Russell and Setchell, 1992). A 26-hydroxylase activity was found in liver, kidney, ovarian, endothelium, mitochondria and so forth (Smith, 1987; Smith, 1996). In the baboon, it seemed that the hepatic 26-hydroxylase was induced by dietary cholesterol (Hasan & Kushwaha, 1993)

Sterol 27-hydroxylase (CYP27A1) and cholesterol 24-hydroxylase (CYP24A1) are P450 enzymes expressed in liver and macrophages, and neural cells of the brain and retina, respectively (Björkhem *et. al*., 1998; Brown and Jessup, 2009) They catalyse the hydroxylation reactions to form 27- and 24 –HCs. Cholesterol 25-hydroxylase (Ch25h) is the enzyme responsible for generating 25-HC and it is expressed at very low levels. Nevertheless, it is interesting, since its product (25-HC) regulates the sterol regulatory element binding protein (SREBP) for cholesterol synthesis. 24-, 25- and 27- HCs are generated by enzymatic side-chain hydroxylation of cholesterol (Rozner and Garti, 2006).

However, oxysterols are also produced as intermediates in the biosynthesis of bile acids or steroid hormones. In terms of their systemic presence, the most abundant oxysterols in the human serum are 24(S)-, 27-, 7α-, and 4β- hydroxycholesterol (OHC), which are generated by reactions catalysed by mitochondrial or ER cholesterol hydroxylases belonging to the cytochrome P450 family (Olkkonen, 2009).



26-hydroxycholesterol*



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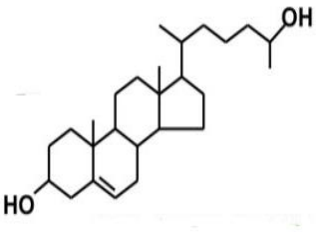
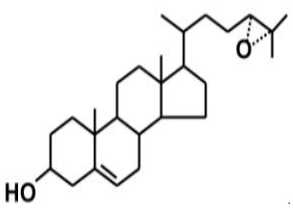
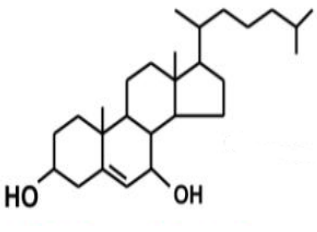


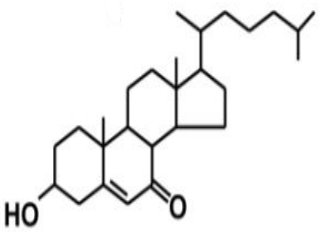
27-hydroxycholesterol *



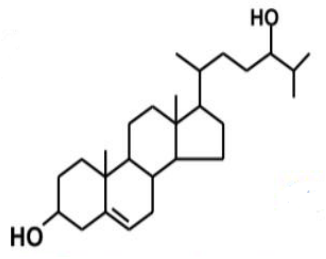
24(S),25-epoxycholesterol *



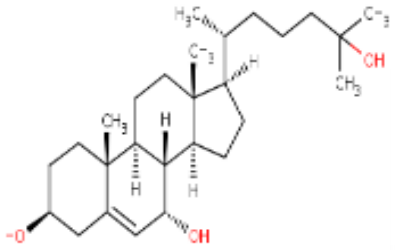
7β-hydroxycholesterol**



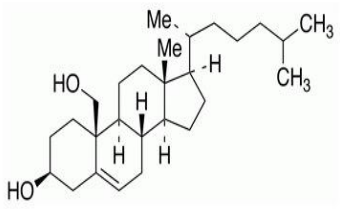
7-ketocholesterol**



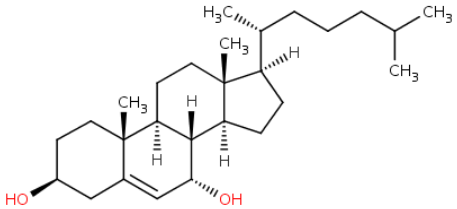
24(S)-hydroxycholesterol*



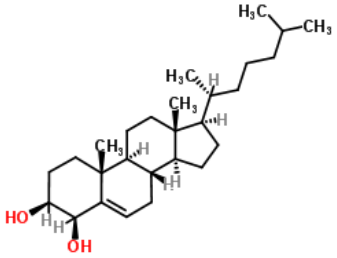
7α,25-dihydroxycholesterol



19-hydroxycholesterol



7α-hydroxycholesterol*



4β-hydroxycholesterol*

Fig. 3 Some oxysterols structures which are related with Cancer or Alzheimer disease or with cholesterol homeostasis. *Oxidized enzymatically. **Oxidized by auto-oxidation. Underlined means those oxysterols can be absorbed from the diet (exogenous source).

3.2.3 Absorption, transport and excretion

Oxysterols are more lipophilic than cholesterol and easily pass membranes and the blood brain barrier (Björkhem *et al.*, 2002; Björkhem, 2013). Compared to cholesterol, dietary or biliary excreted oxysterols are absorbed more quickly in the intestine, have faster plasma clearance and are quickly collected by tissues. The major oxysterols in the circulation are transported and distributed by lipoproteins (Björkhem *et al.*, 2002).

Once the oxysterols enter the enterocyte, they can follow different routes:

- Return to the intestine by ABCG5 and ABCG8.

  Part of the cholesterol is secreted back into the intestinal lumen by the transporters ABCG5 and ABCG8 which are a subfamily of ATP-binding cassette transporters. Sterol efflux activities of ABCA1 and ABCG1 modulate macrophage expression of inflammatory cytokines and chemokines as well as lymphocyte proliferative responses (Yvan-charvet *et. al*. 2010).

- Esterification and distribution.

Most oxysterols measured in plasma and tissues are esterified (Staprans *et. al.,* 2003), suggesting that they are good substrates for ACAT in cells and LCAT in circulation (Gill *et al.*, 2008). Once the ACAT in the enterocyte reacts with oxysterols, they are incorporated to chylomicrons and later to Very Low Density Lipoproteins (VLDL), Low Density Lipoproteins (LDL) and High Density Lipoproteins (HDL) (Staprans *et al.,* 2003; 2005). Hence, oxysterols can be transported to different cells of the organism. They can be also transported by albumin.

- Oxysterols can be metabolized or degraded to other compounds, mainly in the liver.

Cholesterol sulfotransferase enzyme (SULT2B1b) usually sulfates the 3β-hydroxil group of cholesterol, but oxysterols also have been found to be substrates of this enzyme (Fuda *et al*., 2007). SULT2B1b is expressed in retina, skin, platelets, liver and other tissues; hence, this route could be an important oxysterol excretion pathway (Higashi *et al*., 2004; Yanai *et al*., 2004; Fuda *et al*. 2007).

Most of the oxysterols can be only eliminated from cells through specific membrane lipid transporters as a consequence of their hydrophobicity. Some ATP-binding cassette transporters are involved in oxysterol excretion, such as ABCA1 and ABCG1 are mainly located in the macrophages and liver to transport oxidized sterols and other molecules out of the cell (Brown & Jessup, 2009).

3.2.4 Biological importance

The first oxysterols were found in atheromatous plaques, suggesting the implication of oxysterols in atherogenic processes (Brooks *et. al*., 1966). COPs present a diverse profile of biological activities, including effects on sphingolipids metabolism, platelet aggregation, apoptosis, and protein prenylation. They also play an important role in the cholesterol homeostasis, which appears to be controlled in part by a complex series of interactions of oxysterol ligands with various receptors, such as the oxysterol binding protein, the cellular nucleic acid binding protein, the sterol regulatory element binding protein (SREBP), the LXR nuclear orphan receptors, and the low-density lipoprotein receptor (Schroepfer Jr, 2000) Figure 7 collect all oxysterol functions as a summary of their activities.

Because of their properties, they are implicated in the etiology of disease states including atherosclerosis (a chronic inflammatory disease as well as a disorder of lipid metabolism), neurodegenerative, inflammatory diseases and Cancer.

3.2.5 Biological activities of oxysterols

1. Cytotoxicity

Different oxysterols have been shown to be cytotoxic to *in vitro* cultured fibroblasts, monocyte/macrophages, endothelial cells and smooth muscle cells (Smith and Johnson, 1989). Cytotoxicity has been demonstrated for all the B-ring cholesterol oxides, the 25-hydroxycholesterol and the epimeric cholesterol hydroperoxides (Smith and Johnson, 1989; Smith, 1996). One mechanism responsible for this cytotoxicity is the insertion of cholesterol

oxides into the plasma cell membrane, largely demonstrated by incubating cultured cells with oxysterols (Smith and Johnson, 1989; Maraschiello, 1998).

Because of their homology, oxysterols are also inserted in the membrane and depending on the composition of the phospholipid matrix, they may induce or inhibit membrane permeability (Sottero *et. al.*, 2009). The presence of oxysterols (highly membrane soluble) into the plasma membrane, affects the phase transition behaviour of plasma membrane and induce local disordering effects. The oxidation of membrane cholesterol can also decrease the ratio of cholesterol to phospholipids and leads to cell lysis. The consequence of such physical changes is an increased plasma membrane permeability toward calcium and sodium cations, inhibition of the hexose transport and inhibition of the Na+, K+ - and Ca^{2}+, Mg^{2}+- ATPase. The increase in intracellular calcium could activate several Ca^{2}+ - dependent proteins and activate mechanisms leading to cell death. Indeed, 7β-hydroxycholesterol, 7-ketocholesterol and 25-hydroxycholesterol have been shown to induce apoptosis of smooth muscle cells, human monocytes and thymocytes. (Maraschiello, 1998). 7α- and 7β-HC, 7-KC, 20α- and 25-HC were found to be potent inhibitors of cellular sterol biosynthesis. This inhibition is accompanied by the suppression of DNA synthesis, inhibition of cell growth and decrease in the molar ratio of cholesterol to phospholipids with further consequences on membrane permeability. (Maraschiello, 1998). 7KC, 7βOHC. And 24OHC, could favour neurodegeneration by their abilities to induce mitochondrial dysfunctions, oxidative stress, and cell death, associated with increases in cytosolic calcium levels (Zarrouk *et. al* 2014).

2. Oxysterols are implicated in the Hedgehog signalling pathway

The Hedgehog (Hh) signalling pathway plays a fundamental role in embryonic development, physiological processes and stem-cell renewal. Certain oxysterols, such as 20(S)-and 22(S)-OHC induce the osteoinductive effects through the activation of Hh signalling in pluripotent mesenchymal cells.

Exogenous oxysterols have been shown to be strong agonist of the cancer-related Hedgehog (Hh) signalling pathway (Hanahan, D., & Weinberg, R. a. (2011). Nevertheless, endogenous 24-OHC and 27-OHC are associated with Hh activity in a dosage and time dependent matter when cells are treated with Hh/Smoothened (SMO) antagonist (Olkkonen, 2012).

3. Oxysterols regulate oestrogen receptor function

ER-α/β are members of nuclear hormone superfamily that mediate a number of physiological processes. Oestrogens exert protective effects on the blood-vessel wall, regulating the cardiovascular system. It has been found that 27-OHC directly antagonizes the transcriptional and non-transcriptional functions of the oestrogen receptors, resulting in a loss of the cardioprotective function of oestrogen. This oxysterol act as an endogenous selective oestrogen receptor modulator based on its cell-type specific proestrogenic action (Zhou 2013).

4. Oxysterols and cholesterol homeostasis

Several oxysterols are associated with the cholesterol homeostasis through different mechanisms becoming regulatory factors, such as:

a) Sterol regulatory element binding protein (SREBP) blockade

It has been shown that oxysterols can block SREBP-SCAP by their union with Insig protein. Specifically, 24(S), 25-epoxycholesterol is bound to the Insig element and inhibits the synthesis of cholesterol (Lund *et. al*., 1998). 27-HC production in response to active cholesterol activates Insig and, hence, leads to the retention of inactive SREBP in the ER (Steck & Lange 2010).

b) Inhibition and degradation of 3-hydroxy-3-methil-glutaryl-CoA (HMG-CoA) reductasa

Oxysterols are much more potent than cholesterol itself in suppressing the activity of HMG-CoA reductasa, thus thought to act as feedback signals for the inhibition of cholesterol biosynthesis (Maraschiello, 1998). 27-hydroxycholesterol seems to degrade this enzyme, inhibiting cholesterol production (Steck and Lange 2008) and statins inhibit HMG-CoA reductasa (Poirot, 2013). At least in human fibroblasts, a small fraction of excess (hence, active) cholesterol must first be converted to 27-HC to trigger this proteolysis. The oxysterol then associates with the resident ER protein, Insig, which, in turn, binds to and marks HMGR for ubiquitination and subsequent proteasomal digestion. Consequently, the inactivation of HMGR responds sharply to small increments in the level of plasma membrane cholesterol at its rest point.

c) Oxysterols and Liver X Receptors (LXR)

Side-chain oxysterols regulate the abundance of cellular sterols through the activation of LXRs (α and β) which are the master regulators of several metabolic pathways, including lipid and cholesterol metabolism as well as inflammation and innate immunity (El Kharrasssi, *et. al*. 2013). These transcription factors promote the expression of members of the ATP-binding cassette (ABC) transporter super-family which expel cholesterol from cells. LXR regulates the expression of genes such as ABCA1 and apoE for cholesterol efflux. Members of ABC family transporters are implicated in the excretion of sterols and oxysterols from the cell. When 24(S), 25-HC binds LXR, cholesterol excretion is stimulated. (Chen *et. al*., 2007). In the brain, LXR agonists have been shown to regulate lipoproteins synthesis and cholesterol efflux in astrocytes and in microglia (El Kharrasssi, *et. al*. 2013). Furthermore, they also participates in brain inflammatory processes.
The physiologically most important endogenous LXR ligands are 24(S),25epoxycholesterol, 24(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, 20(S)-hydroxycholesterol and 27-hydroxycholesterol ((Olkkonen *et. al.* 2012). Exogenous oxysterols such as 7α-OHC and 7β are not ligands of LXRs (Maraschiello, 1998).

d) Oxysterols and sterol transport

A number of lipid transfer proteins (LTP) are suggested to transfer sterols between cellular compartments through non-vesicular pathways. 24(S)-HC and 27-HC could transport sterols to the liver regulating their incorporation to HDL (Björkhem *et. al*. 1994;

1998; Lund *et al*., 1996). 7-KC inhibits the export of cholesterol to HDL, accumulating cholesterol in cells (Jessup *et al.*, 2002) supporting the formation of atheromatous plaques.

Oxysterol-binding protein (OSBP), a cytosolic protein, is a receptor for endogenous oxysterols.

The ORP, OSBP homologues, have been implicated in many cellular processes including lipid metabolism, cell signalling, vesicular trafficking and non-vesicular sterol transfer (Zhou 2013). Figure 4 shows a schematic representation of the biological effects of ORP. It is demonstrated that ORPs have the capacity to stimulate cholesterol transfer in live cells (Olkkonen *et al*. 2012). ORPs can bind oxysterols, cholesterol, and/or phospholipids, and are implicated in inter-organelle lipid transport and signalling. The common feature for all ORPs is the core lipid binding ORD domain at the C-terminus, which contains the conserved OSBP signature motif EQVSHHPP. A majority of ORPs in mammals carry long N-terminal extensions containing a pleckstrin homology (PH) domain. The PH domains of ORPs have been found to bind phosphoinositides (PIPs) which control the subcellular localization of proteins. (Zou, 2013).

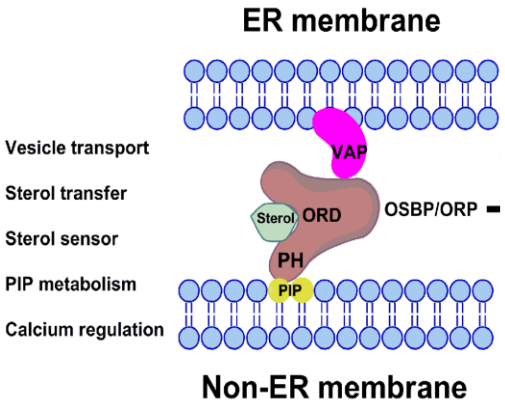


Fig 4. A schematic presentation summarizing the biological functions of ORPs. The localization of ORPs at a membrane contact site is depicted. ER, endoplasmic reticulum; VAP, VAMP associated protein anchoring the ORP at the ER; ORD, OSBP-related (ligand binding) domain; PHD, pleckstrin homology domain; PIP, phosphatidylinositol phosphate. [You Zhou, 2013].

Several ORPs are known to localize and function at membrane contacts sites (MCS) at which ER is closely apposed with other organelles. MCS have roles in lipid synthesis/transport, calcium fluxes, and signaling processes (Olkkonen *et.al* 2012). Upon addition of 25-hydroxycholesterol, most of the OSBP became concentrated in the Golgi apparatus (Ridgway et. al. 1992)

3.3 Pathophysiological implications of Oxysterols

Oxysterol levels are increased in pathophysiologic conditions such as macrophage foam cells, atherosclerotic lesions, cataracts, osteoporosis, multiple sclerosis, and Alzheimer's disease. AD is a common, aging-related dementing disorder characterized by progressive neuronal degeneration, gliosis, and the accumulation of intracellular inclusions (neurofibrillary tangles) and extracellular deposits of β-amyloid in discrete regions of the basal forebrain, hippocampus, and association cortices. Hypercholesterolemia is a risk factor for AD (Vaya & Schipper 2007/ Glenner, G G; Wong, C. W. (1988).).

Oxysterols, which are cholesterol-derived ligands of the LXRs and oxysterol binding proteins strongly regulate the processing of amyloid precursor protein (APP) (Vaya & Schipper 2007).

The cytotoxic potential of oxysterols can contribute to the disease processes. For example 27-hydroxycholesterol that is specifically produced by nerve cells has been investigated as a potential biomarker for neurodegenerative disorders such as Alzheimer's disease and also is being linked with breast cancer because of its activity as an endogenous selective estrogen receptor modulator.

Oxysterols enhance the inflammatory reactions by stimulating synthesis of cytokines, tumor necrosis factor α (TNF-α), interleukins and other inflammatory proteins that causes the progression of many chronic diseases. (S. Sabuncuo, 2014). They are incorporated into the biological membranes and modify them. This fact is important and directly related with their cytotoxicity.

3.3.1 Cytotoxicity and apoptotic effects

Oxysterols are associated with the initiation and progression of major chronic diseases including atherosclerosis, neurodegenerative processes, diabetes, kidney failure, and ethanol intoxication (Sottero *et al*., 2009). They provoke an imbalance of the ratio between oxidative and reductive biochemical reactions (oxidative stress) which acts on all organism levels from cell signalling to disease expression through up-regulation or inflammation, apoptosis and fibrosis. Apoptosis or programmed cell death (*PCD*) is physiological mechanism, which removes cells when they have completed their life cycle or when they are genetically damaged. Cell stress stimulates pro-apoptotic signaling pathways that activate caspases and proteases and cause mitochondrial dysfunction (Claudio Giovannini *et.al.* 2007/Elmore, S. 2007).

The induction of apoptosis occurs via two major pathways: the death receptor-dependent (extrinsic) pathway (Fig. 5) or the mitochondrial (intrinsic) pathway (Fig. 6). Oxysterols can induce apoptosis through both pathways.

Oxysterols may increase intracellular levels of reactive oxygen species (ROS), induce modification of cell proteins and alter various signaling pathways and gene expression (Lordan, *et al* 2009). The most cytotoxic oxysterols are: 7-hydroxy, 7-keto and triol derivatives. Table 1 shows some effects of a selected oxysterols in the apoptotic pathways.

Several cytotoxic routes have been proposed for oxysterols:

- perturbation of the intracellular calcium levels,
- Intracellular ROS overproduction
- Mitochondrial and Lysosomal membranes modifications
- Polyamine metabolic perturbations

Some oxysterols, such as 7-ketocholesterol and 7β-hydroxycholesterol have the ability to trigger cell death, activate inflammation and modulate lipid homeostasis. (Anderson & Borlak 2006). 7β-HC induces the permeabilization of mitochondrial membrane and the release of endonuclease G to the nucleus, provoking DNA fragmentation and cell death. 7β-HC also enhances ROS formation in the mitochondria, which possibly destabilizes the lysosomal membrane. (Otaegui-Arrazola *et al*., 2010).

On the other hand, it has been proven that 7-KC is associated with apoptotic characteristics like enhancing superoxide anion products and it also has autophagic characteristics (Zarrouk, N *et.al.* 2013). Table 1 shows other examples of oxysterols which are directly or indirectly implicated in the apoptotic pathways by induction of proapoptotic ligands like TNF or by regulation of receptors such as Fas.

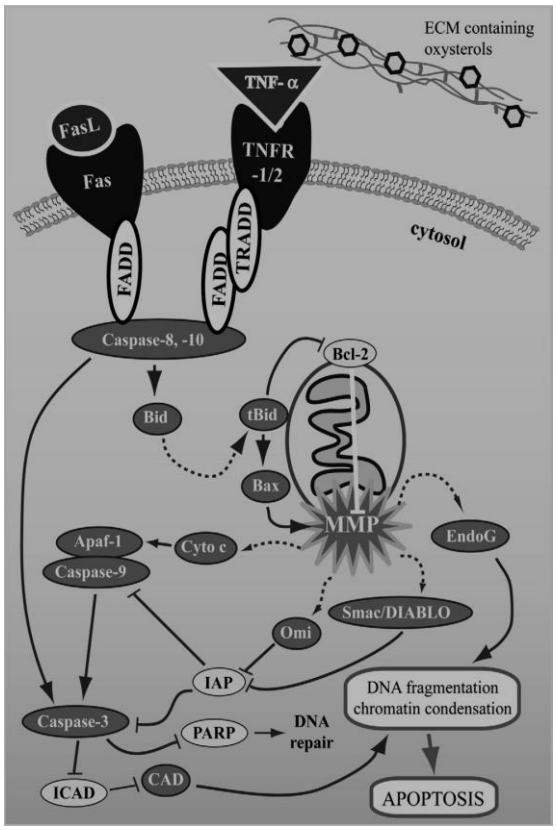


Fig. 5: The death receptor (extrinsic) pathway. ⇀, activation; , translocation; ⊣, inhibition. Apaf-1, apoptosis-protease activating factor-1; Cyto c, cytochrome c; FADD, Fas-associated death domain; ICAD, inhibitor of CAD; tBid, truncated Bid; TRADD, TNFR-associated death domain.
[Source: Lordan *et.al*. 2009].

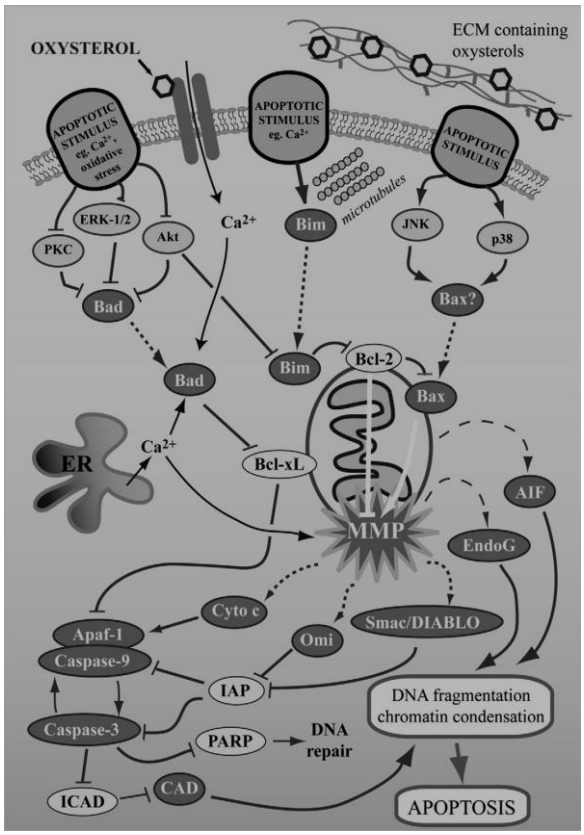


Fig.6: The mitochondrial (intrinsic) pathway. ⇀, activation. , translocation; ⊣ inhibition. Apaf-1, apoptosis protease activating factor-1; cyto c, cytochrome c; ICAD, inhibitor of CAD. [Source: Lordan *et al.* 2009].

In 2009, Lordan and coworkers concluded that apoptotic processes induced by oxysterols depend largely on the cell type and the oxysterol in question. They also suggested that some oxysterols, as 7-keto, 7β-OH and 25-Oh, induce both apoptotic pathways, assuming a cross-talk between them. Moreover, oxysterols may cause oxidative stress and perturbations in intracellular Ca^{2+} initiating factors of apoptotic transduction within the cell (Lordan *et al*. 2009). In addition, oxysterols have been reported to display some degree of cytotoxicity against cancer cells, and to increase the sensitivity of tumor cells to other cytotoxic agents (Dias *et al.*, 2014).

Table 1. Intervention of oxysterols in the apoptotic pathways. Those oxysterols could be considered as a "control points" of apoptosis and they could be used as targets for therapeutic intervention to prevent the programmed cell death (Lordan *et al.*, 2009).

Oxysterols	7β-OH	25-OH	B-epoxide	7-keto	5,6-secosterol	22-OH
Implication in apoptosis	Up-regulation of Fas and FasL and apoptosis of smooth muscle cells of vascular origin. Induction of TNF in human monocytes and THP-1 macrophages	Up-regulation of Fas and FasL and apoptosis of smooth muscle cells of vascular origin.	May implicated in PKC activation. Associated with decrease of Bcl-2.; caspases 2, 3 and 8.	Induces TNF expression and secretion. Induce apoptosis of PC12 neuroendocrine cells via ROS-mediated activation of NF-kB and Akt/PKB pahways.	Induce cell senescence at high concentrations, causes sustained ERK1/2 activation and cellular proliferation at low concentrations. Induces apoptosis by both intrinsic and extrinsic pathways.	Induction of TNF in human monocytes and THP-1 macrophages.

3.3.2 Pro-inflammatory effects and immunity

Inflammation has been recently associated with several chronic diseases, such as obesity, atherogenesis and Alzheimer. Oxysterols have been proven to be implicated in the up-regulation in the expression of various inflammatory molecules, including adhesion molecules, growth factors, cytokines and chemokines and also it has been proved that certain oxysterols have anti-inflammatory effects (Otaegui-Arrazola *et al.*, 2010).

The innate immune system regulates adaptive immunity via oxysterols. Macrophage derived oxysterols suppress IgA production by B cells. Upon activation of their toll-like receptors, macrophages produce it, and it is found in trace amounts in normal plasma. It has been found that 25HC affects innate immune response via Stat 1 (Sabuncuo, S., 2014) and it has been proposed to have an antiviral role. 25-HC is induced in macrophages upon LPS or virus stimulation. 7 α, 25-diHC is a ligand of the G-protein coupled receptor EBI2, which is important to localize B cells and CD4+ dendritic cells in lymphoid organs and to produce a normal antibody response. 27-oxygenated metabolites of cholesterol are consistently high in human monocytes, including 27-HC, 7α, 27-diHC, 3β-HCA and 7α-hydroxy-3-oxocholesterol-4-enoic acid. (Griffiths J. *et al.*, 2013).

Interleukin-8 (IL-8), which might be proatherogenic by recruiting T lymphocytes and monocytes in the arterial subendothelial space and by inhibiting expression of local tissue inhibition of metalloproteinase-1, is regulated by various oxysterols basically through a calcium-dependent phenomenon involving MEK/ERK ½ pathway and activation of AP-1 (Otaegui-Arrazola *et al*. 2010).

The retinoic acid receptor-related orphan receptors (RORs), including the α, γ, β, are important nuclear receptors, their function is the transcriptional regulation of genes involved in development. Metabolism, and immunity RORα regulates cerebellum development, bone formation and immune response. RORγ plays an essential role in the development of lymphoid tissues and T cells. Their activities could be suppressed by 7α-OHC or 24(S)-OHC, respectively. Moreover, it has been found that 24(R)-OCH and 24(S),25 –EPOX regulate RORγ selectively (Olkkonen *et.al.* 2012).

Soroosh and co-workers found that oxysterols are agonist ligands of orphan nuclear receptor RAR-related orphan receptor gamma t (RORyt) and drive Th17 cell differentiation (Soroosh *et al*. 2014). Because of its essential role in driving IL-17 production, RORγt represents a potential therapeutic target for autoimmune diseases. They presented evidence that 7β, 27-dihydroxycholesterol (7β,27-OHC) and 7α, 27-OHC are RORγt agonist ligands and may serve as potential endogenous RORγt ligands in promoting the differentiation of mouse and human CD4+ Th17 cells.

3.3.3 Oxysterol Related Proteins and disease implications

While much is known about the interaction between the regulatory factors (LXR, HMG-CoA and SREBP-Insig) and cholesterol, only little has been studied about the mechanism to deliver the cholesterol or oxysterol to its appropriate compartments. Oxysterol-binding protein/oxysterol-binding protein-related protein (OSBP/ORP) family may regulate such process by binding oxysterol and/or cholesterol and by functioning as a cholesterol sensor or cholesterol transporter (Koriyama *et.al..* 2012/ Raychaudhuri, S., & Prinz, W. A. 2012.). Some of these ORPs are thought to be involved in pathophysiological processes.

- **ORP1L**, localize at late endosomes, forms a RILP-Rab7-ORP1L complex and is involved in both protein and lipid transport functions of the late endocytic compartments. It is expressed predominantly in macrophages, brain and lung but it is also found in colon, kidney, and liver (Olkkonen *et al*. 2006).

- **ORP1S and ORP2.** ORP2 presents on LD and has a functional role in the regulation of neutral lipid metabolism, possibly as a factor that integrates the cellular metabolism of triglycerides (TG) with that of cholesterol. ORP1S is expressed predominantly in skeletal muscle and heart. ORP2 is expressed ubiquitously in mammalian tissues. Highest mRNA levels of ORP2 are present in specific parts of the central nervous system (cerebellum, pituitary gland, pons, and putamen) as well as in leukocytes, placenta, and pancreas

- **ORP3** is highly expressed in epithelial, neuronal and hematopoietic cells, as well as in certain forms of cancer. ORP3 may play an important role in efficient directed membrane trafficking and it has been demonstrated a new function of ORP3 in machinery that controls the actin cytoskeleton, cell polarity and cell adhesion. (Lehto, Mayranpaa *et al*. 2008).

- **ORP4** mRNA and protein expression overlapped partially with OSBP and were restricted to brain, heart, muscle and kidney. An interaction with intermediate filaments inhibits the intracellular cholesterol transport pathway mediated by vimentin. ORP4 promotes the survival of rapidly proliferating cells (M. Charman et. al. 2014).

- **ORP5** localizes to the ER and may cooperate with Niemann-Pick C1 (NPC1) to mediate the exit of cholesterol from endosomes/lysosomes. ORP5 expression is related to invasion and a poor prognosis in pancreatic cancer patients (Ishikawa, S *et al*. 2010).

- **ORP6** shows the highest expression in brain and skeletal muscle (Lehto, Tienari *et al*.2004) and it was identified as one of the candidate genes that are possibly involved in the regulation of high-density lipoprotein (HDL) cholesterol levels.

- **ORP7** shows the highest expression in the gastrointestinal tract (Lehto, Tienari *et al*.2004) modulates the effect of its ligand, 25-OHC, on proteasome-dependent degradation of GS28, which may result in modulation of Golgi transport functions. Its polymorphisms are associated with serum total and LDL-cholesterol.

- **ORP8** is expressed at the highest levels in macrophages, liver, spleen, kidney, and brain and it is localized in the ER via its C-terminal transmembrane domain. ORP8 negatively regulates ABCA1 expression and macrophage cholesterol efflux and also has the capacity to modulate lipid homeostasis and SREBP activity, probably through an indirect mechanism required Nup62 (Zhou, Li *et al*. 2011).

- **ORP9** and ORP11 are dimerized and may act as an intracellular lipid sensor or transporter (Zhou, Li *et al*. 2010) and further influence metabolic diseases.

- **ORP10** was shown to associate dynamically with microtubules, being consistent with its involvement in intracellular transport or organelle positioning. ORP10 suppresses hepatic lipogenesis and very-low-density lipoprotein production) and it is genetically associated with both TG (triglycerides) and LDL cholesterol level.

- **ORP11** is present at the highest levels in human ovary, testis, kidney, liver, stomach, brain, and adipose tissue. ORP11 dimerizes with ORP9 and localizes at the Golgi-late endosome interface (Zhou, Li *et al*. 2010). ORP11 gene is associated with LDL cholesterol levels, hyperglycemia /diabetes as well as with metabolic syndrome per se.

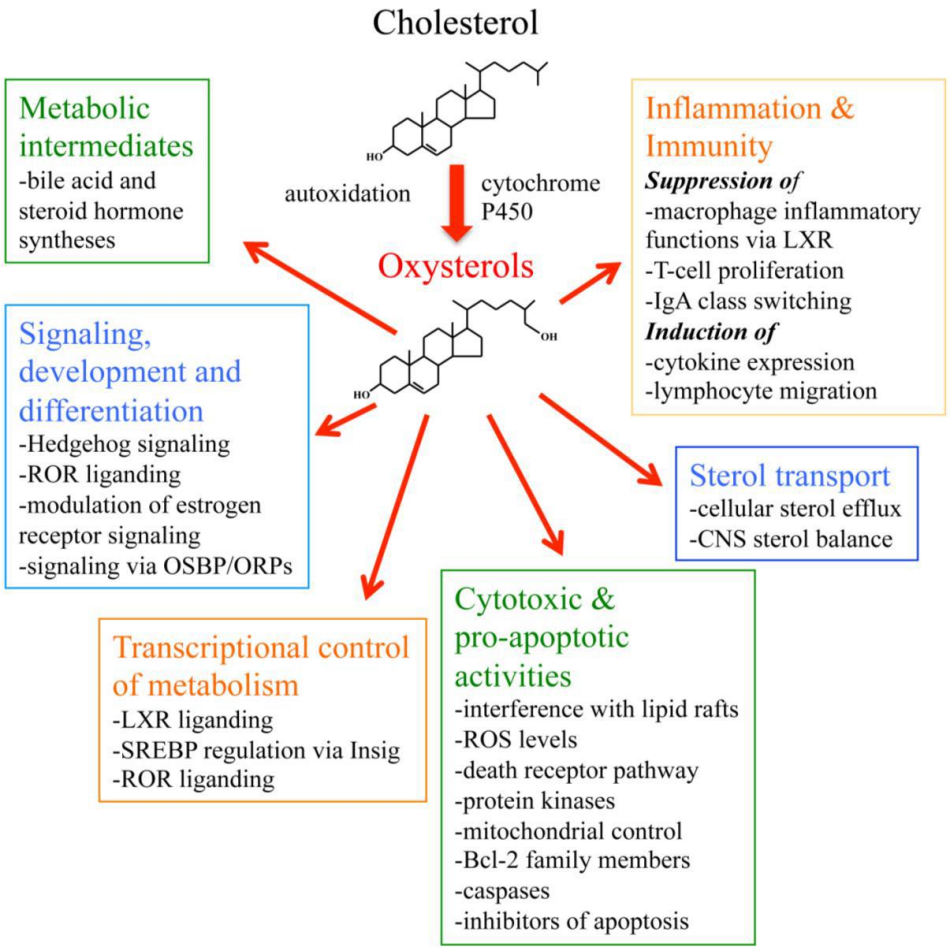


Figure 7. A schematic presentation summarizing the major functions and biological activities of oxysterols. Abbreviations used: ROR, retinoic acid receptor-related orphan receptor; OSBP, oxysterol-binding protein; ORP, OSBP-related protein; LXR, liver X receptor; SREBP, sterol regulatory element binding protein; Insig, insulin-induced gene; ROS, reactive oxygen species; Bcl-2, B-cell lymphoma 2; IgA, immunoglobulin A; CNS, central nervous system [Source: Olkkonen *et al*. 2012].

4. METHODOLOGY

I have used different keywords, which are indicated above, to search information on the websites such as NCBI (PubMed), Science direct, Journals from Elsevier related with Steroid biochemistry and molecular biology, Journals of lipids research and biochemistry, Nature (for some definitions and also to find reviews), Journals about Alzheimer disease and Cancer. I also visited the webpage called "pubs.acs.org" into the biochemistry space, "aocs.org" and "biochemj.org" and finally "The European Network for Oxysterol Research (ENOR)" although it is in construction you can look the abstracts of the symposiums…

Keywords used for the search**:** Oxysterols, inflammation, apoptosis, cancer & oxysterols, AD & oxysterols, cholesterol, cholesterol biosynthesis, cholesterol oxidation, atherosclerosis, ORP/OSBP, name of some oxysterols such as 27-hydroxycholesterol, 7-ketocholesterol…

The PhD thesis of Ciriaco Maraschiello (1998) was also consulted and used as a main reference to obtain details about the chemistry of cholesterol and lipid oxidation and about the origins of the oxysterols including their implication in atherosclerosis. Books of biochemistry were also consulted for the understanding of cholesterol processes and its biology.

5. DISCUSSION

Because of their biochemistry (the way that they are originated, their chemistry, their biological activities and the pathways that they are involved in), oxysterols could be directly or indirectly targeted as an innovative strategy for therapeutic treatment of diseases such as Cancer or the Alzheimer's disease.

Oxysterols are present in mammalian tissues at very low concentrations, as mixtures accompanied by a high excess of cholesterol. However, they are found enriched in pathologic structures such as macrophage foam cells, atherosclerotic lesions, cataracts, and gall stones. The cytotoxic and pro-apoptotic actions of oxysterols are suggested by play a role in the disease processes involved. Oxysterols have been implicated in the pathology of degenerative diseases such age-onset macular degeneration and Alzheimer's disease. Several oxysterols have a potent regulatory activity on cellular cholesterol homeostatic machineries. In addition, and as described in the literature review, oxysterols are believed to act as endogenous regulators of gene expression in lipid metabolism and as signalling molecules with key roles in developmental, differentiation and inflammation processes (Olkkonen *et.al*, 2012).

A) Are oxysterols implicated in cholesterol homeostasis?

As described in the literature review section, oxysterols can interact with LXR, SREBP and HMG-CoA reductasa, which are key regulators in the homeostasis of cholesterol. Another experiment made by Olkkonen, revealed the interaction of OSBP with the cholesterol homeostasis, suggesting that OSBP regulates cellular lipid homeostasis because when he silenced OSBP by RNA interference, it resulted in increased cellular amount and cholesterol efflux activity of ABCA1 (cholesterol transporter), in the absence of effects on the ABCA1 mRNA level or LXR activity. It seems that OSBP opposes the activity of LXR by destabilizing ABCA1 (Olkkonen *et al*. 2012).

In a study made by Berrodin and co-workers, 5α, 6α-epoxycholesterol was identified as endogenous modulator of Liver X Receptor Activity (LXR) that, in time, is a key regulator of cholesterol homeostasis, lipid metabolism, and keratinocyte differentiation. There are natural ligands that activate LXRs including 25-hydroxycholesterol 27-hydroxycholesterol, 22(R)-hydroxycholesterol, 20(S)-hydroxycholesterol and 24(S),25-epoxycholesterol (Berrodin *et al*. 2010) that stabilize ABCA1, regulating the cholesterol efflux. Bai and his team, evaluated the role of 25HC on lipid metabolism by overexpressing the gene encoding oxysterol sulfotransferase (SULT2B1b). The conclusion of the study was that sulfation of 25HC by SUlT2B1b plays an important role in the maintenance of intracellular lipid homeostasis via LXR/SREBP-1c signaling pathway in human aortic endothelial cells (Bai *et al*. 2012). So it has been proven that another oxysterol is involved in cholesterol homeostasis.

However, other publications support that cholesterol itself is the most important player in its own homeostasis and other sterols, including oxysterols, play an ancillary role. Oxysterols, particularly those oxidized on their side-chain, are potent activators of LXR, eliminating cholesterol excess (Brown 2012) and believed to be the naturals ligands for these receptors and also accelerate degradation of HMGR. Furthermore, it is known that they stimulate cholesterol efflux, powering off SREBP activation via Scap and Insig. 24, 25-EC plays an important role in the acute regulation of cholesterol synthesis being a modulator and also monitoring this synthesis. Brown demonstrated another role of this oxysterol in the cholesterol homeostasis by inhibiting the ultimate step catalyzed by DHCR24, which is the one enzyme in the cholesterol biosynthesis pathway not required for 24,25EC generation (Brown 2012). This fact could be interesting because DHCR254 could be a "control point" and may have particular relevance in the brain.

Side-chain oxidized oxysterols also have a high capacity to affect critical genes in cholesterol turnover in vitro. Most of the published in vitro experiments with oxysterols are highly unphysiological, however. Mouse models studied in Björkem's laboratory, with high or low levels of 27-hydroxycholesterol, have little or no disturbances in cholesterol homeostasis. 24S-hydroxycholesterol is an efficient ligand to LXR and suggested to be important for cholesterol homeostasis in the brain. Nevertheless, the overexpression of this oxysterol only had a modest effect on cholesterol turnover in *in vivo* experiments. Because of that, Björkem and his team concluded that oxysterols are not the master regulators of cholesterol homeostasis *in vivo* suggested previously (Björkem, 2014). This is the most up to date information about this question. Therefore, although it is true that Oxysterols are involved in the process, it seems to be that they just form part of the process of the regulation of cholesterol, but they are not the most important regulators of the process.

B) Oxysterols as direct or indirect target for therapeutic indication:

York and Bensinger hypothesize that the chemotactic signals of oxysterols may outweigh the anti-proliferative effects, and imply that inhibition of oxysterols could provide a novel therapeutic target for both the generation of efficient antitumor immune responses and the inhibition of growth-promoting myeloid tumor-infiltrating cells (York & Bensinger 2013). Based on this idea, the two diseases are analyzed separately as an attempt to assess the relevance of this moieties in the treatment of some important diseases such as Cancer and Alzheimer.

B.1. Oxysterols and Alzheimer disease

Do oxysterols really influence the progression of Alzheimer? Referring to neurological disorders (CNS), it has been suggested that in AD, glial heme oxygenase-1 (HO-1) induction may transduce ambient noxious stimuli into altered patterns of cholesterol and oxysterol metabolism. This may affect neuronal membrane turnover, plasticity and survival and thereby influence diseases progression. Manipulation of central sterol homeostasis by administration of oxysterol-mimetic compounds and/or pharmacological control of glial HO-1 expression may constitute a

novel therapeutic strategy in patients with AD and other CNS conditions implicating derangements in tissue cholesterol homeostasis (Vaya & Schipper 2007).

One of the hallmarks of AD is the deposition of extracellular plaques composed predominantly of 4kDa amyloid-β peptide (Aβ). It has been found that β-amyloid, the toxic peptide in neurons of Alzheimer's disease patients, binds oxysterols and catalyzes its oxidation to 7β-hydroxycholesterol (Nelson & Alkon 2005). Oxysterols, which are cholesterol-derived ligands of the liver X receptors (LXRs) and oxysterol binding proteins (OSBP), strongly regulate the processing of APP. They suggest that OSBP-1 could play a role in linking cholesterol metabolism with intracellular APP trafficking and Aβ production, and more importantly indicate that OSBP1 could provide an alternative target for Aβ-directed therapeutic (Zerbinatti *et al*. 2008). Inhibiting OSBP1 activity it could influence the APP trafficking and Aβ production. It has been found an oxysterol which is able to reverse the OSBP1 activity, it is 25-hydroxycholesterol. Numerous lipoprotein receptors and apolipoproteins are expressed in the brain, so the key remains in find a receptor which direct the oxysterol supplied into the brain and inhibits the Aβ deposition by influencing on the APP trafficking. Another oxysterol is involved in the homeostasis of cholesterol in the brain, it is 27-hydroxycholesterol. Cholesterol is converted into this COP, this way the homeostasis is achieved. This fact could be useful in term of using this oxysterol for therapies against AD.

Presence of the blood-brain barrier (BBB) is critical for cholesterol metabolism in the brain, preventing uptake of lipoprotein-bound cholesterol from the circulation. In contrast to cholesterol, 27-hydroxycholesterol is able to pass the BBB. In patients with AD, 27OH accumulates. This accumulation may be consequence of the fact that one important enzyme involved in the metabolism of 27-OH, CYP7B1, is located in neuronal cells, that are reduced in number in the brain of AD patients. It is discussed that inhibitors of CYP27 may be used as a therapy for neurodegenerative diseases (Björkhem & Cedazo-Minguez, 2014).

Traditionally, 24S-HC is related with apoptosis and necroptosis,a form of programmed cell death, and it is considered the most abundant oxysterol in the brain maintaining cholesterol homeostasis (Brown 2012) but in this study they found that primary human brain cells, particularly astrocytes, also have the capacity to synthetize 24,25-EC which suggests that neurons reduce costly cholesterol biosynthesis to focus on neurotransmission, by relying on astrocyte-derived cholesterol. Scientists proposed to use oxysterols to drive dopaminergic neurogenesis from stem cells via an LXR-dependent mechanism, which is speculated to involve 24,25EC as the LXR activator. (Noguchi, Saito & Urano, 2013). This hypothesis could be tested by overexpressing OSC, which effectively and selectively abolishes 24,25EC synthesis, and decreases LXR-mediated transcription. It down-regulates APP trafficking via enhancement of the complex formation of APP with up-regulated glucose-regulated protein 78, an ER chaperone. By this mechanism, 24(S)-OHC supresses amyloid-β (Aβ) production in human neuroblastoma SH-SY5Y cell. Furthermore, 24(S)-OHC at sub-lethal concentrations induces adaptive response via transcriptional activation of liver X receptor signalling pathway, thereby protecting neuronal cells against the forthcoming oxidative stress induced by 7-ketocholesterol (Noguchi, Saito &

Urano, 2013). High concentrations of 24(S)-OHC induce neuronal cell death by necroptosis. This caspases independent cell death by 24(S)-OHC is not inhibited by antioxidants (Noguchi, Saito & Urano, 2013). Enzyme LCAT convert 24OH-C in 24OH-CE (cerebrosteryl ester). LCAT activity in CN, expressed as 24OH-CE/24OH-C ratio, could be a new therapeutic target, and can be used as a biomarker for AD (when ratio is decreased AD would be detected) (La Marca et. al, 2013). This oxysterol. 24(S)-OHC is an efficient suppressor of cholesterol synthesis, so it could be considered to have a regulatory role in the brain.

LXRβ is an oxysterol-dependent nuclear receptor that controls lipid homeostasis, water transport, immune response and neural development. LXRβ agonists may have beneficial effects in therapy as a target for treatment of various forms of neurodegeneration by modulating the cytotoxic functions of microglia (Jan-Ake Gustafsson, 2013). 22 S-HC is an antagonist of LXR and 22 R-HC is an endogenous agonist of LXR. LXR antagonists as therapeutic agents in the context of demyelinating neurodegenerative disorders (Gondcaille *et.al.* 2013). Furthermore, 24, 25-EC is a potent ligand in the developing mouse midbrain. Both, cholic acid (an LXR endogenous ligand) and 24, 25-EC, promote neural development: cholic acid increase survival and neurogenesis of Brn3a-positive red nucleus neurons; and 24, 25-EC promote dopaminergic neurogenesis and also differentiation of embryonic stem cells.

B.2. Oxysterols and Cancer

Certain oxysterols were proposed to be carcinogenic (Bischoff, 1963; Bischoff, 1969). Because of their pro-apoptotic and cytotoxic effects, oxysterols could be applied to avoid carcinogenesis. Otherwise, it could be that carcinogenic cells are just using that oxysterols intelligently…The cytotoxicity activity of oxysterols could be used to find anticancer moieties. Recent studies link cholesterol metabolism to breast cancer. Certain cholesterol metabolites can promote or, surprisingly, suppress breast cancer (Poirot, 2014/Maddika *et.al.* 2007).

Oxysterols may be involved in tumor initiation by enhancing the production of ROS/RNS. Furthermore, tumor promotion may be enhanced by oxysterols through upregulated expression of proteins such as COX-2-leading to the alteration of cellular phenotypes. In addition, certain oxysterols can support cancer progression through the induction of migration. Oxysterols may exert their effect by binding to specific proteins and activating signaling cascades (Jusakul *et al.* 2011).

How to make a target of the carcinogenic cells and how to stop that synthesis (which seems to be favored) in carcinogenic cells? The University of North Carolina, demonstrated that Kes-1, a key regulator in membrane trafficking, act as a sterol-regulated rheostat for TGN/endosomal phosphatidylinositol 4-phosphate signaling. Kes-1 has dual lipid-binding activity, on one hand it integrates endosomal lipid metabolism with target of rapamycin complex-1 (TORC-1)-dependent proliferative pathways and on the other hand, transcriptional control of nutrient signaling (Villasmil *et al.* 2012). Kes-1 antagonizes ATF-4-dependent transcriptional programmes that may have major implications in cancer biology.

Studies from 2013 reveled that tumor-derived oxysterols can serve to subvert the immune system by recruiting protumorigenic neutrophils into the tumor microenvironment. The consequence is the generation of proangiogenic factors and matrix metalloproteinase proteins that provide a tumor a significant growth and survival advantage. Tumors are experts at co-opting inflammatory pathways, and dampening host immunity to their growth and survival advantage. Tumor-derived oxysterols were found to down-regulate the chemokine (C-C motif) receptor 7 (CCR7) in an LXR-dependent manner, this mechanism is dependent of activation of LXR-α in DCs (York & Bensinger 2013). In this review the authors referenced another study which demonstrated that tumors produce an array of hydroxycholesterol species, in particular 22-HC and 27-HC, in sufficient quantities to activate LXR in tumor tissue as well as at distant tissues sites of the host, such as BM. Oxysterols generated by tumor cells are necessary for the continuous recruitment of neutrophils to the tumor site. This recruitment appeared to be dependent on the binding of 22-HC oxysterols to the GPCR CXCR2 and was independent of LXR activity (York & Bensinger 2013). Inactivation of oxysterol generation by tumors via the ectopic expression of the cholesterol sulfotransferase 2B1b (SULT2B1b) significantly attenuated neutrophil infiltrate and tumor growth (Motz & Coukos 2011).

In recent days, a lot of discoveries has been made in the field of oxysterols. One study carried by Gold and her co-workers demonstrated that 25HC amplifies the activation of immune cells by mediating the recruitment of the AP-1 components FBJ osteosarcoma oncogene (FOS) and Jun proto-oncogene (JUN) to the promoters of a subset of Toll-like receptor-responsive genes; and increases the production of immune mediators (Gold *et al*. 2014). 25HC is produced by immune cells in response to infection and it has a role in foam cell formation.

Certain oxysterols can support cancer progression through the induction of migration. Oxysterols may exert their effect by binding to specific proteins and activating signaling cascades and COPS also have mutagenic effects by damaging DNA, so they may play a key role in the different stages of carcinogenesis. They also generate the cellular response in tumor tissue through LXRs that activate target genes in the inflammatory process (Medica & Sabuncuo 2014).

6. CONCLUSIONS

Oxysterols are still nowadays an open field for investigation. Despite the fact that they were identified 60 years ago, little is still known about their metabolism or their physiological functions. There are a lot of experiments made *in vitro* but the truth is that there is a lack of evidence of the biological activities of oxysterols in relevant *in vivo* experiments: a lot of conclusions drawn from the published results are not really "translatable" to humans. Much still needs to be done to demonstrate the implications of oxysterols in the pathophysiology of certain diseases and to claim them as direct or indirect target for therapeutic treatment. It is probably not possible to act directly on oxysterols as they might be the result of the natural process of aging. Oxysterols are indeed part of natural physiologic processes but when

their production is exaggerated or becomes unregulated, they might contribute to accelerate some pathophysiological processes related to neurodegenerative diseases, cardiovascular diseases and cancer.

The following points could be considered as the main biological characteristics of oxysterols including some suggestions for potential therapeutic indications:

- Most of the biological implications of oxysterols are related to their cytotoxicity, apoptotic and pro-inflammatory effects. Oxysterols are also involved in cholesterol homeostasis, they play important roles in the stimulation of LXR, degradation of HMG-CoA reductasa, sterol transport or in the SREBP.
- Both cholesterol and oxysterols affect properties of membranes and regulate sterol homeostasis through interaction with effector proteins. Oxysterols play a role in cholesterol homeostasis but are not the sole regulators of this important organic process.
- The accumulation of oxysterols can induce apoptosis, necrosis and cytokine production as the result of an inflammatory process. Numerous studies suggested that oxysterols are involved in the pathophysiology of various types of cancer, including cancers of the colon, lung, skin, breast and bile ducts.
- Oxysterols could be involved in fertility, e.g. oxysterol nuclear receptors liver X receptor (LXR α and β) are supposed to be key in maintaining both integrity and functions of the testis. In addition, cholesterol autoxidation and the related oxysterols may play a role in sperm function that is known to be highly affected by oxidative stress (Zerbinati *et al*. 2013/ Maqdasy *et.al*. 2014).
- Oxysterols as lipid-signaling molecules can have pleiotropic effects on the fate and function of the immune system. These effects range from the regulation of immune cell survival and proliferation to chemotaxis and antiviral immunity (A.G. York, 2013).
- Certain oxysterols can support cancer progression through the induction of migration. Oxysterols may exert their effect by binding to specific proteins and activating signaling cascades. Oxysterols also have mutagenic effects by damaging DNA, so they may play a key role in the different stages of carcinogenesis. They also generate the cellular response in tumor tissue through LXRs that activate target genes (Medica & Sabuncuo 2014). Using synthetic molecules that act as specific antagonists or agonists of oxysterols receptors could represent a potential strategy for therapeutic treatment. The same approach could be used for Alzheimer's disease.
- The interaction between sterols and sphingolipids may proportionate a new therapeutic action against Alzheimer's disease, using a genetic manipulation (Gulati *et al*. 2011). The role of the oxysterols here would be considered as mediators of this cross-talk between cholesterol and sphingolipids through the oxysterol binding proteins. Altering the ORP expression could also be a potential target for disease treatment (Ngo *et al*. 2010).

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