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Cholesterol and oxysterol sulfates: Pathophysiological roles and analytical challenges

Running title: Analysis and pathophysiological roles of oxysterol sulfates

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Abstract

Cholesterol (Chol) 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 are 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.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.15227

Introduction

Sulfate-based lipids (SL) represents a wide range of lipid classes spanning across low to high molecular weight compounds (Dias, Ferreira, et al., 2019) with key functions d 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 are 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 mostly studied lipid class among the other glycerol-, sphingosine- or taurine-derived lipids (Mueller *et al.*, 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) act as a signalling molecule (Shi et al., 2014), pregnenolone sulfate (PregS) and dehydroepiandrosterone sulfate (DHEAS) are neuroactive and more membrane transporters are uncovered for cellular uptake of sulfated sterols (Fietz et al., 2013). Among other sulfated sterols, CS is the most reported and ubiquitously distributed sterol in mammalian tissues (Strott & Higashi, 2003). In addition to sulfation by sulfotransferases, cholesterol (Chol) and its precursors undergo enzymatic or free radical driven oxidations, resulting in oxidised derivatives (oxysterols).

Recent research in to oxysterols has identified many biological targets (Griffiths & Wang, 2019) despite their abundance being ~10–1000 fold lower when compared to cholesterol in cells and biological fluids (Dias, Borah, 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 Chol in biological systems. This review brings together current understanding of sulfated Chol and oxysterols and analytical challenges in measuring their biological levels.

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 hydroxyls 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 Chol generating oxysterols which can be further sulfated at 3-position resulting in 24(*S*)-hydroxycholesterol-3-sulfate (24HC3S), 25-hydroxycholesterol-3-sulfate (25HC3S), (25*R*)-26-hydroxycholesterol-3-sulfate (26HC3S), 20(*S*)-hydroxycholesterol-3-sulfate (20HC3S) and 22(*R*)-hydroxycholesterol-3-sulfate (22HC3S) (Cook, Duniec-Dmuchowski, Kocarek, Runge-Morris, & Falany, 2009; Javitt, Lee, Shimizu, Fuda, & Strott, 2001). The additional hydroxyl group acquired by these oxysterols allows the formation of disulfated derivatives, such as 24(*S*)-hydroxycholesterol-3,24-disulfate (24HCDS), 25-hydroxycholesterol-3,25-sulfate (25HCDS) and (25*R*)-26-hydroxycholesterol-3,26-disulfate (26HCDS). Oxysterols that are formed by free radical attack, namely 7α-hydroxycholesterol (7αHC), 7β-hydroxycholesterol (7βHC), 7-ketocholesterol (7KC), epoxy cholesterols [5β,6β-epoxycholesterol (5,6βEC) and 5α,6α-epoxy cholesterol (5,6αEC)], which can then be converted into the corresponding sulfated derivatives (**Figure 1**).

The family of SULTs consist of membrane-related enzymes, mainly localised in the Golgi apparatus, and cytosolic enzymes (Falany, 1997). The SULTs cytosolic enzymes have been associated with the metabolism of endo- and xenobiotics 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, and this review will focus on the members of 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).

SULT2A1

In humans, SULT2A1 has been primarily linked to sulfation of DHEA; however, it is also responsible for the sulfation of other steroid substrates such as pregnenolone (Preg), androgens and bile acids (Gamage et al., 2006; Kong, Yang, Ma, Tao, & Bjornsson, 1992; Otterness 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, & Weinshilboum, 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 is 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). In 2002, observations by Thomae *et al*. suggested an ethnic-specific variation in the expression and activity of SULT2A1 among Caucasian and African American individuals (Thomae et al., 2002), that likely contributes to the high inter-individual variability of DHEAS.

SULT2B1a and SULT2B1b

The subfamily of SULT2B, including its two splice variants, namely SULT2B1a and SULT2B1b, are widely distributed in human tissues and are able to metabolise sterol-like structures (Javitt et al., 2001). Both isoforms originate from the alternative splicing of the SULT2B1 gene localised 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). In 2001, Javitt *et al*. reported that SULT2B1b is expressed in tissues responsive to hormones in a higher fashion than SULT2B1a (Javitt et al., 2001). In fact, the B1b isoform preferentially acts on Chol, whereas the B1a isoform catalyses the sulfation of Preg, but not Chol (H. Fuda et al., 2002). The expression of the isoform B1b is usually several-fold higher than the isoform B1a (Falany, 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 (Falany et al., 2006; Geese & Raftogianis, 2001; He, Meloche, Dumas, Frost, & Falany, 2004). Double knockout Sult2b1$^{-/-}$ mice models are viable and show significant decrease in their CS/Chol

ratio compared with their wild-type counterparts (Wang, Beck-García, Zorzin, Schamel, & Davis, 2016), suggesting that low level of CS may form by other SULTs. CS-deficient mice displayed a heightened sensitivity to a self-antigens (Wang et al., 2016). Systemic upregulation of SULT2B1b inhibited lipogenesis by sulfonating and deactivating the LXR-activating oxysterols 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).

Metabolism of sterol sulfates

The cleavage of the sulfate moiety of 3β-hydroxysteroid sulfate is catalysed by membrane-bound microsomal steroid sulfatase (STS) (Conary, Nauerth, Burns, Hasilik, & von Figura, 1986). The gene encoding human STS is located on the distal short arm of the X-chromosome (Yen et al., 1988) 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-tumoral oestrogen and androgen levels and therefore, linked to steroid hormone-dependent tumour growth (Nardi et al., 2009). Studies by Zaichuk *et al*. in 2007 showed that oestrogen regulates the transcription of STS~~s~~ in breast carcinoma (Zaichuk, Ivancic, Scholtens, Schiller, & Khan, 2007).

X-linked ichthyosis, a disease clinically characterised by skin peeling localised 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 implications 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).

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 also detected in other biological fluids such as urine, bile, seminal plasma and 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 Chol, CS also represents a biosynthetic precursor of several bioactive steroids. In this scenario, the sulfoconjugation reaction may represent a key step in the formation of a readily available hydrophilic form of Chol. CS has shown to regulate the Chol homeostasis by negative regulation of the key enzyme in Chol synthesis pathway, 3-hydroxy 3-methylglutaryl-CoA reductase (HMG-CoA reductase) indirectly (Williams, Hughes-Fulford, & Elias, 1985) and block the esterification of cholesterol directly by inhibiting the activity of lecithin-cholesterol acyltransferase enzyme (Nakagawa & Kojima, 1976). Indeed, CS can be subjected to several enzymatic 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, the complete understanding of the nature of CS interactions, CS trafficking and the signalling pathways in which it could be involved is still elusive.

Intra- and extra-cellular trafficking of CS is one of the most unexplored characteristics except for sex hormones sulfates (e.g. 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 compart 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).

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. In 1998, Sato *et al.* correlated this pathologic condition with the ability of CS to inhibit serine proteases involved

in cell dissociation, a key feature in skin development (Sato, Denda, Nakanishi, Nomura, & Koyama, 1998). 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 nineties (Ito, Iwamori, Hanaoka, & Iwamori, 1998; Iwamori, Iwamori, & Ito, 1997; Iwamori, Suzuki, Kimura, & Iwamori, 2000). The inhibitory behaviour of CS towards these pancreatic enzymes has been related to its protective role at the gastrointestinal mucosa level. In addition, it is noteworthy to underline that the inhibition of these enzymes occurred in a non-specific fashion. In other words, the interaction between the two molecular partners is based only on the physico-chemical properties of CS and the presence of an anion binding region on the tertiary structure of the target protein.

In 1999, the ability of CS to inhibit serine proteases was extended by Iwamori *et al*. to thrombin and plasmin (Iwamori, Iwamori, & Ito, 1999). 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).

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 stereotypically involves a multitude of mediators and many different cell types. 5-Lipoxygenase (5-LO) is involved in the production of leukotrienes, soluble mediators of the inflammatory state and immune system functionality. In particular, leukotrienes play a pivotal role in asthma and bronchitis. When a Ca^{2+} influx takes place, 5-LO binds 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. (Aleksandrov et al., 2006), demonstrated the inhibitory behaviour of CS towards 5-LO in a cell-free assay. Here, CS has been found to decrease 5-LO interaction with the nuclear membrane in a cell-based assay upon stimulation, thus decreasing leukotriene biosynthesis.

In 2016, Wang *et al*. demonstrated the relevance of CS as a modulator of T-cell receptor (TCR) functionality (Wang et al., 2016). The TCR is a multisubunit membrane receptor which

includes an antigen-recognition domain composed of the TCRα and β (or γ and δ) 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 has been related to the nanoclustering of several TCRs. Chol is able to interact with TCRβ thus promoting TCR nanoclustering. Conversely, CS can disrupt TCR clusters by interfering in the Chol-TCRβ interaction. Interestingly, the Chol /CS ratio is a variable parameter during T cell development and differentiation (Wang et al., 2016).

Dedicator of cytokinesis protein 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. In 2018, Sakurai *et al.* 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 (Sakurai et al., 2018). In particular, the 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 polipeptidic counterpart.

Role of CS as a ligand in signalling pathways

In 2004, Kallen *et al.* reported the crystal structure of CS with the nuclear receptor retinoic acid-related orphan receptor α (RORα) (Kallen, Schlaeppi, Bitsch, Delhon, & Fournier, 2004). Since RORα could be implicated in the control of Chol homeostasis, the Authors set up crystallization trials both with Chol 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^{370}) with the consequent displacement of several water molecules which were instead present in the interaction with Chol. Even though the crystal

studies unambiguously pointed out 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 RORα endogenous ligand (Han et al., 2014; Kim et al., 2008; Zenri et al., 2012).

CS has been also found to have an important role in the substrate specificity of phosphatidylinositol 3-kinase (PtdIns-3K) (Woscholski, Kodaki, Palmer, Waterfield, & Parker, 1995) Phosphatidylinositol (3,4,5)-trisphosphate (PIP3), produced by PtdIns-3K' s activity, is associated with the signalling pathway of several growth factors and it is considered a secondary messenger. Phosphatidyinositol diphosphate (PIP2) is the preferred substrate of PtdIns-3K *in vivo*, inside the cell. Conversely, phosphatidyinositol monophosphate and phosphatidylinositol are the preferred substrates of PtdIns-3K in cell-free systems. In 1995, Woscholski *et al.* 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 (Woscholski et al., 1995).

Oxysterols sulfates and their receptors

Oxysterols are bioactive lipids which share the 27-carbons skeleton with Chol 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. G protein-coupled receptors, 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 proposed that oxysterol sulfates were bioactive molecules that acted as selective ligands with biological outcomes. **Table 1** lists oxysterols sulfates with reported cellular activities, but not all oxysterol sulfates detected by analytical techniques are 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 enzymatic activity of STS is able to afford the parent oxysterol in its active form. In 2001, Song *et al.* demonstrated that 5α,6α-epoxycholesterol-3-sulfate (5,6αECS) and 7-ketocholesterol-3-sulfate (7KCS) were able to bind both nuclear receptors

LXRα and LXRβ inhibiting their activation 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 out by testing two closely related sulfated oxysterols, 5β,6β-epoxycholesterol-3-sulfate (5,6βECS) and 6-ketocholestanol-3-sulfate, in the same assays. As both of the latter compounds failed in modulating LXRs activation, the authors speculated that the antagonistic behaviour of 5,6α-ECS and 7KCS towards LXRs was independent of their physiochemical properties (e.g. amphiphilicity) (Song et al., 2001).

In 2009, Cook *et al.* reported that the endogenous LXRs 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 LXRs target gene whose expression increases in the presence of agonists. Accordingly, the sulfation of LXRs endogenous agonists can be considered a negative feedback mechanism able to control LXRs activation (Cook et al., 2009).

Also 25-hydroxycholesterol (25HC), another endogenous LXRs agonist, can be converted into an antagonist when sulfated at 3β-OH. 25HC3S was identified by Ren *et al*. in 2007 first in hepatocytes nuclei. 25HC3S has been found to decrease the expression of SREBP-1 target genes (e.g. HMG-CoA reductase) with a consequent overall decrease of Chol 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, namely 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 Chol biosynthesis and lipogenesis (e.g. Fatty acid synthase and Acetyl-CoA carboxylase-1) (Leyuan Xu et al., 2010). By contrast, Zhang *et al.* demonstrated that 25HC3S up-regulated several genes involved in hepatic cells proliferation (Zhang et al., 2012). In a $LDLR^{-/-}$ mouse model overexpressing SULT2B1b with 25OHC

supplementation increased 25OHCS levels and it was demonstrated that endogenous 25HC3S is 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 (e.g. NAFLD) by Durect corporation. In 2012, 25HC3S has been also 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.*, showing the selection of a partial-agonistic conformation of the receptor by the ligand (Yang et al., 2019).

One of Ren's group discoveries has been the identification of the sulfolipid 25HCDS in rat hepatocytes. Like 25HC3S, 25HCDS was able to reduce Chol levels and to negatively regulate immune responses at transcriptional level probably interfering with LXRs, SREBPs and PPARγ (Ren et al., 2014). However, since no proof of concept regarding the exact mechanism of action of 25HCDS has been reported yet, the latter hypothesis remains elusive.

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 et al., 2015a).

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 with potential prognostic value during clinical evaluation (Meng et al., 1997). Later, Acimovic *et al*. suggested that sulfation could act as a protective mechanism against the accumulation of oxysterols in circulation (Acimovic et al., 2013). A glimpse into the panel of oxysterol sulfates was expanded by Sanchez-Guijo *et al.* 2015a 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 samples of RLXI patients (Sánchez-Guijo et al., 2015a). 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 et al., 2015a), very little is known about the predominant oxysterol sulfates circulating in fluids and accumulated in cells/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 (Weerachatyanukul, 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 knowledge on the oxysterol signature in normolipidemia and normoglycemia conditions and their basal levels is 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 sulfotransferases (Hirotoshi Fuda et al., 2007) leading up to the formation of oxysterol sulfates.

The concentration values reported in the literature for oxysterol sulfates are still scarce and require corroboration as levels reported for 24-hydroxycholesterol-3-sulfate-24-glucuronide ranges 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 22.5-46 ng/mL (~46.7-95.4 nM) when compared to levels below 2.5ng/mL (<LOQ) in healthy male donors (Sánchez-Guijo et al., 2015a). The disparity of values found could be attributed to

differences in the characteristics of the individuals included in the study groups as well as to 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 pipeline including sample collection, storage, extraction, fractionation, separation, detection and quantification steps. For instance, sample pre-treatment 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 pre-treatment 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 pre-treatment 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, Hukkanen, Turpeinen, Mattila, & Tolonen, 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 case serum samples were used, Helmschrodt *et al*. suggested the addition of antioxidant, butylated hydroxytoluene (0.05%) to increase the stability of oxysterols. Another aspects that are often ignored include the freeze-thaw cycles, often required for biochemical and chemical analysis appear not to affect the levels CS (Sánchez-Guijo, Oji, Hartmann, Traupe, & Wudy, 2015b). Storage up to 3 months led to the same conclusions (Hautajärvi et al., 2018; Helmschrodt et al., 2013; Sánchez-Guijo et al., 2015b). However, the number of freeze-thaw cycles has shown to decrease the level of oxysterols (Helmschrodt et al., 2013).

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 1). 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 the extraction of predominant lipid classes (Reis et al., 2013). In the case of structurally similar compounds, the extraction performance of Chol and CS in two of the most popular LLE solvent mixtures is similar, though solvent systems with a higher dielectric constant (ε) extracted higher amounts of CS compared to Chol (MeOH:$CHCl_3$ (2:1, v/v)), whereas solvent mixtures of lower ε with more hydrophobic character were more efficient towards the extraction of Chol 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 distinct. The 24HC and 25HC isomers eluted prior to the 7-ketocholesterol (7KC) and 4β-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 impact 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 ACN-$ZnSO_4$ (4:1, v/v) followed by C_{18} SPE fractionation (Sánchez-Guijo et al., 2015a) resulted in complete recovery (100.6%).

While the presence hydroxy group affects the hydrophobicity of the oxysterol moiety and may impact on the performance during the extraction step, the presence of the sulfate and hydroxy groups in oxysterols sulfates also impacts on the detection approaches that can be used to detect and quantify oxysterol sulfates. Unlike oxysterols that 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) the presence of the sulfate group facilitates the detection of oxysterol sulfates in the negative ion mode through mass spectrometry-based approaches. Because oxysterols sulfates occur in residual levels in biological samples, detection of oxysterols sulfates is often achieved by targeted detection approaches such as 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 Sanchez-Guijo and colleagues established 1ng/mL as the limit of detection of oxysterol sulfates in MRM detection approaches (Sánchez-Guijo et al., 2015a).

Contrarily, the presence of the hydroxy group has no influence on the efficacy of ionisation and hence on the detection step. As ionisation of oxysterol sulfate occurs by removal of hydrogen atom at the sulfate group, the ionisation 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 et al., 2015a) 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 literature reported, oxysterols which are structurally related compounds of oxysterol sulfates account for less than 1% of total Chol in hyperlipidemia (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.

Concluding Remarks

In summary, it is clear that CS and oxysterol sulfates act as key players of many biological pathways influencing human health and disease. While CS has been extensively

studied, only a handful of research focused on oxysterol sulfates. The lack of a more complete panel of oxysterol sulfate standards commercially available and the poor knowledge on the optimal conditions for the extraction, detection and quantification of oxysterol sulfates from biological matrices has hampered the complete understanding on 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 that 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.

Competing Interests' Statement: None

Authors declare no conflicts of interest.

Acknowledgements

AR acknowledges the support from UIDB/50006/2020 with funding from FCT/MCTES through national funds. IHKD acknowledge the support from Royal Society, UK. HKID and LD acknowledge funding from Alzheimer's research UK midlands network.

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
5,6αECS: 5α,6α-epoxycholesterol-3-sulfate
5-LO: 5-Lipoxigenase
7KC: 7-ketocholesterol
7KCS: 7-ketocholesterol-3-sulfate

ACN: Acetonitrile
CS: Cholesterol sulfate
DHEA: Dehydroepiandrosterone
DHEAS: Dehydroepiandrosterone sulfate
DOCK2: Dedicator of cytokinesis protein 2
EDTA: Ethylenediaminetetraacetic acid
HMG-CoA reductase: 3-hydroxy 3-methylglutaryl-CoA reductase
HPLC: High Performance Liquid Chromatography
IκBα: NF-κB inhibitor
LLE: Liquid-liquid extraction
LXRα: Liver X receptor alpha
LXRβ: Liver X receptor beta
Mincle: Macrophage inducible Ca2+-dependent lectin receptor
MRM: Multiple Reaction Monitoring
NF-κB: Nuclear Factor-κB
PAPS: 3'-phosphoadenosine 5'-phosphosulfate
PIP2: Phosphatidyinositol diphosphate
PIP3: Phosphatidylinositol (3,4,5)-trisphosphate
PPARγ: Peroxisome proliferator-activated receptor gamma
Preg: Pregnenolone
PregS: Pregnenolone sulfate
PtdIns-3K: Phosphatidylinositol 3-kinase
RLXI: Recessive X- linked ichthyosis
RORα: Retinoic acid-related orphan receptor α
SL: Sufate-based lipids
SPE: Solid Phase Extraction
SREBP-1: Sterol Regulatory Element-Binding Protein-1
STS: Steroid Sulfatases
SULTs: Sulfotransferases
TCR: T-cell receptor
TNFα: Tumor Necrosis Factor alpha

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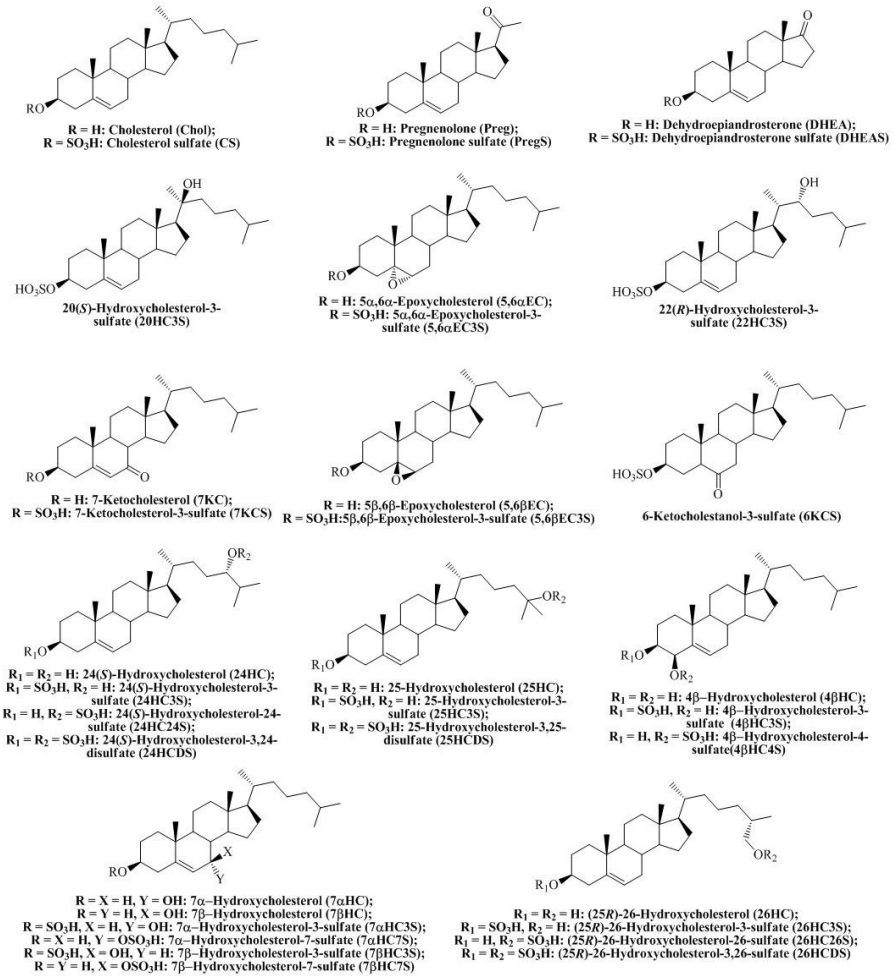

Figure 1: Structures of sterol and oxysterol sulfates

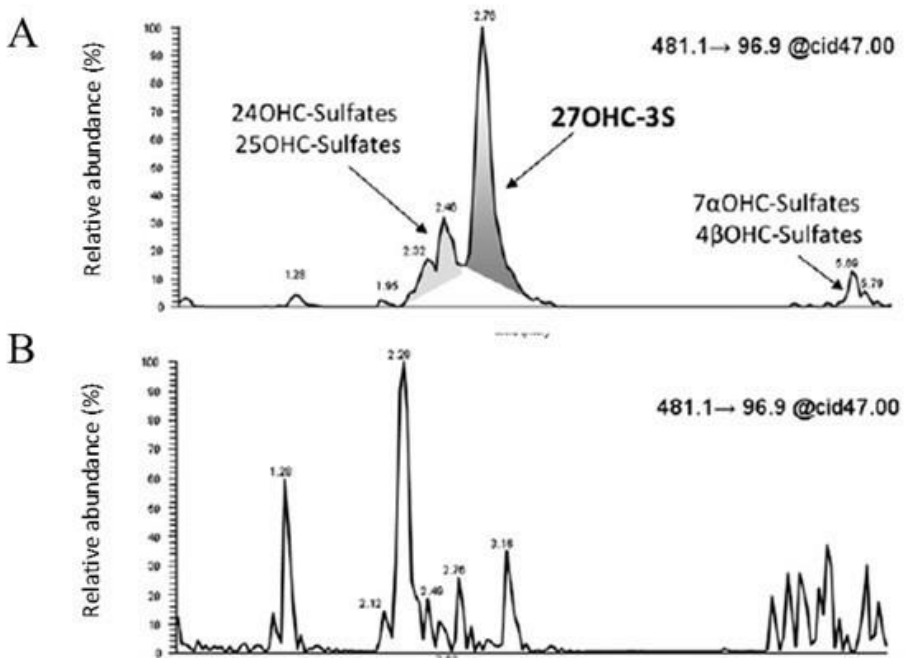

Figure 2: Chromatographic separation of oxysterol sulfates in serum samples from RLXI patient (A) and healthy control subject (B) using targeted multiple reaction monitoring (MRM) detection mode. This data was originally published in the Journal of Lipid Research. Sánchez-Guijo A, et. al. High levels of oxysterol sulfates in serum of patients with steroid sulfatase deficiency. J Lipid Res. 2015;56(2):403–412. © the American Society for Biochemistry and Molecular Biology.

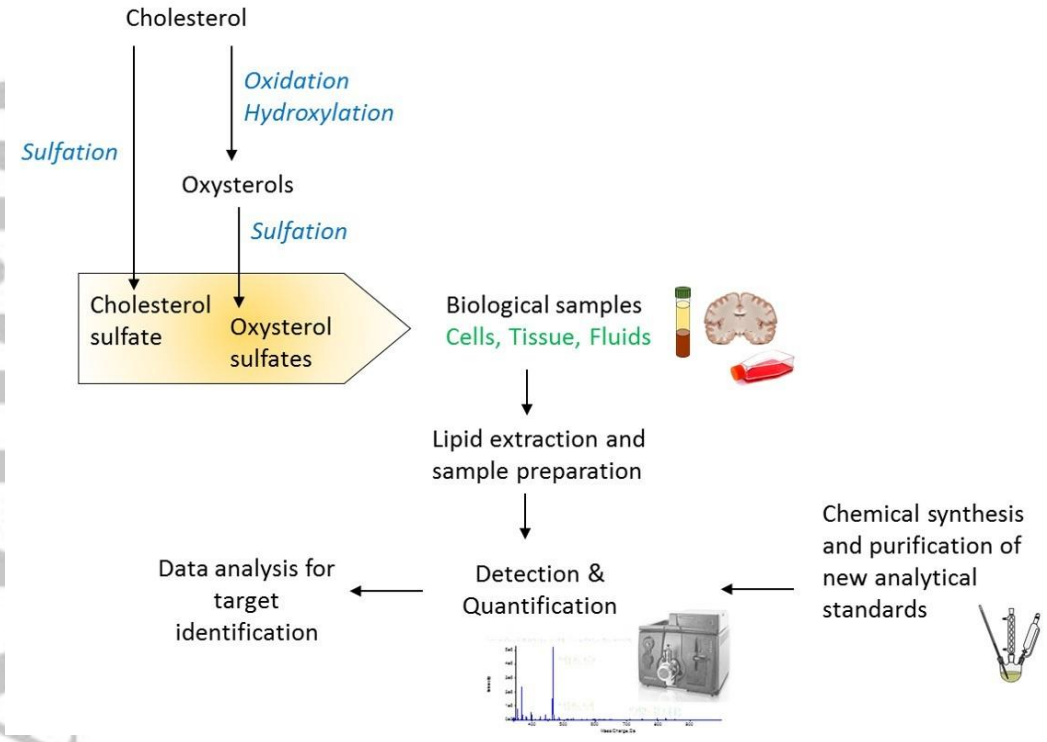

Figure 3: Overview of cholesterol sulfate and oxysterol sulfate analysis

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 sensitise cells to apoptosis	0.6 -100 µM	(Segala et al.2013) (Warns, Marwarha, Freking, & Ghribi, 2018)
	Neuroblastoma cell line, SHSY-5Y cells	No effect on cell viability	10 µM	(Marwarha, Rhen, Schommer, & Ghribi, 2011)
	Human embryonic kidney 293 cells	Attenuates the 26HC-induced increase in α-synuclein expression Inhibit transactivation of reporter genes by LXR	4-20 µM	(Song, Hiipakka, & Liao, 2001)
7KCS	Human embryonic kidney 293 cells	Reduce cytotoxicity induced by 7ketoC	5 nM	(Hirotoshi Fuda, Javitt, Mitamura, Ikegawa, & Strott, 2007)
	Human retinal pigment epithelial cell line, ARPE-19	Attenuates ABCA1 and VEGF inductions by 7ketoC	0-20 µM	(Moreira, Larrayoz, Lee, & Rodríguez, 2009)
	Human embryonic kidney 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)
25OHC3S	Hepatocytes	Inhibits the LXR/SREBP signalling pathway, regulates lipid metabolism, inflammatory responses, and cell proliferation	0-25 µM	(Ren et al., 2014; Ren et al., 2007), (Ren & Ning, 2014)

	Human monocytic cell line, THP-1	Attenuates inflammatory response via PPARγ signalling	0-50 μM	(Ma et al., 2008) (L. Xu et al., 2012),

Table 2. Analytical strategies employed in the collection, extraction, and analytical approach in the detection and quantification of cholesterol sulfate and oxysterols in human plasma samples.

	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 quartenary amine column	GC-FID (TMS derivatives), n.s.	Muskiet et al., 1983
	Sodium (citrate)	LLE with MeOH (80%)	HPTLC coupled to densitometry, n.s.	Przybylska et al., 1995
	-	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 et al., 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 (acetylated derivatives), n.s.	Delfino et al., 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 (μmol/L): 0.02	Fong et al., 2013

Oxysterols	Serum/plasma	Protein ppt ACN-$ZnSO_4$ followed by fractionation by SPE (SepPak cartridge)	LC-MS/MS, LOQ (ng/mL): 80	Sanchez-Guijo et al., 2015b
	Serum (-)	SPE extraction with MeOH in Strata-X (33µm) cartridges	LC-(ESI)MS detection and quantification by SIM in QqQ, LOQ (ng/mL): 5	Lee et al., 2016
	Plasma (K_2EDTA)	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 (ng): 0.3-5	Dzeletovic et al., 1995
	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 (ng/mL): 0.02	Murakami et al., 2000
	Plasma (-)	LLE with CH2Cl2:MeOH (1:1, v/v) aided by ultrasonic bath homogenization (10min).	LC-(APCI)MS detection and quantification by MRM in QTrap, LLOQ (ng/mL): 1	McDonald et al., 2012
	Plasma (EDTA)	LLE with MeOH:iso-propanol (1:1, v/v)	LC-(APCI)MS detection and quantification by MRM in QqQ, LLOQ (ng/mL): 0.5	Helmschrodt et al., 2013
	Plasma (EDTA)	LLE with ethanol, followed by alkaline hydrolysis and extraction with $CHCl_3$:MeOH (2:1, v/v) and	GC-MS of TMS derivatives, n.s.	Graya et al., 2018

		SPE fractionation in a silica column		
	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 (pg/mL): 18-253	Dias et al., 2018
	Plasma (K_2EDTA)	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 cal curves built with deuterated standards, LLOQ (ng/mL): 0.5-2	Hautajärvi et al., 2018

n.s. not stated