Cholesterol and oxysterol sulfates: Pathophysiological roles and analytical challenges

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Cholesterol and oxysterol sulfates are important regulators of lipid metabolism, inflammation, cell apoptosis, and cell survival. Among the sulfate-based lipids, cholesterol sulfate (CS) is the most studied lipid both quantitatively and functionally. Despite the importance, very few studies have analysed and linked the actions of oxysterol sulfates to their physiological and pathophysiological roles. Overexpression of sulfotransferases confirmed the formation of a range of oxysterol sulfates and their antagonistic effects on liver X receptors (LXRs) prompting further investigations how the changes to oxysterol/oxysterol sulfate homeostasis can contribute to LXR activity in the physiological milieu. Here, we aim to bring together for novel roles of oxysterol sulfates to their physiological and pathophysiological roles. Overexpression of sulfotransferases confirmed the formation of a range of oxysterol sulfates and their antagonistic effects on liver X receptors (LXRs) prompting further investigations how the changes to oxysterol/oxysterol sulfate homeostasis can contribute to LXR activity in the physiological milieu. Here, we aim to bring together for novel roles of oxysterol sulfates, the available techniques and the challenges associated with their analysis. Understanding the oxysterol/oxysterol sulfate levels and their pathophysiological mechanisms could lead to new therapeutic targets for metabolic diseases.

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Abbreviations: 24HC, 24(S)-hydroxycholesterol; 24HC3S, 24(S)-hydroxycholesterol-3-sulfate; 24HCDS, 24(S)-hydroxycholesterol-3,24-disulfate; 25HC, 25-hydroxycholesterol; 25HC3S, 25-hydroxycholesterol-3-sulfate; 25HCDS, 25-hydroxycholesterol-3,25-disulfate; 26HC, (25R)-26-hydroxycholesterol; 26HC26S, (25R)-26-hydroxycholesterol-26-sulfate; 26HC3S, (25R)-26-hydroxycholesterol-3-sulfate; 27HC, 27-hydroxycholesterol; 5α,6α-ECS, 5α,6α-epoxycholesterol-3-sulfate; 5-LO, 5-lipoxygenase; 7KC, 7-ketocholesterol; 7KCS, 7-ketocholesterol-3-sulfate; ACN, acetonitrile; CS, cholesterol sulfate; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DOCK2, dedicator of cytokinesis protein 2; HMG-CoA reductase, 3-hydroxy 3-methylglutaryl-CoA reductase; LLE, liquid–liquid extraction; LXRs, liver X receptor α; LXRβ, liver X receptor β; Mincle, macrophage inducible Ca2+-dependent lectin receptor; MRM, multiple reaction monitoring; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PIP2, phosphatidylinositol diphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PregS, pregnenolone sulfate; RLXI, recessive X-linked ichthyosis; RORs, retinoic acid-related orphan receptor α; SL, sulfate-based lipids; SPE, solid phase extraction; SREBP-1, sterol regulatory element-binding protein-1; STS, steroid sulfotases; SULTs, sulfotransferases; TCR, T-cell receptor.

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1 | INTRODUCTION

Sulfate-based lipids (SL) represent a wide range of lipid classes, from low to high molecular weight compounds (Dias et al., 2019) with key functions in many aspects of human health and disease (Hu et al., 2007; Merten, 2001; Suzuki et al., 2003). The biotransformation of lipids by sulfation and desulfation reactions is fundamental to many cellular pathways. SL represent a diverse class of lipids including sulfate-, sulfonate-, and thiol- or thioether-based lipids (Dias, Ferreira, et al., 2019). In humans, steroid sulfates represent a highly abundant and extensively studied lipid class among the other glycerol-, sphingosine-, or taurine-derived lipids (Mueller, Gilligan, Idkowiak, Arlt, & Foster, 2015). Steroid sulfates were traditionally viewed as inactive precursors as they require active transport into cells via organic anion transporters. However, recent research suggests that these derivatives have active roles. For example, cholesterol sulfate (CS) acts as a signalling molecule (Shi et al., 2014), pregnenolone sulfate (PregS) and dehydroepiandrosterone sulfate (DHEAS) are neuroactive and more membrane transporters have been uncovered for cellular uptake of sulfated steroids (Fietz et al., 2013). Among other sulfated steroids, CS is the most reported and ubiquitously distributed sterol in mammalian tissues (Strott & Higashi, 2003). In addition to sulfation by sulfotransferases, cholesterol and its precursors undergo enzymic or free radical driven oxidations, resulting in oxidized derivatives (oxysterols).

Recent research in to oxysterols has identified many biological targets (Griffiths & Wang, 2019) despite their abundance being ~10- to 1,000-fold lower when compared to cholesterol in cells and biological fluids (Dias et al., 2019; van Meer, Voelker, & Feigenson, 2008). Some of these oxysterols have been reported to be sulfated, and new biological functions of oxysterol sulfates are emerging. In fact, research groups who have focused their attention on oxysterol sulfates found that these molecules are key mediators in the cellular processes, such as attenuation of the inflammatory response (L. Xu et al., 2012), and the regulation of lipid metabolism via SREBP (sterol regulatory element-binding protein-1; Bai et al., 2012; Ma et al., 2008; Ren et al., 2007). Oxysterol sulfates show dynamic ways of activating, inhibiting, or shuttling of cholesterol in biological systems. This review brings together current understanding of sulfated cholesterol and oxysterols and analytical challenges in measuring their biological levels.

2 | BIOSYNTHESIS OF STEROL SULFATES

The biological activities of sterol sulfates are regulated by the balanced activity between steroid sulfotransferases and steroid sulfatases that catalyse the formation and hydrolysis of steroid sulfates, respectively (Purohit, Potter, Parker, & Reed, 1998). The biosynthesis of sulfated lipids is mediated by a large family of sulfotransferases (SULTs) that catalyse the transfer of sulfate groups from a 3′-phosphoadenosine-5′-phosphosulfate (PAPS) donor compound to an acceptor molecule with aromatic or aliphatic hydroxyl functional groups (Falany, 1997). The transfer of the sulfate group by SULTs at 3-position of the main sterols results into mono-sulfated sterols such as CS, PregS, and DHEAS (Figure 1). The cytochrome P450 (CYP) enzymes catalyse the addition of hydroxyl group to the side chain of cholesterol generating oxysterols which can be further sulfated at 3-position resulting in 24(S)-hydroxycholesterol-3-sulfate (24HC3S), 25-hydroxycholesterol-3-sulfate (25HC3S), (25R)-26-hydroxycholesterol-3-sulfate (26HC3S), 20(S)-hydroxycholesterol-3-sulfate (20HC3S), and 22(R)-hydroxycholesterol-3-sulfate (22HC3S). Oxysterols that are formed by free radical attack, namely, 7α-hydroxycholesterol (7αHC), 7β- hydroxycholesterol (7βHC), 7-ketocholesterol (7KC), and epoxy cholesterol (5β,6β-epoxycholesterol [5β,6βEC] and 5α,6α-epoxycholesterol [5α,6αEC]), which can then be converted into the corresponding sulfated derivatives (Figure 1).

The family of SULTs consist of membrane-related enzymes, mainly localized in the Golgi apparatus and cytosolic enzymes (Falany, 1997). The cytosolic SULTs have been associated with the metabolism of endobiotic and xenobiotic substrates while the membrane-bound enzymes are primarily involved in sulfation of tyrosyl protein residues (Nowell & Falany, 2006). So far, four families of human cytosolic SULTs have been identified: SULT1, SULT2, SULT4, and SULT6. As enzymes of the SULT2 family have been associated with the sulfation of oxysterols, this review will focus on this group (Lindsay, Wang, Li, & Zhou, 2008). Members of the SULT2 family are divided into two subfamilies, SULT2A and SULT2B, based on their amino acid sequence and encoded by the two corresponding genes, SULT2A1 and SULT2B1 (Gamage et al., 2006).

2.1 | SULT2A1

In humans, SULT2A1 has been primarily linked to sulfation of DHEA, although it is also responsible for the sulfation of other steroid substrates such as pregnenolone, androgens, and bile acids (Gamage et al., 2006; Kong, Yang, Ma, Tao, & Bjornsson, 1992; Ottemness et al., 1992). The SULT2A1 isoform is highly expressed in human liver, foetal adrenal glands, adult adrenal cortex, and small intestine (Nowell & Falany, 2006; Thomae, Eckloff, Freimuth, Wieben, & Weinschilboum, 2002). As a result, endogenous and orally administered steroids undergo sulfation by SULT2A1 as part of their metabolism. In particular, DHEAS obtained from DHEA by SULT2A1 serves as a precursor in the synthesis of androgens and oestrogens in human peripheral tissues (Mortola & Yen, 1990). The circulating endogenous levels of DHEAS are known to decrease with age and therefore associated with age-related diseases such as osteoporosis, muscle loss, vaginal atrophy, fat accumulation, hot flashes, skin atrophy, Type 2 diabetes, and cognitive deficits (Orentreich, Brind, Vogelman, Andres, & Baldwin, 1992). Observations by Thomae et al. (2002) suggested an ethnic-specific variation in the expression and activity of SULT2A1.
among Caucasian and African American individuals that is likely to contribute to the high interindividual variability of DHEAS.

2.2 | SULT2B1a and SULT2B1b

The subfamily of SULT2B, including its two splice variants, namely, SULT2B1a and SULT2B1b, is widely distributed in human tissues and is able to metabolize sterol-like structures (Javitt et al., 2001). Both isoforms originate from the alternative splicing of the SULT2B1 gene localized to chromosome band 19q13.3, approximately 500 kb telomeric to the location of SULT2A1 (Her et al., 1998). In the gene for SULT2B1, exon 1A encodes a unique amino-terminal end for the B1a isoform and additional 48 amino acids, compared to the B1b spliced variant (H. Fuda, Lee, Shimizu, Javitt, & Strott, 2002). Javitt et al. (2001) reported that SULT2B1b is expressed to a greater extent
than SULT2B1a, in tissues responsive to hormones. In fact, the B1b isoform preferentially acts on cholesterol, whereas the B1a isoform catalyses the sulfation of pregnenolone, but not cholesterol (H. Fuda et al., 2002). The expression of the isoform B1b is usually several-fold higher than the isoform B1a (Falaney, He, Dumas, Frost, & Falany, 2006) and widely distributed in many tissues including human liver, trace amounts in brain, prostate, placenta, breast, lungs, platelets, and kidney (Falaney et al., 2006; Geese & Raftogianis, 2001; He, Meloche, Dumas, Frost, & Falany, 2004). Double knockout Sult2b1−/− mice are viable and show significant decrease in their CS/cholesterol ratio compared with their wild-type counterparts (Wang, Beck-García, Zorzín, Schamel, & Davis, 2016), suggesting that low level of CS may be formed by other SULTs. CS-deficient mice displayed a heightened sensitivity to self-antigens (Wang et al., 2016). Systemic up-regulation of SULT2B1b inhibited lipogenesis by sulfonating and deactivating the liver X receptor (LXR)-activating oxyesters in LDLR−/− mice (Bai et al., 2012) and overexpression of hepatic SULT2B1b sensitized the mice to drug-induced liver damage (An et al., 2019) and inhibition of gluconeogenesis (Shi et al., 2014).

3 | METABOLISM OF STEROL SULFATES

The cleavage of the sulfate moiety of 3β-hydroxy steroid sulfate is catalysed by membrane-bound microsomal steroid sulfatase (STS; Conary, Naurerth, Burns, Hasilik, & von Figuera, 1986). The gene encoding human STS is located on the distal short arm of the X-chromosome (Yen et al., 1998) and ubiquitously expressed in many human tissues including placenta, breast, skin, lungs, ovaries, adrenal glands, and brain (Reed, Purohit, Woo, Newman, & Potter, 2005). STS have been associated with high intra-tumour oestrogen and androgen levels and therefore linked to steroid hormone-dependent tumour growth (Nardi et al., 2009). Studies by Zaichuk, Ivancic, Scholtens, Schiller, and Khan (2007) showed that oestrogen regulates the transcription of STSs in breast carcinoma.

X-linked ichthyosis, a disease clinically characterized by skin peeling localized in the anterior and posterior areas of upper and lower extremities, is caused by a mutation in the enzyme STS. Patients with recessive X-linked ichthyosis not only display a significant increase in CS in squamous keratinizing epithelia but also exhibit effects in overall lipid metabolism and mental retardation (Elias, Williams, Choi, & Feingold, 2014). In healthy epidermis, CS is produced by the action of SULT2B1b and desulfated in the outer epidermis, thus contributing to epidermal differentiation, maintenance of barrier function and desquamation. As a consequence of STS deficiency, CS levels could exceed 10% of the total lipid mass in epidermal cells (Rizner, 2016).

4 | CHOLESTEROL-3-SULFATE

Besides being the most abundant steroidal sulfoconjugate present in human plasma, with an average concentration of 2 μM (Meng, Griffiths, Nazer, Yang, & Sjövall, 1997), CS has also been detected in other biological fluids, such as urine, bile, seminal plasma, and in many tissues, as described previously (Castellanos, Hernandez, Tomic-Canic, Jozic, & Fernandez-Lima, 2020; Drayer & Lieberman, 1967; Lopalco et al., 2019; Strott & Higashi, 2003). Even though CS is typically considered the hydrophilic excretion form of cholesterol, CS also represents a biosynthetic precursor of several bioactive steroids. In this context, the sulfoconjugation reaction may represent a key step in the formation of a readily available hydrophilic form of cholesterol. CS regulates cholesterol homeostasis, indirectly, by negative regulation of the key enzyme in cholesterol synthesis pathway, 3-hydroxy 3-methylglutaryl-CoA reductase (HMG-CoA reductase) (Williams, Hughes-Fulford, & Elias, 1985) and, directly, blocks the esterification of cholesterol by inhibiting the activity of lecithin-cholesterol acyltransferase enzyme (Nakagawa & Kojima, 1976). Indeed, CS can be subjected to several enzymic transformations carried out by microsomal cytochromes (e.g., CYP11A1, also referred to as cholesterol side-chain cleavage enzyme) in order to obtain sulfated precursors of sex hormones. During the last decades, the role of CS as a signalling molecule has been investigated (Sakurai et al., 2018; Shi et al., 2014; Wang et al., 2016), although many questions remain unanswered. For example, understanding of the nature of CS interactions, CS trafficking, and the signalling pathways in which it could be involved is still incomplete.

Intracellular and extracellular trafficking of CS is one of the most unexplored characteristics except for sex hormone sulfates, such as PregS and DHEAS.. Indeed, the latter compounds were found to be suitable substrates of the plasma membrane transporter, sodium-dependent organic anion transporter SOAT (SCL10A6; Grosser et al., 2018). Interestingly, Liou et al. demonstrated the binding of CS to the lysosomal cholesterol transporter Niemann-Pick disease type C2 protein (NPC2), a key protein involved in cholesterol transport from the lysosomal compartment after the endocytic uptake of low-density lipoproteins (Liou et al., 2006). The interaction between NPC2 and CS was demonstrated both by a chromatographic shift assay and by competition assay. It is noteworthy to mention that CS was unable to interact with the functional analogue Niemann-Pick disease type C1 protein (NPC1) according to a scintillation counting binding assay (Infante et al., 2008).

5 | CHOLESTEROL-3-SULFATE AND ITS RECEPTORS

As described above, recessive X-linked ichthyosis has been related to a deficiency in cholesterol sulfatase expression with subsequent accumulation of CS. Sato, Denda, Nakanishi, Nomura, and Koyama (1998) correlated this pathological condition with the ability of CS to inhibit serine proteases involved in cell dissociation, a key feature in skin development. As a matter of fact, Ito et al. demonstrated the direct inhibition of several hydrolytic enzymes by CS (e.g., pancreatic elastase, trypsin, chymotrypsin, thrombin, plasmin, and DNAse I) in the late '90s (Ito, Iwamori, Hanaoka, & Iwamori, 1998; Iwamori, Iwamori, &
and plasmin. As these 5-Lipoxygenase (5-LO) mediators and many different cell types. Inflammation is a complex multistep biological response of body tissues to harmful stimulations which typically involves a multitude of involved in the production of leukotrienes (LTs), soluble mediators of the inflammatory state, and immune system functionality. In particular, LTs play a pivotal role in asthma and bronchitis. When a Ca\(^{2+}\) influx takes place, 5-LO binds to the nuclear membrane where it can convert arachidonic acid into the bioactive leukotrienes. As a constituent of cell membranes, CS can modulate the function of several proteins, including 5-LO, directly interacting at the membrane level. Aleksandrov et al. (2006), demonstrated the inhibitory behaviour of CS towards 5-LO in a cell-free assay. Here, CS decreased 5-LO interaction with the nuclear membrane in a cell-based assay upon stimulation, thus decreasing LT biosynthesis.

Wang et al. (2016) showed CS to act as a modulator of T-cell receptor (TCR) functionality. The TCR is a multisubunit membrane receptor which includes an antigen-recognition domain composed of the TCR\(\alpha\) and \(\beta\) (or \(\gamma\) and \(\delta\)) heterodimer and a signalling domain, typically three CD3 dimers. Although TCR binds its corresponding peptide - MHC ligands - with extremely weak affinity, it is well-known that a single molecule of its ligand is able to activate the T cell. The low affinity and the high sensitivity of this receptor have been related to the nanoclustering of several TCRs. Cholesterol is able to interact with TCR\(\beta\), thus promoting TCR nanoclustering. Conversely, CS can disrupt TCR clusters by interfering in the cholesterol-TCR\(\beta\) interaction. Interestingly, the cholesterol/CS ratio is a variable parameter during T cell development and differentiation (Wang et al., 2016).

The ability of CS to inhibit serine proteases was extended by Iwamori, Iwamori, and Ito (1999) to thrombin and plasmin. As these two proteases are involved in blood clotting and fibrinolysis, respectively, CS can be considered an endogenous modulator of homeostasis of the blood clotting system within the vascular network by a presumably non-specific irreversible mechanism. Moreover, CS has been found to promote divalent cation-independent adhesion of both activated and inactivated platelets, although the mechanisms by which CS exert these prothrombotic activities are not clear (Merten, 2001).

### 5.1 Role of CS in inflammation and the immune system

Recent research found that CS play a significant role in the control of inflammation by modulating key targets (Aleksandrov et al., 2006). Inflammation is a complex multistep biological response of body tissues to harmful stimulations which typically involves a multitude of mediators and many different cell types. 5-Lipoxygenase (5-LO) is involved in the production of leukotrienes (LTs), soluble mediators of the inflammatory state, and immune system functionality. In particular, LTs play a pivotal role in asthma and bronchitis. When a Ca\(^{2+}\) influx takes place, 5-LO binds to the nuclear membrane where it can convert arachidonic acid into the bioactive leukotrienes. As a constituent of cell membranes, CS can modulate the function of several proteins, including 5-LO, directly interacting at the membrane level. Aleksandrov et al. (2006), demonstrated the inhibitory behaviour of CS towards 5-LO in a cell-free assay. Here, CS decreased 5-LO interaction with the nuclear membrane in a cell-based assay upon stimulation, thus decreasing LT biosynthesis.

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The protein, dedicator of cytokinesis 2 (DOCK2), is a guanine nucleotide exchange factor which plays a key role in immune surveillance and immune responses by regulating the chemotaxis and the activation of leukocytes. Sakurai et al. (2018) demonstrated that CS is highly expressed in Harderian gland, an orbital gland that produces the lipids that form the oily layer of the tear film in the eye of Sult2b1\(^{-/-}\) mice, was able to inhibit the action of DOCK2. In particular, a direct interaction between CS and DOCK2 has been confirmed by a cell-free surface plasmon resonance binding assay (Sakurai et al., 2018). Human tear film also contains a high level of CS (Lam et al., 2014), and it is possible that CS limit ocular surface inflammation by inhibiting DOCK2.

CS has been also reported as an endogenous ligand of macrophage inducible Ca\(^{2+}\)-dependent lectin receptor (Mincle), an innate immune receptor involved in skin allergic inflammation (Kostarnoy et al., 2017). In the studies reported above, the specific interaction of CS with the corresponding target protein was not proven, and in most cases, the observed activity of CS was attributed to its amphiphilic nature without identifying a proper binding pocket/site on the polypeptidic counterpart.

### 5.2 Role of CS as a ligand in signalling pathways

Kallen, Schlaeppi, Bitsch, Delhon, and Fournier (2004) reported the crystal structure of CS with the nuclear receptor retinoic acid-related orphan receptor \(\alpha\) (RORX). As RORX could be involved in the control of cholesterol homeostasis, the authors set up crystallization trials in both with cholesterol and CS. Both lipids co-crystallized with the ligand-binding domain of the receptor-interacting at the same level. Remarkably, CS showed an increased affinity due to the interaction of the sulphate group with key polar residues of the ligand-binding pocket (Gln\(^{289}\), Tyr\(^{290}\) and Arg\(^{279}\)) with the consequence displacement of several water molecules which were present in the interaction with cholesterol. Even though the crystal studies unambiguously identified the interaction of this orphan nuclear receptor with CS, evidence of this interaction in vivo is still lacking. Indeed, even if the activation of this nuclear receptor occurs upon stimulation with CS, the latter is considered so far only a putative RORX endogenous ligand (Han et al., 2014; Kim et al., 2008; Zenri et al., 2012).

CS has an important role in the substrate specificity of PI3K (Woscholski, Kodaki, Palmer, Waterfield, & Parker, 1995). Phosphatidylinositol (3,4,5)-trisphosphate (PIP\(_3\), produced by the action of PI3K, is associated with the signalling pathway of several growth factors, and it is considered a secondary messenger. Phosphatidylinositol bisphosphate (PIP\(_{2}\)) is the preferred substrate of PI3K in vivo, inside the cell. Conversely, phosphatidylinositol monophosphate and phosphatidylinositol are the preferred substrates of PI3K in cell-free systems. Woscholski et al. (1995) demonstrated that the characteristic substrate specificity of this enzyme in vivo could be restored in the presence of CS, pointing out its potential relevance as an interacting partner inside the cell.
Oxysterols are bioactive lipids which share the 27-carbon skeleton with cholesterol and differ from the latter by the presence of extra oxygenated functional groups, apart from the 3β-hydroxyl group. In addition to being biosynthetic precursors of bile acids and sex hormones, they serve as selective ligands towards several targets (e.g., GPCRs, enzymes, nuclear receptors, and other membrane and cytosolic proteins). Similarly, their sulfoconjugates have been found to act as modulators of different targets. Traditionally, oxysterol sulfates have been viewed as detoxification derivatives of oxysterols that are synthesized for excretion. However, recent work has proposed that oxysterol sulfates were bioactive molecules that act as selective ligands with biological outcomes. Table 1 lists oxysterol sulfates with reported cellular activities, but not all oxysterol sulfates detected by analytical techniques have been investigated for their biological action, showing a gap in the oxysterol research field.

Oxysterol sulfoconjugation occurs mainly by the cytosolic PAPS-dependent enzyme SULT2B1b, also referred to as hydroxysteroid sulfotransferase. This metabolic transformation is generally reversible, as the enzymic activity of STS is able to provide the parent oxysterol in fotransferase. This metabolic transformation is generally reversible, as the enzymic activity of STS is able to provide the parent oxysterol in

### Table 1: Cellular activities and tested concentration ranges of oxysterol sulfates in human cell models

| Oxysterol       | Cell type                        | Outcome                                                                 | Tested Concentration range | References                                                                 |
|-----------------|----------------------------------|-------------------------------------------------------------------------|-----------------------------|---------------------------------------------------------------------------|
| 5,6αECS         | Colorectal Cancer cell line, Caco-2 | Accumulation sensitizes cells to apoptosis                               | 0.6–100 μM                  | Segalà et al. (2013); Warns, Marwartha, Freking, & Ghribi (2018)          |
|                 | Neuroblastoma cell line, SHSY-5Y cells | No effect on cell viability                                              | 10 μM                      | Marwarha, Rhen, Schommer, & Ghribi (2011)                                  |
|                 | HEK 293 cells                     | Attenuates the 26HC-induced increase in α-synuclein expression           | 4–20 μM                    | Song, Hiiipakka, & Liao (2001)                                             |
| 7KCS            | HEK 293 cells                     | Reduce cytotoxicity induced by 7ketoC                                    | 5 nM                       | Hirotsuhi Fuda, Javitt, Mitamura, Ikekawa, & Strott (2007)                |
|                 | Human retinal pigment epithelial cell line, ARPE-19 | Attenuates ABCA1 and VEGF induction by 7ketoC                        | 0–20 μM                    | Moreira, Larrayoz, Lee, & Rodriguez, (2009)                               |
|                 | HEK 293 cells                     | Inhibit transactivation of reporter genes by LXR                       | 4–20 μM                    | Song et al. (2001)                                                        |
| 24HC3S/24HCDS   | Hepatocytes                       | LXR antagonists                                                         | 20 μM                      | Cook et al. (2009)                                                        |
| 25HC3S          | Hepatocytes                       | Inhibits the LXR/SREBP signalling pathway, regulates lipid metabolism, inflammatory responses, and cell proliferation | 0–25 μM                    | Ren et al. (2007); Ren et al. (2014); Ren & Ning (2014)                  |
|                 | Human monocyct cell line, THP-1  | Attenuates inflammatory response via PPARγ signalling                   | 0–50 μM                    | Ma et al. (2008); L. Xu et al. (2012)                                     |

Abbreviations in Table. 5,6αECS, 5α,6α-epoxycholesterol-3-sulfate; 7ketoC, 7-ketocholesterol; 7KCS, 7-ketocholesterol-3-sulfate; 24HC3S, 24(S)-hydroxycholesterol-3-sulfate; 24HCDS, 24(S)-hydroxycholesterol-3,24-disulfate; 25HC3S, 25-hydroxycholesterol-3-sulfate

(7KCS) bind to both nuclear receptors LXRα and LXRβ), inhibiting their activation and acting as antagonists. It is noteworthy that in addition to a cell-based gene transactivation assay, the authors also performed a cell-free coactivator peptide recruitment binding assay in order to demonstrate the direct interaction of 5,6α-ECS and 7KCS with the receptors. Moreover, a structure-dependant ligand recognition mechanism was sought, by testing two closely related sulfated oxysterols, 5(1,6)-epoxycholesterol-3-sulfate (5,6ECS) and 6-ketocholestanol-3-sulfate, in the same assays. As both of the latter compounds failed to modulate LXR activation, the authors speculated that the antagonistic behaviour of 5,6α-ECS and 7KCS towards LXRα was independent of their physiochemical properties (e.g., amphiphilicity).

Cook et al. (2009) reported that the endogenous LXRα agonist 24(S)-hydroxycholesterol (24HC) could be sulfated by three different sulfotransferases, namely, SULT1E1, SULT2A1, and SULT2B1b at the 3-OH or 24-OH positions with different rates and affinities affording 24HC3S, 24(S)-hydroxycholesterol-24-sulfate, and 24HCDS. Surprisingly, 24HC3S and 25HC24S showed a remarkable antagonistic behaviour in a time-resolved fluorescence energy transfer (TR-FRET) LXRα coactivator recruitment assay, suggesting a dramatic switching in ligand properties as the sulfate moiety was introduced in the structure of the parent compounds. Interestingly, SULT2B1b is a target gene for the LXRα, whose expression increases in the presence of agonists. Accordingly, the sulfation of endogenous agonists of LXRα can
be considered a negative feedback mechanism to control the activation of LXR.

Also 25-hydroxycholesterol (25HC), another endogenous LXR agonist, can be converted into an antagonist when sulfated at 3β-OH. 25HC3S was identified by Ren et al. in 2007 first in hepatocyte nuclei. 25HC3S decreased the expression of SREBP-1 target genes, such as HMG-CoA reductase, with a consequent overall decrease of cholesterol levels. Moreover, its administration to human hepatocytes resulted in reduced SREBPs, in particular, SREBP-1, expression, and maturation. Hence, 25HC3S was found to decrease NF-κB nuclear levels by increasing cytosolic levels of its inhibitor IκBα. Thus, repressing TNFα-induced inflammatory response in HepG2 cells. Interestingly, its parent compound, 25HC, elicited the opposite activity (Leyuan Xu et al., 2010). In the same paper, the antagonistic behaviour of 25HC3S towards LXRs was demonstrated. Indeed, 25HC3S was able to decrease the expression of LXR target genes involved in cholesterol biosynthesis and lipogenesis, such as fatty acid synthase and acetyl-CoA carboxylase-1: Leyuan Xu et al., 2010). By contrast, Zhang et al. (2012) demonstrated that 25HC3S up-regulated several genes involved in the proliferation of hepatic cells. In an LDLR−/− mouse model, overexpressing SULT2B1b with 25OHC supplementation increased 25OHC3S levels, and endogenous 25HC3S was a crucial regulator of lipid biosynthesis mediating inhibitory effects to the LXR-SREBP-1c signalling pathway (Bai et al., 2012).

According to its biological profile, acting as an inhibitor of LXR and SREBP-1c signalling pathways (Bai et al., 2012) as well as to its anti-inflammatory properties (Leyuan Xu et al., 2010; L. Xu et al., 2012), 25HC3S is currently evaluated in phase II clinical trial for its potential application in liver diseases, such as NAFLD) by Direct Corporation. In 2012, 25HC3S was found to act as a PPARγ agonist in THP-1 macrophages, where it can suppress inflammatory responses by increasing IκBα transcriptionally. Indeed, IκBα bears a PPAR response element (PPRE) sequence on its promoter (L. Xu et al., 2012). Although no co-crystallized structures are available, recently, the binding mechanism of 25HC3S to PPARγ was simulated in silico by Yang et al. (2019), showing the selection of a partial-agonist conformation of the receptor by the ligand.

One of Ren’s group discoveries has been the identification of the sulfolipid 25HCDS in rat hepatocytes. Like 25HC3S, 25HCDS reduced cholesterol levels and depressed immune responses at transcriptional level, probably interfering with LXRs, SREBPs, and PPARγ (Ren et al., 2014). However, as no proof of concept regarding the exact mechanism of action of 25HCDS has been reported yet, the latter hypothesis remains to be confirmed.

7 | ANALYTICAL STRATEGIES IN THE ANALYSIS OF PLASMA OXYSTEROL SULFATES: CURRENT CHALLENGES

Most of the findings reported on oxysterol sulfates in cells and tissues have been carried out using the commercially available 25HC3S standards (Bai et al., 2012; Ma et al., 2008; Ren et al., 2007; Leyuan Xu et al., 2010; L. Xu et al., 2012; Y. Xu et al., 2013), but exploratory studies have shown that the panel of oxysterol sulfates in circulation may in fact be broader (Meng et al., 1997; Ren et al., 2014; Sánchez-Guijo, Oji, Hartmann, Schuppe, et al., 2015).

One of the first studies focused on the screening of oxysterol sulfates in biological fluids described the presence of elevated levels of a compound compatible with the presence of a glucuronidated cholestenediol sulfate in serum and urine samples of children with severe cholestatic liver disease (Meng et al., 1997). The authors were able, after extensive sample handling and derivatisation steps, to identify and characterize it as the glucuronidated form of the 24HC3S, by fast atom bombardment mass spectrometry using glycerol as a matrix compound (Meng et al., 1997). The authors also reported the occurrence of oxysterol glycine and taurine conjugates, though sulfation seemed to be the main detoxification route in cholestatic liver disease and without potential prognostic value during clinical evaluation (Meng et al., 1997). Later, Acimovic et al. (2013) suggested that sulfation could act as a protective mechanism against the accumulation of oxysterols in the circulation. A glimpse into the panel of oxysterol sulfates was expanded by Sánchez-Guijo, Oji, Hartmann, Schuppe, et al. (2015) who reported the presence of the 27-hydroxycholesterol sulfate (27HCS, otherwise known as (25R)-26-hydroxycholesterol-3-sulfate) and found that 27HCS was not the only sulfated steroid derivative that was consistently elevated in serum of patients with recessive X-linked ichthyosis (Sánchez-Guijo, Oji, Hartmann, Schuppe, et al., 2015). This compound was among a wider panel of oxysterol sulfates (Figure 2) including isomers containing the hydroxyl group at the 25-, 4-, and 7-position of cholesterol moiety and even disulfated compounds.

Despite the evidence for a wider panel of oxysterol sulfates in circulation provided by these exploratory studies (Meng et al., 1997; Sánchez-Guijo, Oji, Hartmann, Schuppe, et al., 2015), very little is known about the predominant oxysterol sulfates present in body fluids and accumulated in cells and tissues, their basal levels, and any variations introduced with age, gender, and ethnicity in health and disease, despite the common knowledge that SL gather at the surface of lipid-raft domains (Weerachayankul, Probodh, Kongmanas, Tanphaichitr, & Johnston, 2007) and contribute to cell–cell communication processes (Honke, 2017; Strott & Higashi, 2003). On the other hand, structurally related compounds such as oxysterols are widely studied, and the oxysterol signature in normolipidaemic and normoglycaemic conditions and their basal levels are known (Dias et al., 2018; Grayaa et al., 2018; McDonald, Smith, Stiles, & Russell, 2012; Murakami, Tamasawa, Matsui, Yasujima, & Suda, 2000; Narayanaswamy et al., 2015). Oxysterols are predominantly found esterified to fatty acids (Dzeletovic, Breuer, Lund, & Diczfalusy, 1995) and are thought to be substrates for sulphotransferases (Fuda et al., 2007), leading the formation of oxysterol sulfates.

The published concentrations of oxysterol sulfates are still scarce and require corroboration as the levels reported for 24-hydroxycholesterol-3-sulfate-24-glucuronide range from 2−18 μM, measured in cholestatic liver disease by FAB-MS (Meng et al., 1997), whereas the levels of 27HC3S in patients with steroid
sulfatase deficiency range between 22.5 and 46 ng·ml\(^{-1}\) (~47–95 nM) when compared to levels below 2.5 ng·ml\(^{-1}\) (<LOQ) in healthy male donors (Sánchez-Guijo, Oji, Hartmann, Schuppe, et al., 2015). The disparity of values found could be attributed to differences in the characteristics of the individuals included in the study groups, and to differences in experimental and methodological conditions adopted, supporting the need for further investigation. Accurate knowledge on the basal levels of oxysterol sulfates in health and disease are intimately related to the experimental conditions chosen during the analysis process, including sample collection, storage, extraction, fractionation, separation, detection, and quantification steps. For instance, sample pretreatment strategies are paramount in the discovery and validation of lipid-based markers in biological samples. Sample collection tubes, freeze–thaw cycles, and storage conditions are often a major source of variability that affect not only the stability of samples but also the overall recovery and fingerprint of plasma lipids (Gonzalez-Covarrubias, 2013; Hammad et al., 2010; Lee, Kind, Yoon, Fiehn, & Liu, 2014; Sarafian et al., 2014). Work conducted on the analysis of structurally related compounds such as CS and oxysterols (Table 2) reveals a diversity of sample pretreatment strategies (e.g., anticoagulant), extraction solvent system used, and analytical methodology has been largely overlooked.

As shown in Table 2, several different anticoagulants are typically used in the collection of blood samples. Even though there is a lack of studies on the effect of sample pretreatment strategies in the levels of oxysterol sulfates, published results with oxysterols reveal that plasma oxysterol levels collected with K\(_2\)-EDTA and citrate collection tubes differed from those observed in serum samples (Hautajärvi et al., 2018; Reinicke, Schröter, Müller-Klieser, Helmschrodt, & Ceglarek, 2018) supporting the use of EDTA-collection tubes over citrate or heparin tubes, due to the complete and non-reversible chelation of Ca\(^{2+}\) and Mg\(^{2+}\) ions which suppressed oxidative reactions (Reinicke et al., 2018). In cases where serum samples were used, Helmschrodt et al. suggested the addition of an antioxidant, butylated hydroxytoluene (0.05%) to increase the stability of oxysterols. Another aspect that is often ignored is the freeze–thaw cycle often required for biochemical and chemical analysis. Whereas this appeared not to affect the levels of CS (Sánchez-Guijo, Oji, Hartmann, Traupe, et al., 2015), the number of freeze–thaw cycles decreased the level of oxysterols (Helmschrodt et al., 2013). Storage up to 3 months did not affect levels of CS (Hautajärvi et al., 2018; Helmschrodt et al., 2013; Sánchez-Guijo, Oji, Hartmann, Traupe, et al., 2015).

One other aspect that has been largely overlooked is the method of extraction. Extraction of steroid-related compounds is typically conducted by liquid–liquid extraction (LLE) protocols followed by fractionation in solid-phase extraction (SPE) cartridges (Table 2). In fact, LLE protocols remain the most popular method of choice due to their simplicity, cost, sample volume required, extraction efficiency, reproducibility, repeatability, lipidome coverage, and potential for automation, where the overall performance of LLE protocols is very similar in
| Biological matrix (collection tube) | Extraction approach (method and solvent system) | Analytical approach and method performance | Ref. |
|-----------------------------------|------------------------------------------------|------------------------------------------|------|
| Cholesterol sulfate | Plasma (EDTA tube) LLE with MeOH followed by purification on Baker-10 quaternary amine column | GC-FID (TMS derivatives), n.s. | Muskiet, Jansen, Wolthers, Marinkovic-Ilsen, and van Voorst Vader (1983) |
| Sodium (citrate) | Plasma (EDTA tube) LLE with MeOH (80%) | HPTLC coupled to densitometry, n.s. | Przybylska et al. (1995) |
| | Sodium (citrate) LLE with acetone/ethanol (1:1, v/v), followed by purification in silica column and elution with CHCl$_3$/MeOH (1:1, v/v) | GC-MS (TMS derivatives), n.s. | Tamasawa, Tamasawa and Takebe (1993) |
| | Serum LLE with acetone/ethanol (1:1, v/v), followed by purification in acidified NH$_2$ bond Elut cartridge and elution with CHCl$_3$/MeOH (1:1, v/v) | GC-MS (acytelylated derivatives), n.s. | Delfino, Procaccini, Illiano, and Milone (1998) |
| Plasma (lithium heparin) | LLE with MeOH, followed by purification by C18 SPE and elution with CHCl$_3$/MeOH (2:1, v/v) | LC-APCI-MS/MS detection (underivatized) and quantification by MRM in QTRAP 3200, LLOD (0.02μM); | Fong, Tam, and Leung (2013) |
| Serum/plasma | Protein ppt ACN-ZnSO$_4$ followed by fractionation by SPE (SepPak cartridge) | LC-MS/MS, LOQ, 80 ng ml$^{-1}$ | Sánchez-Guijo, Oji, Hartmann, Traupe, and Wudy (2015) |
| Serum (−) | SPE extraction with MeOH in Strata-X (33 μm) cartridges | LC-(ESI)MS detection and quantification by SIM in QqQ, LOQ, 5ng ml$^{-1}$ | Lee, Lee, Hong, Chung and Choi (2016) |
| Oxysterols | Plasma (K$_2$EDTA) Saponification in ethanolic solution, followed by LLE with CHCl$_3$ and purification in silica SPE and elution with 30% iso-propanol in hexane | GC-MS of TMS derivatives, LOD, 0.3–5 ng | Dzeletovic et al. (1995) |
| Plasma (heparin) | Plasma (heparin) LLE with CHCl$_3$/MeOH (2:1, v/v) followed by fractionation in a packed silica column and eluted in ethyl acetate | GC-MS of TMS derivatives, LOD, 0.02 ng ml$^{-1}$ | Murakami et al. (2000) |
| Plasma (−) | Plasma (−) LLE with CH$_2$Cl$_2$/MeOH (1:1, v/v) aided by ultrasonic bath homogenization (10 min). | LC-(APCI)MS detection and quantification by MRM in QTRAP, LLOQ, 1 ng ml$^{-1}$ | McDonald et al. (2012) |
| Plasma (EDTA) | Plasma (EDTA) LLE with MeOH/isopropanol (1:1, v/v) | LC-(APCI)MS detection and quantification by MRM in QqQ, LLOQ 0.5 ng ml$^{-1}$ | Helmschrodt et al. (2013) |
| Plasma (EDTA) | Plasma (EDTA) LLE with ethanol, followed by alkaline hydrolysis and extraction with CHCl$_3$/MeOH (2:1, v/v) and SPE fractionation in a silica column | GC-MS of TMS derivatives, n.s. | Grayaa et al. (2018) |
| Plasma (EDTA) | Plasma (EDTA) LLE with MeOH followed by fractionation by SPE in a HLB Oasis PRIME column | LC-(ESI)MS detection and quantification by MRM in QTRAP 5500, LLOQ, 18-253 pg ml$^{-1}$ | Dias et al. (2018) |
| Plasma (K$_2$EDTA) | Plasma (K$_2$EDTA) Saponification of plasma in ethanolic solution, followed by protein precipitation in ACN (1.5% formic acid) and purification by SPE 96-well plates | LC/ESI-HR-MS detection and quantification against calibration curves built with deuterated standards, LLOQ, 0.5–2 ng ml$^{-1}$ | Hautajärvi, Hukkanen, Turpeinen, Mattila, and Tolonen (2018) |

Abbreviations in Table. n.s., not stated. ACN, acetonitrile. APCI-MS atmospheric pressure chemical ionization; (ESI)MS, electrospray mass spectrometry. ESI-HR-MS, electrospray coupled with high-resolution mass spectrometry. GC-FID, gas-chromatography coupled with flame ionisation detection; HPTLC, high performance thin layer chromatography; LC-APCI-MS/MS, liquid-chromatography tandem mass spectrometry with atmospheric pressure chemical ionization; LLE, liquid–liquid extraction; LLOQ, lower limit of quantitation; QqQ, triple quadrupole analyzer; SIM single ion monitoring; SPE, solid phase extraction; TMS, trimethylsilylation.
the extraction of predominant lipid classes (Reis et al., 2013). In the case of structurally similar compounds, the extraction performance of cholesterol and CS in two of the most popular LLE solvent mixtures is similar, though solvent systems with a higher dielectric constant ($\varepsilon$) extracted higher amounts of CS compared with cholesterol (MeOH: CHCl$_3$ [2:1, v/v]), whereas solvent mixtures of lower $\varepsilon$ with more hydrophobic character were more efficient towards the extraction of cholesterol but not of CS (MeOH:CHCl$_3$ [1:2, v/v]; data not shown).

Based on our previous experience on the extraction of lipids from biological samples, it is clear that organic solvent mixtures have a major impact on the extraction performance (Reis et al., 2013), particularly on the less abundant lipids. Remarkably, the influence of the solvent system in the extraction performance of oxysterol sulfates by LLE protocols has not yet been addressed. Despite this lack of knowledge, the sulfate group confers increased polarity to the oxysterol, though the position of the hydroxy group may also be responsible for changes in hydrophobicity to the oxysterol sulfate moiety and hence potentially have a strong influence on the extractability of oxysterol sulfates in organic solvents. To support this, it was previously shown that the elution of underivatized oxysterol positional isomers under reverse-phase HPLC conditions was very different. The 24HC and 25HC isomers eluted prior to the 7-ketocholesterol (7KC) and $4\beta$-hydroxycholesterol oxysterols (Dias et al., 2018; Grayaa et al., 2018; Narayanaswamy et al., 2015; Reinicke et al., 2018) confirming the distinct hydrophobicity of oxysterol positional isomers. These slight differences in polarity facilitate the chromatographic separation under reverse-phase conditions but could also affect the extraction efficiency of oxysterol sulfates from aqueous biological matrices during the LLE when polar solvent mixtures are used. In the case of oxysterols sulfates, extraction by protein precipitation with acetonitrile (ACN)-ZnSO$_4$ (4:1, v/v) followed by C$_{18}$ SPE fractionation (Sánchez-Guijo, Oji, Hartmann, Schuppe, et al., 2015) resulted in complete recovery (100.6%).

While the presence of hydroxy groups affects the hydrophobicity of the oxysterol moiety and may affect the performance of the extraction, the presence of the sulfate and hydroxy groups in oxysterol sulfates also affects the detection methods that can be used to quantify oxysterol sulfates. In terms of mass spectrometric assays, the oxysterols are usually detected in the positive ion detection mode (Dias et al., 2018; Hautajärvi et al., 2018; Helmschrodt et al., 2013; Mendiara et al., 2018; Murakami et al., 2000), whereas the presence of the sulfate group facilitates the detection of oxysterol sulfates in the negative ion mode. Because oxysterols sulfates occur in residual levels in biological samples, detection of oxysterols sulfates is often
achieved by targeted detection methods such as multiple reaction monitoring (MRM). Due to the specificity of the transitions in MRM approaches, these display an increased sensitivity in the detection step with the advantage of eliminating the contribution of the other sulfated metabolites that may contribute to the overall plasma sulfometabolome and already observed by targeted approaches (Dias, Ferreira, et al., 2019). Previous work by Sánchez-Guijo and colleagues established 1 ng ml\(^{-1}\) as the limit of detection of oxysterol sulfates, using MRM detection (Sánchez-Guijo, Oji, Hartmann, Schuppe, et al., 2015).

Contrarily, the presence of the hydroxy group has no influence on the efficacy of ionization and hence on the detection step. As ionization of oxysterol sulfate occurs by removal of a hydrogen atom at the sulfate group, the ionization efficiency of oxysterol sulfates is similar to that of CS. This was confirmed by the injection of an equimolar mixture of oxysterol sulfates and CS and detection under reverse-phase elution conditions in the negative ion mode (unpublished results).

Regardless of the collection, extraction, and analytical strategy adopted in the analysis of oxysterol sulfate, the values reported (Acimovic et al., 2013; Meng et al., 1997; Sánchez-Guijo, Oji, Hartmann, Schuppe, et al., 2015) show that these are well below the micromolar range generally used in the biological assessment of oxysterol sulfates in cells and tissue (Ren et al., 2007; Leyuan Xu et al., 2010; L. Xu et al., 2012). Based on the published values, oxysterols that are structurally related compounds of oxysterol sulfates account for less than 1% of total cholesterol in hyperlipidaemia (Björkhem et al., 2001; Dias et al., 2018; Reinicke et al., 2018) while oxysterol sulfates (24HC3S and 26HC3S) account for less than 15% of total oxysterols (Acimovic et al., 2013). This could explain why oxysterol sulfates have been largely overlooked by the scientific community.

8 | CONCLUDING REMARKS

In summary, it is clear that CS and oxysterol sulfates act as key players in many biological pathways influencing human health and disease. While CS has been extensively studied, only a handful of papers have focused on oxysterol sulfates. The lack of a more complete panel of oxysterol sulfate standards commercially available and the poor knowledge of the optimal conditions for the extraction, detection, and quantification of oxysterol sulfates from biological matrices has hampered a more complete understanding of the role of oxysterol sulfates. The development of mass spectrometry-based approaches designed for the sensitive detection of oxysterol sulfates are crucial to improve our understanding of the molecular interplay between oxysterols and oxysterol sulfates at cell and tissue levels, as such interactions are of the utmost importance for cholesterol/oxysterol homeostasis (Figure 3). This in turn relies on increased investment of time and resources by synthetic organic chemists to promote the commercial availability of novel oxysterol sulfates to be used as standards.

8.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Cidlowski et al., 2019; Alexander, Fabbro et al., 2019; Alexander, Kelly et al., 2019).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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