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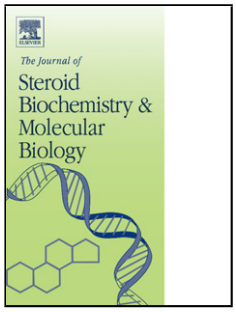

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Localisation of oxysterols at the sub-cellular level and in biological fluids

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Highlights

- Oxysterols are involved in many biological processes including the regulation of cholesterol homeostasis
- The location of oxysterol metabolising enzymes may play an important role in the oxysterol concentration in cellular micro-environments.
- Oxysterols in biological fluids can use as markers to analyse endogenous flaws in cholesterol/oxysterol homeostasis.

List of Abbreviations

20-OHC	20‑hydroxycholesterol
22-OHC	22‑hydroxycholesterol
24-OHC	24‑hydroxycholesterol

25-OHC	25‑hydroxycholesterol
26-OHC	26‑hydroxycholesterol
27-OHC	27-hydroxycholesterol
5, 6-epox	5, 6-epoxy cholesterols
5α, 6α-epox	5α, 6α- epoxy cholesterol
5β, 6β-epox	5β, 6β- epoxy cholesterol
7-KC	7-ketocholesterol
7-K,25-OHC	7-keto-27-hydroxycholesterol
7α-OHC	7α-hydroxycholesterol
7α,25-OHC	7α, 25-dihydroxycholesterol
7α,27-OHC	7α, 27-hydroxycholesterol
7β-OHC	7β-hydroxycholesterol
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ACAT	Acyl-CoA:cholesterol acyl transferase
AD	Alzheimer´s disease
APP	Amyloid precursor protein
BBB	Blood-brain barrier
CH25H	Cholesterol 25-hydroxylase
ChEH	Cholesterol-5, 6-epoxide hydrolase
CNBP	Cellular nucleic acid binding protein
CYP46A1	Cholesterol 24-hydroxylase
CYP46A1	Cytochrome P450 46A1
CYP7A1	Sterol 7α-hydroxylase
CYP27	Sterol 27-hydroxylase
CYP8B1	Cholesterol 12α-hydroxylase
DDA	Dendrogenin A
DHCA	Dihydroxycholestanoic acid
ER	Endoplasmic reticulum
HSD	Hydroxysteroid dehydrogenase
HSD3B7	3β-hydroxysteroid dehydrogenase type 7
LDL	Low density lipoproteins

LXR	Liver X receptor
MS	Multiple Sclerosis
NAFLD	Non-alcoholic fatty liver disease
OSBP	Oxysterol binding protein
PD	Parkinson´s Disease
POPC	1-palmitoyl-2-oleoyl-phosphatidylcholine
SCAP	SREBP cleavage activation protein
SMO	Smoothed receptor
SREBP	Sterol regulatory element binding protein
StAR	Steroidogenic acute regulatory protein
THCA	Trihydroxycholestanoic acid
TSPO	Mitochondrial translocator protein
ROS	Reactive oxygen species
VLCFA	Very long chain fatty acids
WMH	White Matter Hyperintensities

Abstract

Oxysterols are oxidized derivatives of cholesterol that are formed enzymatically or via reactive oxygen species or both. Cholesterol or oxysterols ingested as food are absorbed and packed into lipoproteins that are taken up by hepatic cells. Within hepatic cells, excess cholesterol is metabolised to form bile acids. The endoplasmic reticulum acts as the main organelle in the bile acid synthesis pathway. Metabolised sterols originating from this pathway are distributed within other organelles and in the cell membrane. The alterations to membrane oxysterol:sterol ratio affects the integrity of the cell membrane. The presence of oxysterols changes membrane fluidity and receptor orientation. It is well documented that hydroxylase enzymes located in mitochondria facilitate oxysterol production via an acidic pathway. More recently, the presence of oxysterols was also reported in lysosomes. Peroxisomal deficiencies favour intracellular oxysterols accumulation. Despite the low abundance of oxysterols compared to cholesterol, the biological actions of oxysterols are numerous and important. Oxysterol levels are implicated in the pathogenesis of multiple diseases ranging from

chronic inflammatory diseases (atherosclerosis, Alzheimer's disease and bowel disease), cancer and numerous neurodegenerative diseases. In this article, we review the distribution of oxysterols in sub-cellular organelles and in biological fluids.

Key words: Oxysterols; subcellular; membrane; mitochondria; peroxisomes; fluids

1-Biosynthesis of oxysterols in cells

Oxysterols, oxygenated derivatives of cholesterols, are involved in many biological processes including the regulation of cholesterol homeostasis. While physiological levels of oxysterols are found to be implicated in many biological functions, imbalance of oxysterol homeostasis is reported to have impact on pathophysiology. Oxysterols are implicated as the initiation and progression of many chronic diseases in the literature; osteoporosis, age related macular degeneration, cataract, atherosclerosis, neurodegenerative diseases (e.g. Alzheimer's and Parkinson's diseases, and multiple sclerosis) [1-4]. Chemically, the addition of one or more oxygenated functional groups to 27-carbon cholesterol molecule changes its behaviour and receptor recognition. Oxidation can occur on the cluster of four hydrocarbon rings (A, B, C and D) or on the side chain of the cholesterol molecule. The double bond present on the B ring is the most energetically favourable target of free radical attack and therefore carbon atoms at the positions of 4, 5, 6 and 7 are more susceptible to free radical oxidation. Commonly observed B-ring oxidised oxysterols include hydroxylated compounds [7α- and 7β-hydroxycholesterol (7α-OHC and 7β-OHC)], oxysterols with a ketone group [7-ketocholesterol (7-KC)], epoxy cholesterols [5β, 6β- epoxy cholesterol (5β, 6β-epox), 5α, 6α- epoxy cholesterol (5α, 6α-epox)] and cholestan−3β, 5α, 6β‑triol [5-8].

In addition to ring oxidation, cholesterol undergoes side chain oxidation to generate oxysterols; 20‑hydroxycholesterol (20-OHC), 22‑hydroxycholesterol (22-OHC), 24‑hydroxycholesterol (24-OHC), 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC, formally known as is (25R) 26-hydroxycholesterol [9]). Further oxidation on cholesterol molecules generates oxysterol molecules with two or more different functional groups (e.g. 7α, 25-dihydroxycholesterol, 7α, 27-hydroxycholesterol and 7-keto-27-hydroxycholesterol). The addition of a hydroxyl group to a side chain mainly occurs via enzymatic pathways that mainly regulate cholesterol homeostasis. Two main groups of enzymes; oxidoreductases and transferases, are known to participate in the metabolism of

oxysterols either through neutral or acidic pathways [10]. Most of the oxidoreductases are members of the heme-containing cytochrome P450 (CYP) family of enzymes (Table 1). Among them, cholesterol 7α-hydroxylase (CYP7A1) and sterol 27-hydroxylase (CYP27) were the first to be identified [11]. CYP7A1 is known to initiate the neutral or classical pathway and CYP27 is known to initiate the acidic or alternate pathway of oxysterol metabolism. Griffiths and Wang explained that once 7α position of cholesterol is hydroxylated, oxysterols become substrates for the enzyme HSD3B7 (3β-hydroxysteroid dehydrogenase type 7) and can be converted from their 3β-hydroxy-5-ene to 3-oxo-4-ene forms [12]. Another dehydrogenase enzyme, 11 β-dehydrogenase isoenzyme 1 (11βHSD1) is responsible for the reversible interconversion of 7-KC and 7β-OHC [13]. Non-heme iron-containing oxidoreductase, cholesterol 25-hydroxylase (CH25H), has recently received attention due to the involvement of its product, 25-OHC, in immunity control [14]. However, 25-OHC has been reported to be produced through other cytochromes (CYP3A4, CYP27, and CYP46A1) and also through free-radical reactions [15-17].

Substrate	**Enzyme**	**Oxysterol derivative**
Cholesterol	CYP3A4	4β-OHC
Cholesterol	CYP7A1	7α-OHC
Cholesterol	CYP11A1	20R-OHC
Cholesterol	CYP11A1	22R-OHC
Cholesterol	CYP46A1	24S-OHC
Cholesterol	CH25H	25-OHC
Cholesterol	CYP27	27-OHC
7α-OHC	CYP27	7α,26-diOHC
22R-OHC	CYP11A1	20R,22R-diOHC
24S-OHC	CYP39A1	7α,24S-diOHC
25-OHC	CYP7B1	7α,25-diOHC
27-OHC	CYP7B1	7α,27-diOHC

Table 1: Oxidoreductase enzymes involved in the metabolism of oxysterols

Similar to cholesterol, oxysterols can also be sulfated by sulfotransferases (SULT2B1b, SULT2B1a and SULT2A1) in the position 3 of the steroid ring A. The addition of a very polar sulfate group to the structure of steroids increases their solubility in water and facilitates their urinary excretion. Even though the sulfated cholesterol is widely described in literature, only some oxysterols (7-KC, 24-OHC, 25-OHC and 27-OHC) are reported to be sulfated. Among these, 25-hydroxycholesterol-3-sulfate (25OHC3S) has been described as a biomarker and a therapeutic target for non-alcoholic fatty liver disease [18], metabolic syndrome and obesity [19, 20]. This sulfated oxysterol is initially synthesised by CYP27 in mitochondria. Ren et al. has shown that in primary rat hepatocytes, 25OHC3S is

accumulated in nuclei after the overexpression of a mitochondrial cholesterol delivery protein, StarD1 [21].

2-Cellular distribution of oxysterols

Cells take up cholesterol/oxysterols packed in lipoproteins with help of the LDL receptor (LDLR) family, which comprises of several multifunctional cell surface proteins that binds and endocytose ligands. Even though this is the main route of cholesterol and oxysterol transportation into cells, there are other receptors found to be involved in oxysterol sensing and transport **(Table 2)**. EBI2 (Epstein-Barr-virus-induced G-protein coupled receptor 2, also known as GPR183) is one of the membrane bound receptor known to bind oxysterols. EBI2 has high affinity towards 7α,25OHC and plays an important role by directing the migration of activated B cells to interfollicular and outer follicular regions of secondary lymphoid tissues [22]. Another membrane bound G-protein coupled receptor, GPR17 has affinity to bind 7α-OHC, 22(R)-OHC and 27-OHC [23]. CXCR2 (C-X-C motif chemokine receptor 2) was shown to bind 22(R)-OHC and 4β-OHC that facilitate tumor-infiltrating immune cells [24].

Inside the cell, endosomes containing cholesterol/oxysterol then interact with a complex series of oxysterol receptors such as the oxysterol binding protein (OSBP), OSBP-related proteins (ORPs), the cellular nucleic acid binding protein, the sterol regulatory element binding protein (SREBP), insulin induced gene protein (INSIG) and Niemann-Pick protein C1 (NPC1). The location of these enzymes may play an important role in the oxysterol concentration in cellular micro-environments.

SREBPs located in ER membrane are transported to Golgi through the formation of a complex with an escort protein called Scap. Proteases within the Golgi release a transcriptionally active fragment, which allows the nuclear translocation. The active form of SREBP initiate the transcription of genes encoding 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) and other enzymes involved in the synthesis and uptake of cholesterol [25]. The INSIG acts as a regulatory protein in SREBP pathway and interaction of oxysterols such as 22(R)-OHC, 24(S)-OHC, 25OHC and 27-OHC with INSIG prevent SREBP transportation to Golgi [25]. OSBPs and its related proteins are a diverse class of proteins that are located in ER membrane, nuclear membrane, late endosomes and cytosol [26]. ORPs binds to 25-OHC, 22(R)-OHC and 7-KC to control cholesterol metabolism [26]. The NPC-1 protein is a late endosomal protein that play an important role in the delivery of low density lipoprotein-derived cholesterol and oxysterols to the ER. Within the nucleus, the Liver X receptors (LXR α or β), along with retinoid X receptors (RXRs) regulate gene transcription as heterodimeric complexes.

Both enzymatically and non-enzymatically derived oxysterols act as important intermediates in steroid and bile acid synthesis pathways [4]. This is mainly attributed to the increased hydrophilicity of oxysterols allowing them to move much more rapidly between the intracellular membrane organelles and facilitating receptor accessibility.

However, reliable measurements of oxysterol levels and activities are limited by their low physiological concentrations (approximately 1000 times lower) relative to cholesterol. Reyk et al showed that total oxysterol levels in human macrophage foam cells from atherosclerotic plaques are 42 times lower than total cholesterol [27]. Among the analysed oxysterols, 27-OHC was 100 fold lower than cholesterol and 24-OH or 25-OHC was more than 1000 fold lower. Recent advances in analytical equipment and column chromatography techniques have substantially improved our understanding of low abundant classes of oxysterols [2, 28]. It is important to note that the level of oxysterol distribution may differ according to the cell type, tissue and organism.

2.1-Oxysterols in membranes

It is well known that the presence of cholesterol is a major regulator of phospholipid bilayer fluidity. Cholesterol molecules align themselves with the hydroxyl groups close to the hydrophilic heads of the phospholipids. The sterol ring structure interacts with sections of the hydrocarbon chain closest to the phospholipid head leaving the rest of the tail flexible. This stiffens the membrane and prevents it from acting as a liquid. Therefore, the apolar part of a cholesterol molecule is deeply integrated into the lipid bilayer [29]. It has been suggested that certain membrane structures with domains rich in cholesterol are more energetically favourable than membranes with a more uniform distribution of cholesterol. Cholesterol molecules exhibit perpendicular tilts in membranes which restricts their motion along bilayers at high concentrations. Also, when cholesterol is present at higher concentrations in membranes (50%), the membrane area is significantly reduced [30]. It has also been proven that the presence of oxidised cholesterol, in particular, tail-oxidised sterols, changes the permeability of lipid bilayers drastically as opposed to the presence of cholesterol [31].

Oxysterols within plasma membranes in cells have been known to exert various effects on cell membrane dynamics. In particular, the specific orientation of certain oxysterols has direct effects on permeability. For example in liposomes, 7-KC, 7β-OHC and 7α-OHC are shown to reduce glucose permeability as well as displaying a condensing effect in mixed monolayers. 7-KC, 7β-OHC, 7α-OHC and 25-OHC are all perpendicularly ordered at the membrane interphase, with only 7β-OHC and 7α-OHC being tilted at low surface pressures [4]. Also, 25-OHC does not display a condensing effect in

membranes as it acts as a 'spacer' molecule instead. This is thought to be due to imperfections in the packing of the bilayer core due to the position of the hydroxyl group on 25-OHC, causing increased permeability **(**Figure 1**)**. The translocation of oxysterols from membranes to vesicles has been tracked via radiolabelling, showing that the transfer rate increases in the following order: 7-KC < 7β-OHC < 7α-OHC < 25-OHC [32].

More recently, further work has been undertaken to identify specifically why certain oxysterols behave in a different manner to others in terms of membrane dynamics [32]. It has been shown that ring-oxidised sterols can develop a tilted orientation inside lipid bilayers which greatly disrupts membrane structure and thus interferes with signalling cascades. Among other effects, tail-oxidised sterols breach the permeability barrier of membranes when they undergo reorientation as opposed to cholesterol, by increasing the movement of small solutes and water through the membrane [33].

Studies to understand oxysterol behaviour in phospholipid membranes are ongoing with current computer simulation studies [33, 34]. For example, Olsen et al. explained that when 25-OHC is added to 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) bilayers at low concentrations, it expands the bilayers, increasing the solvent exposure of nearby phospholipids [33]. Their work suggested that oxysterols interfere with phospholipid shielding of cholesterol and increase the availability and exposure of cholesterol on membranes. Change of membrane fluidity and cholesterol/oxysterol composition also changes their interaction with integral and peripheral membrane proteins. Oxysterols are recognised by a family of nuclear receptors including liver X receptors (LXRs). Activated LXRs drive LXR-dependent transactivation of multiple genes involved in cellular cholesterol homeostasis, including ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1), and APOE. LXR activated target genes enhance cholesterol efflux and limit uptake of lipoprotein-derived cholesterol by inducing expression of the E3 ubiquitin ligase IDOL (inducible degrader of the low-density lipoprotein (IDOL). It is suggested that cells mainly export oxysterols as an alternative mechanism to the classical high density lipoprotein (HDL)-mediated reversed cholesterol transport. Nunes et al demonstrated that plasma 27-hydroxycholesterol was increased in the Low HDL subjects as compared to High HDL subjects and cholesterol efflux was lower in Low HDL compared to High HDL, suggesting alternative cholesterol excretion pathway to maintain cellular cholesterol levels [35].

Skin cells have been shown to express high levels of 25-OHC after UV irradiation leading to cell death by pyroptosis and apoptosis via P2X7 receptor pathways [36]. In the plasma membranes of nerve cells, the interactions of 7-KC, 7β-OHC and 24(S)-OHC with the P2X7 receptor has been shown to

activate the ATP-gated P2X7 receptor that triggers the formation of large nonselective membrane pores which results in inflammasome activated cell death [37].

Recent studies have identified new membrane protein interactions with different oxysterols, explaining their diversity and opening new therapeutic avenues. For example, Voisin et al. highlighted a pathway where 6-oxo-cholestan-3β,5α-diol drives breast cancer progression through the interaction between the glucocorticoid receptor [38]. The identification of 5, 6-epoxy cholesterols (5, 6-epox) centred new metabolic pathways, which are being explored. Based on the conjugation products of 5, 6-epox with biogenic amines that are known to interact with cholesterol-5, 6-epoxide hydrolase (ChEH), a hetero-oligomeric complex of two cholesterogenic enzymes that control mammalian developmental programs, led to the identification of the steroidal alkaloid, dendrogenin A (DDA). DDA is a selective inhibitor of cholesterol epoxide hydrolase and it triggers tumour re-differentiation and growth control *in vivo* [39]. Such discoveries reveal the interactions of cholesterol metabolic pathways that lead to the production of a metabolic tumour suppressor and neuroprotective agent.

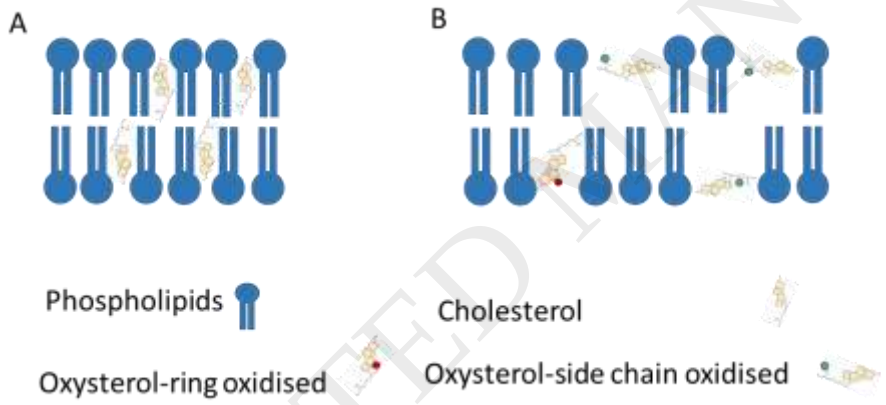

Figure 1: Arrangement of cholesterol and oxysterol within phospholipid bilayer. Polar head group of the cholesterol positions near aqueous environment and its hydrophobic body buried in the bilayer providing tighter packing (**A**). Additional oxidation of the cholesterol molecule alters its polarity distribution (**B**), where the ring axis is tilted towards bilayer surface. This orientation of oxysterols contribute to thinner membranes.

2.2-Oxysterols in membrane lipid micro domains

Raft-like membrane lipid micro domains are small sub domains of plasma membranes which are made up of high concentrations of cholesterol and glycosphingolipids. It was previously thought that

all lipids could diffuse freely within plasma membranes until it was discovered that specific sub domains form these lipid micro domains that can translocate laterally within membranes [40]. Lipid micro domains are thought to be implicated in signal transduction regulation by existing as a signalling platform via the localisation of receptors, effector enzymes, and substrates in a single raft leading to rapid signal transduction due to this close proximity [41]. The association of certain oxysterols on lipid micro domains has shown that 25-OHC acts as a promoter of lipid raft formation and 7β-OHC acts as an inhibitor of lipid micro domain formation [42]. 7-KC is already known to be highly implicated in the development of atherosclerosis as it is a component of oxidised LDL, and researchers used this to determine how incorporation of 7-KC into lipid micro domains impacts calcium ion release in monocytes leading to apoptotic effects. 7-KC treatment causes alterations in lipid micro domains leading to various signalling pathways which ultimately lead to cell death [43]. When neuroblastoma cells were treated with non-toxic levels of 27-OHC, lipid micro domain formation was upregulated in a redox driven mechanism [44].

Other ways in which oxysterols influence membrane dynamics may occur when two or more oxysterols interact with each other. This is due to the altered polarity in oxysterols which changes interactions between the membrane components. Two oxysterols; 7-KC and 25-OHC displayed interactions in model membranes causing changes to membrane properties compared to the presence of cholesterol only [45]. Compression elasticity data shows that the ketone functional group on the steroid nucleus of 7-KC is what interferes with sterol-phospholipid packing [46]. A similar principle is also observed when cell membranes are introduced to 7-KC; this particular oxysterol induces changes in cholesterol efflux, a crucial process in anti-atherogenesis. This efflux was analysed from lipoprotein-loaded THP-1 cells, plasma membrane vesicles, and from artificial, protein-free liposomes. The presence of 7-KC decreases the efficiency at which cholesterol is removed from cells to lipid free apoprotein A-I [47].

Research into the kinetic effects of microsolubilisation of a membrane model (DMPC) vesicles by apolipoprotein A-I found that the formation of discoidal high-density lipoproteins was affected by oxysterol chemical structure. Varying oxysterol chemical structure, as well as sterol concentration and the lipid phase state of DMPC, confirmed that some oxysterols had a similar effect to cholesterol and led to an increased rate of microsolubilisation, whereas other oxysterols acted as inhibitors of this process. Change in the membrane surface due to imperfections in packing of lipid phases is the reason for this variation in microsolubilisation, as some oxysterols have the ability to induce the formation of an ordered lipid phase [48].

2.3-Oxysterols in Endoplasmic Reticulum and Golgi

Cholesterol homeostasis within the cell and balanced ratio of phospholipids to cholesterol, on cell membranes is controlled by the transportation of membrane-bound, SREBP molecular machinery on the ER to the Golgi complex [49]. Multiple *In vitro* studies have shown that oxysterols can control the SREBP pathway [25, 50, 51]. However, it is not yet clear whether these mechanisms are applicable to *In vivo*. Despite being a cholesterol poor environment (3-6% total lipids), compared to cholesterol-rich plasma membranes (30-50% of total lipids) [52], SREBPs sense cholesterol and oxysterol levels on ER membranes. An escort protein called Scap forms a complex with SREBPs aiding transportation of SREBP:Scap from ER to Golgi. Both cholesterol and oxysterols are reported to inhibit this movement by inducing Scap to bind to either of two related ER anchor proteins called Insig-1 and Insig-2 [25, 49]. While the direct binding of cholesterol cause conformational change to Scap resulting Scap:Insig interaction, Oxysterols, at least 25-OHC does not make direct interaction with Scap *in vitro* [53]. Instead, Sun et al demonstrated that oxysterols are directly bind to Insig proteins *in vitro*, suggesting an alternative mechanism on ER membrane to promote Scap:Insig interaction [53, 54].

A remarkable ability of oxysterols to induce rapid changes in cellular cholesterol levels is reported via the action of ER-resident protein acyl-CoA : cholesterol acyl transferase (ACAT) [55]. While the transcriptional pathway to reduce cellular cholesterol level occurs within hours, side-chain oxysterols such as 25-OHC have been shown to reduce the free cholesterol burden by increasing cholesterol esterification within minutes [56]. In addition to direct activation of ACAT, 25-OHC also promoted the movement of plasma membrane cholesterol to an ACAT-accessible pool in the ER [55, 57]. This explains how 25-OHC is not directly bound to SCAP [53] but enables the cell to adapt rapidly within minutes, to excess free cholesterol, well in advance of changes in cholesterol balance mediated by SREBP and LXR target genes [58].

2.4-Oxysterols and mitochondria

Cholesterol is transported to mitochondria from the endoplasmic reticulum (ER) either by direct membrane contact between the ER and mitochondria or mobilised in lipid droplets (cholesterol esters) [59],[60]. In eukaryotic cells, steroidogenic acute regulatory protein (StAR), which is a critical regulator for the synthesis of adrenal and gonadal steroids, is known to transport cholesterol from the outer to inner membrane in mitochondria [59, 61]. Caveolin-1, a protein involved in membrane organization and signalling was located in mitochondria from hepatic cells, and has been

considered as a potential cholesterol importer [62]. However, there is no direct evidence of caveolin-1 transporting cholesterol in mitochondria. Mitochondria contains lower levels of cholesterol when compared to the ER and plasma membrane [63]. Mitochondrial translocator protein (TSPO) that has high affinity for cholesterol, is primarily located in the outer mitochondrial membrane [64]. TSPO plays a functional role in cholesterol transport to the inner mitochondrial membrane. Both StAR and TSPO co-operate in mitochondrial cholesterol trafficking and their functional co-operation was shown to be necessary for steroid hormone biosynthesis in Leydig cells [65, 66].

Cholesterol metabolism in mitochondria is important for the production of bile acids and steroid hormones. Cholesterol is oxygenated in mitochondria by cytochrome P450 enzymes or by reactive oxygen species into oxysterols, which are the intermediates in bile acid and steroid hormone synthesis pathways [67, 68]. Mitochondria initiate acidic (or alternative) synthesis of oxysterols via CYP27, which is located in the inner mitochondrial membrane. CYP27 hydroxylate cholesterol to form 27-OHC and 25-OHC followed by rapid 7α-hydroxylation via microsomal oxysterol 7α-hydroxylase (CYP7B1) [69, 70]. Mutation in the CYP27 cause the establishment of Cerebrotendinous xanthomatosis (CTX), a rare autosomal-recessive lipid storage disease [71]. The lack of this enzyme reduce the level of the end product of the pathway, chenodeoxycholic acid and subsequent upregulation of cholesterol 7α-hydroxylase. As the rate limiting enzyme in classical bile acid pathway, upregulation of 7α-hydroxylase results raised levels of 7α-hydroxy-4-cholesten-3-one and accumulation of cholestenol in cells and tissue. Patients with CTX display a broad range of neurological and non-neurological symptoms with multiple organ involvement [72].

Mitochondrial oxysterol metabolism may play important roles in pathogenesis of diseases such as atherosclerosis, carcinogenesis or Alzheimer's disease, and it is an area of extensive research for developing novel therapies. Mitochondrial CYP27 plays an important role in cholesterol homeostasis. In hepatic cells, CYP27 hydroxylase initiates an alternative pathway of bile acid synthesis, which constitutes 10% of bile acid production [73], while in non-hepatic cells, this enzyme participated in the reverse cholesterol transport to the liver [74]. 27-OHC is the most abundant oxysterol reported in human atheroma, and the CYP27 enzyme that produces this oxysterol has been regarded as a defence mechanism to prevent macrophage cholesterol accumulation [61]. Westman et al. showed that in cholesterol-laden human alveolar macrophages, excess cholesterol was removed from the macrophages as 27-OHC and 3β-hydroxy-5-cholestenoic acid [75].

2.4.1-Mitochondrial membrane microdomains

In mitochondria, raft-like microdomains were associated with membrane scrambling during apoptosis, changes in mitochondrial curvature, recruitment of proteins associated with mitochondrial fission or fusion and mitochondrial oxidative phosphorylation and ATP production [76, 77]. Even though the effects of oxysterol accumulation and their ability to modify membrane properties were previously studied in plasma membranes, there is no evidence of structural effects of oxysterols on mitochondrial membranes. It is possible that the accumulation of oxysterols could also negatively affect mitochondrial microdomain formation and thereby causes mitochondrial dysfunction. Future research to study the relation between oxysterol content and lipid raft domains will help to uncover the mechanisms if, and how oxysterols alter mitochondrial membrane structure and function.

2.4.2-Oxysterol induced mitochondrial dysfunction

Embryonic stem cells are an emerging tool for cell therapy applications and recently, researchers studied the role of oxysterols in mitochondrial function during osteogenic stem cell differentiation [78]. Osteogenic stem cells were treated with a combination of 22(S)-OHC and 20(S)-OHC, and discovered upregulated mitochondrial activities such as intracellular reactive oxygen species production, intracellular ATP content, membrane potential, mitochondrial DNA copy number, mitochondrial biogenesis and increased levels of respiratory proteins complex I-IV.

Dysregulation of cholesterol metabolism in mitochondria and resulting lipotoxicity has been identified in many metabolic diseases such as cancer, heart and liver diseases [79, 80]. Fatty acid and cholesterol accumulation in hepatocytes undergoes lipid peroxidation to form oxysterols that causes impairment of mitochondrial function, biogenesis and depletion of respiratory chain complexes in non-alcoholic fatty liver disease (NAFLD) [81, 82]. A related study showed that when hepatocytes were exposed to the combination of fatty acids (palmitic and oleic) and oxysterol cholestane-3β,-5α,-6β-triol, they showed mitochondrial dysfunction [83, 84]. There is evidence for alterations in the oxysterol profiles and the synergistic interaction of free fatty acids and oxysterols, impairing mitochondrial biogenesis, which underpins the progression of liver disease [82, 83]. 7β-OHC, 7KC and triol concentrations were elevated in NAFLD and were associated with increased H_2O_2 production, uncoupling and impaired ATP homeostasis in mitochondria and induced mitochondria-dependent apoptosis [83, 85-87]. In human and rat hepatocellular tumour cells, there were increased levels of cholesterol in mitochondrial membranes that altered membrane dynamics, contributing to mitochondrial dysfunction [45]. Oxysterols 7β-hydroxysitosterol and 7β-OHC induced

loss of mitochondrial membrane potential, membrane permeability, oxidative stress and apoptosis of human colon cancer, Caco-2 cells [88]. In murine 158N oligodendrocytes, ROS derived 7-KC and 7β-OHC was elevated and induced apoptotic cell death involving mitochondrial depolarization [89]. From previous research, it is clear that oxysterols cause mitochondrial dysfunction by altering mitochondrial transmembrane potential and mitochondrial membrane permeability in human diseases [90-92].

2.5-Oxysterols and Lysosomes

Sterols, including oxysterols, enter the cell via receptor-mediated endocytosis of low density lipoproteins (LDL) and traffic to the lysosomes, which are a major site of non-enzymatic oxysterol formation. Within the lysosome, several oxysterols generated through oxidative processes accumulate [93, 94]. Thus, among these oxysterols, 7KC is found at the highest level in the endosomal and lysosomal compartments [93]. In addition, human pro-monocytic U937 cells treated either with 7-KC, 7β-OHC, or cholesterol-5beta, 6beta-epoxide, show an important accumulation of 7-KC, 7β-OHC. Cholesterol-5β, 6β-epoxide are found in acidic compartments of the cytoplasm (phagolysosomes) associated with membrane whorls designed as ‘myelin figures’ [95-97]. This accumulation of oxysterols in myelin figures has been considered first as a phospholipidosis process to attenuate the cytotoxic effects of oxysterols. It is now suggested that these myelin figures could also include autophagic vesicles (resulting from reticulophagy) involving the fusion of the autophagosome, containing large parts of endoplasmic reticulum, within the lysosome [98]. It is suggested that the process of phospholipidosis would prevent the intracellular accumulation of 7KC which favours lysosomal membrane destabilization and contributes to cell death induction [97]. Altogether, these data suggests for development of a strategy to target cholesterol oxidase in the lysosome to prevent 7KC-induced cell death [99]. This strategy supports that the introduction of novel catalytic functions into the lysosome could be of therapeutic interest to prevent major age related disease associated with increased intracellular 7KC levels such as cardiovascular diseases, age related macular degeneration and Alzheimer’s disease [100].

2.6-Oxysterols and Peroxisomes

Peroxisomes were identified in the 1950s by de Duve and co-workers using fractionation techniques and enzymatic activity analyses [101]. Based on transmission electron microscopy analyses, these structures were called 'micro bodies' [102]. The term peroxisome was introduced in 1965 by

Christian de Duve due to the capacity of these organelles of producing (oxidases) or degrading (peroxidases) hydrogen peroxide (H_2O_2) [103]. Peroxisomes have different shapes; they could be observed on the elongated tubular form (>2 µm in length) or spherical form (0.1-1 µm) including rod shaped [104]. The peroxisome contributes to the most important metabolic processes: beta-oxidation of very long chain fatty acids (VLCFA: C22 and higher), branched chain amino acids and fatty acids (e.g., pristanic acid), bile acid intermediates (Di and trihydroxycholestanoic acid: DHCA, THCA) and long chain dicarboxylic acid, alpha-oxidation of phytanic acid, glyoxylate detoxification, biosynthesis of ether phospholipids (plasmalogens) and metabolism of reactive oxygen species [105-107]. There is evidence that peroxisomes are involved in cholesterol oxidation, biosynthesis and trafficking [108-110]. Using RNA interference directed knockdown of ABCD1 and ACOX1 peroxisomal proteins in 158N murine oligodendrocytes, it was shown that the peroxisomal dysfunctions enhanced oxidative stress [111]. This dysfunction also induced a subsequent disruption of redox homeostasis, which favoured cholesterol auto-oxidation, mainly the intracellular accumulation of oxysterols oxidized at C7 (7-KC, 7β-OHC) [111, 112]. In agreement with these previous data, there were, abnormal levels of 7α-OHC, 7β-OHC, 7-KC, 25-OHC, 27-OHC and 5β-cholestane 3α, 7α, 12α-triol in CRISPR/Cas9-mediated knockout of Abcd1 and Abcd2 genes in murine microglial BV-2 cells as compared to the wild type BV-2 cells [113]. This *in vitro* study on nerve cells provides evidence that the peroxisome is involved in oxysterol homeostasis. As oxysterols are involved in signalling response either by altering cell membrane properties or by binding and activating several receptors [114], it is tempting to speculate that peroxisomal alterations which are only few studied, except in the context of peroxisomopathies leading to severe neurodegenerative diseases [115], could have several impacts on cell activity.

Organelle	**Proteins involved in cholesterol and oxysterol homeostasis**
Membrane	GPRI83/EBI2 GPRI7 CXCR2

	ABCA1, ABCG1 Glucocorticoid receptor P2X7 receptor SMO NMDA NPC1
ER and Golgi	SREBPs SCAP START OSBP OSBP-related proteins (ORPs) INSIG
Nucleus	Nuclear receptor family (LXR, RXR, RAR-related orphan proteins (RORα, RORβ and RORγ) Estrogen receptor α (ERα)
Cytoplasm	OSBP StARD4/5
Mitochondria	StARD1 TSPO
Lysosome	NPC2 Acid lipases
Peroxisome	ABCD1 , ABCD2, ACOX1

Table 2: Sub-cellular distribution of proteins involved in cholesterol and oxysterol homeostasis.

3-Oxysterols in biological fluids

Oxysterols in biological fluids can originate from the diet or through endogenous enzymatic or non-enzymatic reactions. Oxysterols, which are already present in food are formed by auto-oxidation induced by processes such as heat, light exposure, refrigeration or freeze-drying [116, 117].

Oxysterols ingested in food or excreted by the liver to bile are absorbed into the human small intestine through molecularly defined absorption pathways in enterocytes [118]. Together with other lipid forms, free and esterified, they are then incorporated into chylomicrons, VLDL, LDL and HDL for easy transportation.

3.1-Oxysterols in plasma

Different classes of oxysterols generated via both neutral and acidic pathways of bile acid biosynthesis are observed in human plasma [119]. It is estimated that oxysterol concentration in plasma is 1000, or greater-, fold lower than the levels of cholesterol [27]. Despite their low levels compared to cholesterol, it is increasingly convincing that oxysterols are involved in pathophysiology of cardiovascular disease. Oxysterol containing oxidised low-density lipoprotein (oxLDL) play a distinct role in atherosclerotic lesion formation, initiation of endothelial dysfunction, intimal thickening and arterial stiffness due to increasing collagen deposition and calcification [120]. Lipid lowering statin treatments can effectively decrease plasma levels of oxysterols [121]. The role of oxysterols and atherosclerosis [112], vascular ageing[122] [120] and other major chronic diseases [1, 116] are extensively reviewed elsewhere.

In this review, we will focuses on two enzymatically formed oxysterols in human circulation; 27-OHC and 24-OHC in relation to Alzheimer's disease (AD). Neuronal derived 24-OHC is extensively researched in relation to neurodegeneration, compared to hepatically and extrahepatically derived 27-OHC. Unlike cholesterol, polar oxysterols can cross endothelial barriers including tight junction-rich blood-brain barrier (BBB) [123]. Fluctuation to plasma oxysterol levels have been described in neurodegenerative diseases such as Alzheimer´s disease (AD), Multiple Sclerosis (MS) or Parkinson´s Disease (PD) [124].

3.2-Plasma oxysterols in Alzheimer's disease

Case-control studies on patients with early cognitive impairment have shown a small non-significant increase of plasma 24-OHC, 7-KC and 7β-OHC levels [125, 126] and a significant increase of 27-OHC [127] compared with cognitively normal controls. Studies that analysed AD plasma reported significantly higher absolute levels of plasma 24-OHC and 27-OHC than in healthy controls [126, 128-130]. However, other studies did not make the same observation [6, 125, 131]. When AD is stratified by early versus late stage of disease, plasma 24-OHC levels are higher in the early stages compared to later stages of AD [126, 132]. Accordingly, AD patients with longer disease duration had lower levels of oxysterols than patients with subjective cognitive impairment or cognitively healthy controls. In 2012, a longitudinal study reported that cognitively healthy participants with higher

plasma 24-OHC were more likely to develop incident cognitive impairment over 8 years of follow-up and that these differences in plasma oxysterols occurred at least four years prior to the onset of cognitive impairment [133].

It can be argued that part of the controversies reported in cross-sectional studies is due to the observation that oxysterol levels can change longitudinally during the evolution of the disease. Whether increased oxysterol levels in early stages reflect that altered cholesterol metabolism is involved in the pathophysiology of AD or is just a consequence of the degenerative process is still unclear. The decrease in later stages could reflect a selective loss of neuronal cells expressing the enzyme cholesterol 24-hydroxylase, CYP46A1 [134].

Popp et al. found a positive association between plasma 27-OHC levels and soluble amyloid protein precursor (APP)-β levels [129]. However, at present, no strong correlation has been reported between plasma oxysterols and traditional AD biomarkers of amyloid, tau, p-tau [129]. When patients with cognitive complaints are stratified according to the biomarker levels, patients with AD-like pathology, that is, lower levels of amyloid and higher levels of tau, have lower plasma 24-OHC and 27-OHC [135]. In mild cognitive impairment (MCI), 24-OHC/27-OHC correlates with amyloid deposition [136]. This may suggest that the possible correlation between plasma oxysterols and CSF biomarkers in the early stages of the disease disappears as the disease progresses when oxysterol and biomarker levels are completely altered.

The higher rates of atrophy are associated with a progressive reduction of the plasma levels of 24-OHC [137]. A significant correlation of 24-OHC with hippocampal volume [138] or the whole grey matter volume was found in mid-age or aged individuals [137]. In summary, current literature supports that brain cholesterol metabolism is altered in AD and is related to disease severity, neurodegeneration and atrophy.

3.3-Oxysterols in cerebrospinal fluid

Cerebrospinal fluid (CSF) is the body fluid most likely to reflect brain biological changes because of its close physical contact with the brain tissue, representing an easily accessible window to explore biochemical insights into neurological disorders [139]. While approximately 99% of the daily production of the brain oxysterols enters the circulation via the blood brain barrier (BBB), less than 1% enters the CSF [5, 140], with lower oxysterols concentration in CSF than in plasma. However, since plasma oxysterol levels could be altered by exogenous or endogenous cholesterol metabolism, CSF oxysterols are more consistently related to neuronal diseases [141]. Therefore, alterations to CSF lipid content are suggested as biomarkers of neurodegenerative changes.

In case-control studies, patients with mild cognitive impairment show higher CSF 24-OHC and 27-OHC levels than healthy controls [6, 132, 142-145]. Moreover, patients with MCI that progress to AD have higher levels of 24-OHC than stable MCI patients that do not progress to dementia [142]. This increase has also been described in AD patients respect to controls [129-132, 142-145], but not observed in Kolsch´s study [146]. Whether the increased levels of 24-OHC and 27-OHC are just a consequence of neurodegeneration or whether membrane cholesterol metabolism is involved in AD pathophysiology remain to be clarified. Increased levels of 24-OHC and 27-OHC in MCI patients could reflect both AD and cerebrovascular disorders as the underlying pathologies, suggested by Leoni and colleagues [144]. In MCI patients that progress to dementia, the proportion of subjects with altered 24-OHC levels are higher than the proportion of subjects with pathologic levels of $A\beta_{42}$, Tau or P-tau. In contrast, in AD subjects, the fraction of patients with pathological levels of 24-OHC is similar or even lower than the fraction of patients with pathological Tau, P-tau, and $A\beta_{42}$ [142, 144]. While this finding could be related to the loss of 24-OHC producing neurons in the advanced stages it could also suggest that stressed cholesterol metabolism could be involved in the pathophysiology of the early stages. Additionally, decreased activity of CYP46A1 has been also suggested to be implicated in the synaptic and memory dysfunctions caused by Tau pathology [147].

With regard to the APOE genotype, both 24-OHC and 27-OHC increase proportionally to the number of e4 alleles in individuals with cognitive decline [132, 144, 148]. Additionally, there is a positive correlation between levels of APOE and 24-OHC in CSF from patients with AD and MCI [132, 142, 143].

Besga and colleagues reported that CSF 24-OHC levels were differentially associated with the White Matter Hyperintensity (WMH) severity. WMH load represents cerebral white matter lesions most likely related to small vessel disease whose prevalence increases with age and represent myelin damage [149]. In patients with CSF-defined AD-like pathology, CSF levels of 24-OHC was positively associated with WMH severity and in patients without AD-like pathology CSF levels of 24-OHC were negatively correlated with WMH. They suggest that in the CSF AD-like group, the positive correlation between oxysterols and white matter lesions could be understood as an increased elimination of cholesterol from ongoing demyelination [135].

In conclusion, and in the context of all these studies, it can be stated that oxysterol levels in CSF reflect changes in brain cholesterol metabolism in AD and may provide important clues about the underlying cellular mechanisms of disease.

3.4-Oxysterols in urine

Urine contains a large number of endogenous metabolites that reflect the metabolic status of an organism. Since urine is an easily available and inexpensive biological fluid that can be collected over time, it uses as an important source for disease biomarker studies [150]. However, the disadvantage of using urinary oxysterols as biomarkers is that most of the enzymatically generated oxysterols are produced in the early oxygenation reactions in bile acid pathway and later sulfonation and glycosylation steps modify oxysterols for easy urinary excretion. Despite these modifications, urinary oxysterols provide valuable information about the kidney function. Boaz et al. reported high level of urinary 7-KC and 7β-OHC in hemodialysis patients even in the presence of higher plasma alpha-tocopherol levels [151]. Another study by Siems et al., analysed dienes, 7β-OH, β-epoxy, α-epoxy, 20α-OH, α-triol, and 7-KC levels in end-stage renal disease (ESRD) patients and observed high levels of the measured oxysterols [152]. These studies concluded that accelerated oxidative stress and lipid peroxidation burden in kidney disease patients and the possibility of oxysterols exerted atherosclerosis-stimulating effects, which can contribute to the increased cardiovascular risk of ESRD patients.

4-Conclusion

Recent years have seen a rapid growth in the interest for understanding the roles of oxysterols in health and disease, and this has been stimulated by more advanced methods for analysis of oxidized moieties that are present in low abundance. We are beginning to see the data emerging from pre-clinical knockout of CH25H and CYP46A that point to oxysterol regulation of inflammation and neurodegeneration. This emerging field is likely to provide more clues to the roles of lipid metabolism, inflammation and neurodegeneration, and new avenues for therapeutic involvement.

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