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Abstract:	Sulfation pathways have recently come into the focus of biomedical research. For steroid hormones and related compounds, sulfation represents an additional layer of regulation as sulfated steroids are more water-soluble and tend to be biologically less active. For steroid diols, an additional sulfation is possible, carried out by the same sulfotransferases that catalyze the first sulfation step. The steroid disulfates that are formed are the focus of this review. We discuss both their biochemical production as well as their putative biological function. Steroid disulfates have also been linked to various clinical conditions in numerous untargeted metabolomics studies. New analytical techniques exploring the biosynthetic routes of steroid disulfates have led to novel insights, changing our understanding of sulfation in human biology. They promise a bright future for research into sulfation pathways, hopefully too for the diagnosis and treatment of several associated diseases.

Steroid disulfates - Sulfation double trouble

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Abbreviations:

ER+, estrogen-receptor positive/containing; ICC, intraclass correlation coefficient; OATP, organic anion transporting polypeptide; PAPS, 3'-phospho-adenosine-5'-phosphosulfate; PAPSS1/2, PAPS synthase 1 or 2; PORD, P450 oxido-reductase deficiency; STS(D), steroid sulfatase (deficiency); SULT(2A1), sulfotransferase (type) 2A1

Abstract

Sulfation pathways have recently come into the focus of biomedical research. For steroid hormones and related compounds, sulfation represents an additional layer of regulation as sulfated steroids are more water-soluble and tend to be biologically less active. For steroid diols, an additional sulfation is possible, carried out by the same sulfotransferases that catalyze the first sulfation step. The steroid disulfates that are formed are the focus of this review. We discuss both their biochemical production as well as their putative biological function. Steroid disulfates have also been linked to various clinical conditions in numerous untargeted metabolomics studies. New analytical techniques exploring the biosynthetic routes of steroid disulfates have led to novel insights, changing our understanding of sulfation in human biology. They promise a bright future for research into sulfation pathways, hopefully too for the diagnosis and treatment of several associated diseases.

Introduction

Sulfation – the addition of a sulfate moiety to a biological acceptor molecule – is a central pathway in metabolism, becoming more recognized in recent years (Gunal, Hardman, Kopriva et al., 2019). For larger biopolymers such as proteins or carbohydrates, it is common to be sulfated at more than one site. One example is the N-terminus of the cytokine receptor protein CCR5; it needs to be sulfated twice to serve as anchor point for its natural ligand CCL5 and for HIV (Abayev, Rodrigues, Srivastava et al., 2018). Another example is the widely used anti-coagulation medication heparin, a glycosaminoglycan which is known as one of the most highly sulfated biomolecule per weight (Taylor, Hogwood, Guo et al., 2019).

However, for low-molecular-weight molecules sulfation at a single site has been conventional; the dynamic single-site sulfation and desulfation of steroid hormones has been reviewed recently (Foster and Mueller, 2018,Mueller, Gilligan, Idkowiak et al., 2015). **Mono-sulfation** makes steroids more soluble and hence sulfated steroids by far outweigh unconjugated steroids in circulation (Mueller et al., 2015). Interestingly, steroid mono-sulfates may still be substrates for downstream steroid processing enzymes (Klymiuk, Neunzig, Bernhardt et al., 2018); hence, the sulfate "cap" would act as a protective group in organic synthesis and direct steroid metabolism to the non-sulfated part of the molecule. However, there is an exception to the rule of mono-sulfated steroids.

Certain steroid hormones feature two hydroxyl groups at opposing ends of the molecule. These can be sulfated at both sites, giving rise to **steroid disulfates**, also known as bis-sulfates. Different disulfated steroid species were first detected in human urine and plasma many decades ago (see **Table 1**) (Foster and Mueller, 2018), the first description dating from 1962 (Pasqualini and Jayle, 1962). The biological role of steroid disulfates remained unknown until today. Reports about these disulfates and other conjugated steroids soon declined; for the decades to come, any conjugate of a steroid was hydrolyzed before analysis by gas-chromatography or liquid-chromatography coupled to mass spectrometry in a standard clinical diagnostic lab (Shackleton, 2010). Disulfates became a forgotten steroid species.

In recent years, new analytical procedures allowed the analysis of bodily fluids by mass spectrometry to be specific for the detection of conjugated steroid species (Marcos and Pozo, 2016). Disulfates are detected for estrogens like estriol disulfate, androgens such as androstanediol disulfate, progestogens like pregnanediol disulfate and corticosteroids including tetrahydrocortisol disulfate (McLeod, Waller, Esquivel et al., 2017). Various steroid disulfates have been discovered or re-discovered; now in the context of large cohort studies (see **Table 2**) or as potential biomarkers in clinical studies including for carcinoma of the adrenal cortex (Sun, Kunzke, Sbiera et al., 2019). This review article attempts to give a comprehensive overview of steroid disulfates. It will cover their biosynthesis and analytics in bodily samples, alongside summarizing recent ideas about the biological function of steroid disulfates.

Steroid pseudosymmetry and promiscuous enzymes make twofold steroid sulfation possible

A number of steroids contain two hydroxyl groups. One is nearly always seen at C3 in the A ring, because nearly all steroids are derived from cholesterol with an OH group at that very position. A second hydroxyl group may be at the D ring itself (seen in estradiol or androstenediol) or in a hydrocarbon extension attached to C17 (**Figure 1A**). In most cases, the rest of the steroid molecule is relatively flat and hydrophobic. These steroid double alcohols (di-ol's) have been called

pseudosymmetric (Mueller et al., 2015,Gangloff, Shi, Nahoum et al., 2003,Lathe and Kotelevtsev, 2014) - they look very similar, when turned around by 180° (**Figure 1B**).

The biosynthesis of disulfated steroids involves the same set of **sulfotransferases** (SULTs) that are responsible for the formation of mono-sulfated metabolites. For androstenediol – a direct conversion product of the weak androgen DHEA – this is most predominately the sulfotransferase SULT2A1 (Mueller et al., 2015). Falany and his co-workers provided a very detailed analysis about the mechanism and consequences of sterol disulfation (Cook et al. 2009). They established for the cholesterol metabolite 24-hydroxycholesterol, that the sulfotransferases SULT2A1 and SULT1E1 were able to sulfate both at the 3- and 24-position, while SULT2B1b could only sulfate the 3-position (Cook, Duniec-Dmuchowski, Kocarek et al., 2009). This sulfation was still beneficial to prevent the toxicity of some oxysterols (Fuda, Javitt, Mitamura et al., 2007). However, only SULT2A1 formed the doubly sulfated disulfate at significant rates (Cook et al., 2009). High levels of SULT2A1 can be found in the adrenal cortex, small intestine and liver in humans (Falany, Macrina and Falany, 2004).

In their substrate-binding site, cytoplasmic sulfotransferases such as SULT2A1 show a high degree of plasticity. They accept a wide variation of substrates, due to flexible binding loops around the substrate binding pocket (Berger, Guttman, Amar et al., 2011,Atkins, 2020). At times it can be difficult to understand substrate specificity on a molecular level, even when looking at 3D structures of enzyme-substrate complexes (Hirschmann, Krause, Baruch et al., 2017). Substrate promiscuity may be linked to the flexibility of the substrate binding loops; crystallographic temperature factors (B factors) correlate with that flexibility of the protein (**Figure 2**) (Carugo, 2018). When both concepts are combined, they allow high accuracy modeling of substrate docking to sulfotransferases (Cook, Wang, Falany et al., 2013).

Where steroid pseudosymmetry and **sulfotransferase promiscuity** meet, steroid disulfates are made (Gunal et al., 2019). This means a suitable tissue needs to be able to make or uptake relevant steroid-diol precursors and, at the same time, express a certain subset of sulfotransferases. For estradiol disulfate, this includes the sulfation genes SULT2A1 and SULT1E1 (Sun et al., 2019). Two different sulfotransferases are most likely required here because one of the hydroxyl groups is an aromatic, the other an aliphatic alcohol. SULT2A1 seems to be especially suited to form disulfates of diol steroids, but also of xenobiotic diols (Falany and Falany, 2007). It is likely that the degree of pseudosymmetry of the steroid substrate is a major determining factor of whether a respective steroid disulfate can be made in a single cell or whether cooperation between different tissues – alongside import and export events – are required for that double sulfation.

Other factors might facilitate double steroid sulfation as well, such as a direct physical interaction of the PAPS-dependent human SULT2A1 enzyme and the PAPS-producing PAPSS2 enzyme (Mueller, Idkowiak, Gesteira et al., 2018), which may influence the stability of sulfation pathway proteins (Brylski, Ebbinghaus and Mueller, 2019). Indirect or allosteric effectors may also modulate the sulfation activity of sulfotransferase enzymes themselves: the cyclo-oxygenase-2 inhibitor celecoxib shifts the preferential sulfation site of SULT2A1 from C3 to C17 within the estradiol molecule (Wang and James, 2005,Ambadapadi, Wang, Palii et al., 2017), and the widely used pain relief acetaminophen (paracetamol) inhibited SULT2A1 function (Cohen, Cirulli, Mitchell et al., 2018).

Finally, the promiscuous binding properties of sulfotransferases can interfere with their own catalytic activity, as substrates may also bind in non-productive conformations and hence give rise to substrate inhibition at higher concentrations (Mueller et al., 2015,Gamage, Tsvetanov, Duggleby et al., 2005). Double sulfation is not limited to steroids, other diols with a degree of pseudosymmetry can form

disulfates as well (Nakano, Ogura, Takahashi et al., 2004). In summary, processes towards generation of steroid disulfates are complex and most likely non-linear.

An important consideration for steroid disulfates is their half-life, dictated by enzymes. Put differently, are these doubly sulfated steroid species still substrates for the **steroid sulfatase** (STS) enzyme? There are not many studies around to answer this question comprehensively. Falany and coworkers established for 24-hydroxycholesterol-3,24-disulfate that double sulfation, followed by selective desulfation leads to a terminal product that is resistant to reactivation by STS (Cook et al., 2009). Placental STS hydrolysis of the sulfates was investigated, and it was found the 3, 24-disulfate was hydrolyzed to an **STS-resistant** 24-mono-sulfate at approximately the same concentration as the disulfate in the initial reaction (Cook et al., 2009). This also suggests that the 3-mono-sulfate group in the mono-sulfate and disulfate was not resistant and was hydrolyzed by STS. This means 3-sulfation is a reversible reaction but that forming the 24-sulfate or 3,24-disulfate is a **terminal reaction** as the 24-sulfate was not hydrolyzed (Cook et al., 2009). This fueled the idea that a second sulfation step represented an additional regulatory step or an irreversible step towards inactivation of the steroid molecule (Mueller et al. 2015).

One idea is that steroid disulfates themselves or desulfated metabolites represent poorer substrates for the steroid sulfatase enzyme (Cook et al., 2009,Falany and Falany, 2007). STS hence plays an important role in the biological sulfation-desulfation balance. Omics approaches indicate that STS is expressed predominantly in the placenta and to a lesser extent in the cerebral cortex, lungs, stomach, gallbladder, urinary bladder and adipose tissue (Foster and Mueller, 2018,Uhlen, Fagerberg, Hallstrom et al., 2015). Expression information for STS always has to be treated carefully, as only the co-expression of the formyl-glycine generating enzyme, also known as sulfatase modifying factor 1 (SUMF1), ensures biosynthesis of the fully active enzyme; very rare mutations in the *SUMF1* gene may also cause apparent STS deficiency (Adang, Schlotawa, Groeschel et al., 2020,Staretz-Chacham, Schlotawa, Wormser et al., 2020).

A very interesting concept about human sulfotransferase/sulfatase pathways is that not necessarily all enzymes involved need to be of human origin. Several bacterial strains with sulfatase activity have been isolated from human and mouse intestine microbiomes (Van Eldere, Robben, De Pauw et al., 1988); meaning the gut microbiota is likely an important player in human sulfation pathways.

Supposed molecular functions of disulfated steroids

Traditionally, steroid hormones are thought to be able to penetrate biological membranes freely. Although this assumption might be outdated now (Okamoto, Viswanatha, Bittar et al., 2018), at least a fraction of non-conjugated steroids will diffuse through biological membranes (McManus, Bohn, Alyamani et al., 2019). However, membrane permeability dramatically changes upon sulfate addition to the steroid molecule. As negatively charged, low-molecular-weight metabolites, these steroid sulfates now require a dedicated class of transporters called the **organic anion transporting polypeptides** (OATPs) to pass across membranes (Doring, Lutteke, Geyer et al., 2012). Furthermore, the twofold sulfation of a steroid makes a massive difference here (Foster and Mueller, 2018). Estradiol-3-sulfate and estradiol-17β-sulfate are both substrates for the sodium-dependent organic anion transporter (SOAT) SLC10A6; this transporter no longer however accepts estradiol-3,17β-disulfate as a cargo (Grosser, Bennien, Sanchez-Guijo et al., 2018). The human organic anion transporter 4 (OAT4) which is present in the kidney and the placenta, but not the adrenal gland, may still accept estradiol-3,17β-disulfate as a substrate (Zhou, Illsley and You, 2006). For another OATP,

OATP1B1, the four amino acids Asp70, Phe73, Glu74, and Gly76 have been identified as essential for estradiol-3-sulfate transport (Li, Hong, Huang et al., 2012); it would be interesting to identify analogous residues in the other transporters. An issue with transport is its directionality. While sodium-dependent transport always seems to be directed towards the cell, some transport systems might run bidirectional under special circumstances. Other transporters are members of the ATP-binding cassette family, managing the efflux of sulfated steroids from cells (Iliev, Braun, Sanchez-Guijo et al., 2020).

Taken together, **the second sulfation step may confine the steroid disulfate within a cell** (such as a cell of the adrenal cortex), if neither steroid sulfatase nor alternative transporters are present.

Relatively little is known about the functions of steroid disulfates within the cell. Most likely, the disulfated steroid hormones will no longer bind to and activate their corresponding steroid **nuclear receptors**. Less predictable is the effect of steroid sulfates on the growing and diverse group of non-genomic receptors. A summary of our current knowledge is presented in **Figure 3** and described in more detail in the accompanying text.

Coupled sulfation and desulfation processes may create **desulfation-resistant steroid sulfate** end-products. An example for this is the stable, STS-resistant 24-hydroxycholesterol-24-mono-sulfate that does not bind to the nuclear receptor of the corresponding sterol (Cook et al., 2009). However, this compound was actually proven to be a potent and stable **antagonist** for the liver X receptor (LXR) (Cook et al., 2009); even though the molecular mechanism for this antagonistic effect is not known. Other sterols may do the opposite. Cholestan disulfate, a derivative of solomonsterol A, acts as a potent pregnane-X-receptor (PXR) **agonist** (Sepe, Ummarino, D'Auria et al., 2012). Cholestan disulfate as a PXR activator might be useful in treating inflammation or liver fibrosis (Sepe et al., 2012). Oxysterols continue to reveal new functions; the doubly sulfated cholesterol metabolite 5-cholesten-3β,25-diol disulfate was recently shown to potently inhibit cholesterol synthesis (Ren, Kim, Kakiyama et al., 2014). As steroid hormones classically signal via nuclear hormones, any action of steroid disulfates on nuclear receptors or other transcription factors might result in feedback loops to upregulate sulfation enzymes again (Dalla Valle, Toffolo, Nardi et al., 2006,Li, Renaud, Klaassen et al., 2016).

Kroiss and coworkers suggested a potentially new fate for steroid disulfates (Sun et al., 2019). They identified different sulfated estradiol species as putative prognostic markers for carcinomas of the cortex of the adrenal gland. Estradiol-3,17β disulfate was among these molecular markers, and cells which contained this disulfate were reported to display a novel form of vesicle or **vacuole with an amorphous material**. These vacuoles were absent in samples with a high content of estradiol mono-sulfates (Sun et al., 2019). These findings immediately give rise to more questions than answers. Maybe they represent a sink for disulfated steroids, that could even form a route for degradation/recycling via standard cellular mechanisms, such as autophagy or the lysosomal pathway.

Steroid disulfates with their hydrophobic backbone and two negative charges could also dock specifically to certain biological structures. Hints into this avenue come from the molecular action of steroid based sulfatase inhibitors with two sulfamate moieties (Andring, Dohle, Tu et al., 2019). Potter and colleagues explored the potential of the bis-sulfamate compound STX140 (shown in the structural panel in **Figure 4**) as a potential treatment for triple-negative breast cancer (Andring et al., 2019). Structurally and chemically, STX140 is related to Irosustat (STX64) which has a high potential for treating breast, prostate and endometrial cancers (Potter, 2018,Thomas and Potter, 2015). STX140 is an STS inhibitor, but also an anti-tumor multi-targeting agent. STX140 directly competes at the tubulin binding site to cause microtubule depolymerization and leads to cell apoptosis (Meyer-Losic, Newman,

Day et al., 2013). STX140 is anti-angiogenic which is an important factor for suppressing tumor growth, and it also directly binds to and inhibits the carbonic anhydrase IX protein, an established marker for aggressive cancers.

However, one needs to be cautious when comparing a steroid disulfate with a steroid bis-sulfamate. The disulfate bears two negative charges at physiological pH value, while the bis-sulfamate is hardly de-protonated at these conditions. Still, STX140 structurally is very similar to estradiol-3,17β-disulfate (El-Gamal, Semreen, Foster et al., 2016). Please compare these molecules within the structure panel **Figure 4**. Hence, estradiol disulfate may turn out to bind to a diverse set of proteins similar to the steroidal STX140 compound. An example for such specific binding of a steroid disulfate to an enzyme unrelated to steroidogenesis is the inhibition of kynurenine aminotransferase II enzymes by estradiol disulfate (Jayawickrama, Nematollahi, Sun et al., 2017), discussed in more detail further below.

Doubly sulfated steroids in medical diagnostics

Untargeted metabolomic studies of increasingly larger sample sizes have highlighted disulfated steroids in recent years. While they do not tend to give molecular connections, these studies show interesting correlations between levels of certain disulfates and a wide range of conditions. We have summarized these studies in **Table 2**. Reviewing these studies was made challenging by the use of non-specified abbreviations and by reporting unusual steroids that we will discuss in a dedicated section below (see **Figure 5**). Selected steroid disulfates are displayed in **Figure panel 4** due to their known biological importance – such as estradiol disulfate – or due to their abundance in the literature. **Table 2** lists steroids such as 5α-androstan-3β,17β-diol disulfate and androsten-3β,17β-diol disulfate, that are mentioned 11 and 15 times, respectively. They are shown in **Figure 4A and B**. We discuss these studies together with recent analytical improvements.

Building upon recent improvements in ease of detection, Pozo and coworkers analyzed steroid disulfates in urine for **prenatal diagnosis** of steroid biosynthetic disorders (Pozo, Marcos, Khymenets et al., 2018). Primarily, the researchers were interested in detecting known insufficiencies of estriol synthesis (Shackleton, Marcos, Arlt et al., 2004,Shackleton, Marcos, Malunowicz et al., 2004). In STS deficiency which caused estriol insufficiency, six of the different disulfates measured by the constant-ion-loss method were markedly increased, compared to controls. In P450 oxido-reductase deficiency, the urinary steroid 5α-pregane-3β,20α-diol disulfate was found in much higher quantities compared to controls (Pozo et al., 2018). A very recent study found lower levels of 5α-androstan-3α,17α-diol disulfate to predict **term fetal growth restriction** (Sovio, Goulding, McBride et al., 2020). On the contrary, raised levels of 5α-pregnan-3β,20α-diol disulfate predicted the increased risks of **pre-eclampsia** (Ross, Baer, Ryckman et al., 2019).

The direct detection of steroid disulfates could have great potential in the diagnosis of these neonatal pathologies, there is one caveat however when it comes to the diagnosis of neonate conditions. Similar to the general rewiring of steroidogenesis shortly after birth (Reisch, Taylor, Nogueira et al., 2019), steroid sulfation also changes greatly in this time (Oikarinen, Kaar and Ruokonen, 1980).

As early as 1974, elevated levels of 5α-pregnane-3α,20α-diol and 5β-pregnane-3α,20α-diol disulfates were correlated with **cholestasis** (Laatikainen, Peltonen and Nylander, 1974). Caussy and colleagues suggested a molecular fingerprint for the diagnosis of advanced **liver fibrosis**, based on untargeted serum metabolome profiling (Caussy, Ajmera, Puri et al., 2019). Their set of 10 metabolic markers elevated in patients with liver fibrosis contained six conjugated steroids including 5α-androstan-3β disulfate. They reported a better diagnostic accuracy based on their set of metabolites than with the

previously used FIB-4 index (Caussy et al., 2019). This study is highly clinically relevant as advanced fibrosis is the strongest predictor of liver-related mortality.

Steroid disulfates have since been associated with certain cancer types; one study connected androsten-3β,17β-diol mono-sulfate 1 with overall **breast cancer** risk (Playdon, Ziegler, Sampson et al., 2017). The same study associated a number of sulfated steroids with alcohol-related estrogen-receptor-positive (ER+) breast cancer: androsten-3β,17β-diol disulfate, androsten-3β,17β-diol mono-sulfate 2, 5α-androstan-3α,17β-diol disulfate, 5α-androstan-3β,17β-diol disulfate, dehydroepiandrosterone sulfate (DHEAS) and pregnen-diol disulfate (Playdon et al., 2017). Please note, this study differentiated two mono-sulfates 1 and 2, but did not assign them to the 3β- and 17β-isomers. An involvement of sulfation pathways has been suggested before, as STS expression was identified as an independent predictor of breast cancer recurrence (Utsumi, Yoshimura, Takeuchi et al., 1999). Huang and coworkers linked various sulfated steroids to improved survival in **prostate cancer**: androsten-3β,17β-diol disulfate, 5α-androstan-3α,17α-diol mono-sulfate, 5α-androstan-3β,17β-diol disulfate, pregnen-diol disulfate and pregnenolone sulfate (Huang, Weinstein, Moore et al., 2019). Furthermore, in prospective profiling lower levels of androsten-3β,17β-diol disulfate were associated with lethal disease (Huang, Mondul, Weinstein et al., 2019).

A hypothesis-free metabolomic study on **dry eye disease** associated reduced levels of androsten-3β,17β-diol disulfate and several mono-sulfates with dryness and irritation symptoms, and with dry eye disease itself (Vehof, Hysi and Hammond, 2017). Disulfates are biomarkers for **food intake** too with the intake of nuts and peanut butter linked to reduced levels of androsten-3β,17β-diol disulfate and 5α-androstan-3β,17β-diol disulfate (Ross et al., 2019,Zheng, Yu, Alexander et al., 2014).

Yu and colleagues looked at the association of components of the serum metabolome to all-cause mortality from the Atherosclerosis Risk in Communities (ARIC) Study (Yu, Heiss, Alexander et al., 2016). This was a large prospective cohort study of 1,887 African Americans where over 200 metabolites were measured and analyzed. They found that pregnen-diol disulfate was independently associated with **all-cause mortality**. The same pregnen-diol disulfate has already been associated with **hypertension** (Zheng, Yu, Alexander et al., 2013). Another disulfate, 5-androsten-3β,17β-diol disulfate, was linked to **cardiovascular mortality** (Huang, Weinstein, Moore et al., 2018).

Associations between **alcohol consumption** and steroid disulfates also appear in the literature. Androsten-3β,17β-diol disulfate and 5α-androstan-3β,17β-diol disulfate were associated with elevated alcohol consumption in two studies (Zheng, Yu, Alexander et al., 2014,Guertin, Moore, Sampson et al., 2014). Pallister and colleagues confirmed these findings and added some intronic SULT2A1 variants (Pallister, Jennings, Mohney et al., 2016). A previous study already had reported increased expression of SULT2A1 in rat liver and intestine upon ethanol consumption (Maiti and Chen, 2015). Decreased pregnen-diol disulfate levels, on the other hand, were associated with more rare genetic SULT2A1 variants (Feofanova, Yu, Metcalf et al., 2018). This 2018 study also associated lower levels of androsterone sulfate, 5α-androstan-3β-17β-diol disulfate and epiandrosterone sulfate with *CYP3A43* expression. Dorgan and colleagues associated androsten-3β,17β-diol mono-sulfate, and to a lesser extend 5-androsten-3β,17β-diol disulfate, with elevated alcohol intake (Dorgan, Jung, Dallal et al., 2020).

Together these studies show how disulfates may improve diagnosis for a **wide range of medical conditions**. A caveat may involve the fact that several of these studies were actually based on the same cohort datasets, most prominently the ARIC study, already described above. In any case, detection of sulfated steroids and interpretation of their occurrence is not trivial and should be carried out by specialists.

Sulfation pathways in neurological health

Neurosteroids are an emerging field of research and many sulfated steroids have activity in the brain (Horishita, Yanagihara, Ueno et al., 2014). Sulfation pathways hence have implications upon cognitive functioning, psychiatric disorders and mental health (Davies, 2018,Ratner, Kumaresan and Farb, 2019). An analysis of the ARIC study described above looked at associations with cognition (Bressler, Yu, Mosley et al., 2017) and an increased risk of incident hospitalized **dementia** was associated with these disulfates: androsten-3β, 17β-diol disulfate, 5α-androstan-3β, 17β-diol disulfate and pregnen-diol disulfate. Bressler and coworkers also reported that the associations with androsten-3β, 17β-diol disulfate were replicated in European American ARIC study participants (Bressler et al., 2017).

Steroid disulfates might also have translational potential for the treatment of **schizophrenia**, a highly prevalent global mental health disorder (Saha, Chant, Welham et al., 2005). A 2015 study highlighted inhibitors of kynurenine aminotransferase enzymes as a new paradigm for treating this condition (Jayawickrama, Sadig, Sun et al., 2015). These enzymes are part of the tryptophan pathway and elevated levels of kynurenic acid are found in patients with schizophrenia. A subsequent study from the same group analyzed estrogen and its derivatives for their ability to inhibit kynurenine aminotransferase enzymes (Jayawickrama et al., 2017). Out of this group of related compounds, estradiol disulfate turned out to be the most potent inhibitor of these enzymes with IC_{50} values down to 26 µM for kynurenine aminotransferase enzymes; mono-sulfates and unconjugated estradiol had much weaker effects (Jayawickrama et al., 2017). Based on estradiol disulfate, second-generation kynurenine aminotransferase II inhibitors were developed (Jayawickrama, Nematollahi, Sun et al., 2018). This novel link between sulfation pathways of estrogens and schizophrenia could explain why this condition shows higher prevalence amongst males, where estrogen levels are lower. It may have implications were estrogen levels change, for example with oral contraceptives, pregnancy or the menstrual cycle.

Sulfation pathways were associated with mental health also via the steroid sulfatase (STS) gene, which is highly expressed in placenta, but also found expressed in the brain. Davies and coworkers associated impaired STS function with postpartum mood disorder, with psychosis and behavioral abnormalities (Davies, 2018). The role of steroid disulfates and of other sulfated metabolites in neurological health remains to be further investigated in the future.

Double trouble with previously reported steroid disulfates

This review article was motivated by the notable and ever-increasing number of reports that link the occurrence of steroid disulfates to various clinical outcomes. Most of these steroid disulfates are displayed in panel **Figure 4**. The studies are discussed above and are also summarized in **Table 2**.

One class of metabolites was difficult for us to interpret, the purported 4-en-3-ol steroids. These compounds are not compatible with established knowledge about the reaction mechanism of 3β-hydroxysteroid-dehydrogenase enzymes (as summarized in **Figure 5**) (Thomas, Duax, Addlagatta et al., 2003). The resulting unusual allyl alcohol in 4-en-3-ol's might be chemically possible, but would probably not last long within the human body. It might be highly labile to oxidation to the much more stable 4-en-3-one, resulting in a shorter circulating half-life. Another argument comes from reversibility considerations of this reaction; to reduce the conjugated 4-en-3-one would actually be challenging and to separate these bonds first might facilitate this reduction greatly. "Conjugated" means that the π-orbitals of the p-bonds involved are aligned with each other. Taken together, 4-ene-3-ol compounds are probably not occurring biologically, an opinion shared with at least several steroid

experts. As none of the papers actually described how they analytically would have discriminated between the biologically feasible 5-en-3-ol and the isobaric, but highly unusual 4-en-3-ol, we decided to regard to these compounds as 5-en-3-ol or simply as en-3-ol throughout this text; **Table 2** however also contains a column “reported as” for transparency.

Another problem was that one report (Vehof et al., 2017) listed disulfates 1 and 2 for androsten-3β,17β-diol disulfate. It is chemically impossible to link these sulfates in any other way than to the 3- and 17-hydroxyl groups. The paper still reported slightly different parameters for versions 1 and 2. We have reproduced their nomenclature in the “reported as” column in **Table 2**.

Chemical synthesis and analysis of disulfates

Chemical synthetic access to sulfated steroids has become easier recently. Activating SO_3 through complex formation with a tertiary amine greatly facilitates sulfation (Gill, Male and Jones, 2019). This and other improvements in synthesizing steroid disulfates (Waller and McLeod, 2014) made more sulfated compounds available as analytical standards. This development is not limited to mono-sulfates and disulfates, but also includes glucuronidates and mixed sulfo-glucuronidates (Pranata, Fitzgerald, Khymenets et al., 2019).

Complementary to these synthetic achievements, mass-spectrometry-based detection of conjugates in general – sulfo-conjugates in particular – has been further developed for untargeted sulfo-proteomics. McLeod and coworkers have used a constant ion loss method to detect steroid disulfate metabolites (McLeod et al., 2017), making use of the unique properties of disulfates. A related method allowed a neutral-loss scan and might be more generally applicable for any sulfated metabolite (Kleinenkuhnen, Buchel, Gerlich et al., 2019). The field is ready now for more untargeted sulfo-metabolomics studies.

Conclusion & Outlook

Steroid disulfates are testament to the versatility of sulfation pathways. Steroid disulfates were first mentioned decades ago. However, they became a forgotten class of sulfated metabolites, partially due to the meanwhile established sample work-up for mass spectrometry that included deconjugation steps. Recent developments in mass-spectrometry-based metabolomics have opened up new research avenues for disulfates. Geyer and coworkers already said that the efflux of sulfated steroids out of adrenal cortex cells had not been studied in depth (Grosser et al., 2018).

Certainly, more is to be discovered about the transport and mechanisms of action of steroid disulfates in further research - to elucidate substrate specificity of the STS enzyme towards steroid disulfates and to fully identify the targets and receptors that still accept doubly sulfated steroids. Selectively analyzing disulfates in existing and novel cohort metabolomics datasets will advance the use of steroid sulfates in diagnostic and prognostic tests. This field appears to have great potential, as steroid disulfates have been linked to a variety of clinical conditions. Additionally, it is of special interest to elucidate molecular mechanisms between these conditions and steroid disulfates. Much could be learnt here about sulfation pathways and their system-wide impact; also opening up new treatment options and broadening the use of existing treatments in clinical settings as well. In the end, steroid disulfates might turn out not to be double trouble, but double reward.

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References

[1] Gunal, S., Hardman, R., Kopriva, S. and Mueller, J.W., 2019. Sulfation pathways from red to green, J Biol Chem. 294, 12293-12312.

[2] Abayev, M., Rodrigues, J., Srivastava, G., Arshava, B., Jaremko, L., Jaremko, M., Naider, F., Levitt, M. and Anglister, J., 2018. The solution structure of monomeric CCL5 in complex with a doubly sulfated N-terminal segment of CCR5, FEBS J. 285, 1988-2003.

[3] Taylor, S.L., Hogwood, J., Guo, W., Yates, E.A. and Turnbull, J.E., 2019. By-Products of Heparin Production Provide a Diverse Source of Heparin-like and Heparan Sulfate Glycosaminoglycans, Sci Rep. 9, 2679.

[4] Foster, P.A. and Mueller, J.W., 2018. SULFATION PATHWAYS: Insights into steroid sulfation and desulfation pathways, J Mol Endocrinol. 61, T271-T283.

[5] Mueller, J.W., Gilligan, L.C., Idkowiak, J., Arlt, W. and Foster, P.A., 2015. The Regulation of Steroid Action by Sulfation and Desulfation, Endocr Rev. 36, 526-63.

[6] Klymiuk, M.C., Neunzig, J., Bernhardt, R., Sanchez-Guijo, A., Hartmann, M.F., Wudy, S.A. and Schuler, G., 2018. Efficiency of the sulfate pathway in comparison to the Delta4- and Delta5-pathway of steroidogenesis in the porcine testis, J Steroid Biochem Mol Biol. 179, 64-72.

[7] Pasqualini, J.R. and Jayle, M.F., 1962. Identification of 3beta,21-dihydroxy-5-pregnene-20-one disulfate in human urine, J Clin Invest. 41, 981-7.

[8] Shackleton, C., 2010. Clinical steroid mass spectrometry: a 45-year history culminating in HPLC-MS/MS becoming an essential tool for patient diagnosis, J Steroid Biochem Mol Biol. 121, 481-90.

[9] Marcos, J. and Pozo, O.J., 2016. Current LC-MS methods and procedures applied to the identification of new steroid metabolites, J Steroid Biochem Mol Biol. 162, 41-56.

[10] McLeod, M.D., Waller, C.C., Esquivel, A., Balcells, G., Ventura, R., Segura, J. and Pozo, O.J., 2017. Constant Ion Loss Method for the Untargeted Detection of Bis-sulfate Metabolites, Anal Chem. 89, 1602-1609.

[11] Sun, N., Kunzke, T., Sbiera, S., Kircher, S., Feuchtinger, A., Aichler, M., Herterich, S., Ronchi, C.L., Weigand, I., Schlegel, N., Waldmann, J., Candida Villares Fragoso, M., Whitsett, T.G., Gill, A.J., Fassnacht, M., Walch, A. and Kroiss, M., 2019. Prognostic Relevance of Steroid Sulfation in Adrenocortical Carcinoma Revealed by Molecular Phenotyping Using High-Resolution Mass Spectrometry Imaging, Clin Chem. 65, 1276-1286.

[12] Gangloff, A., Shi, R., Nahoum, V. and Lin, S.X., 2003. Pseudo-symmetry of C19 steroids, alternative binding orientations, and multispecificity in human estrogenic 17beta-hydroxysteroid dehydrogenase, FASEB J. 17, 274-6.

[13] Lathe, R. and Kotelevtsev, Y., 2014. Steroid signaling: ligand-binding promiscuity, molecular symmetry, and the need for gating, Steroids. 82, 14-22.

[14] Cook, I.T., Duniec-Dmuchowski, Z., Kocarek, T.A., Runge-Morris, M. and Falany, C.N., 2009. 24-hydroxycholesterol sulfation by human cytosolic sulfotransferases: formation of

monosulfates and disulfates, molecular modeling, sulfatase sensitivity, and inhibition of liver x receptor activation, Drug Metab Dispos. 37, 2069-78.
[15] Fuda, H., Javitt, N.B., Mitamura, K., Ikegawa, S. and Strott, C.A., 2007. Oxysterols are substrates for cholesterol sulfotransferase, J Lipid Res. 48, 1343-52.
[16] Falany, J.L., Macrina, N. and Falany, C.N., 2004. Sulfation of tibolone and tibolone metabolites by expressed human cytosolic sulfotransferases, J Steroid Biochem Mol Biol. 88, 383-91.
[17] Berger, I., Guttman, C., Amar, D., Zarivach, R. and Aharoni, A., 2011. The molecular basis for the broad substrate specificity of human sulfotransferase 1A1, PLoS One. 6, e26794.
[18] Atkins, W.M., 2020. Mechanisms of promiscuity among drug metabolizing enzymes and drug transporters, FEBS J. 287, 1306-1322.
[19] Hirschmann, F., Krause, F., Baruch, P., Chizhov, I., Mueller, J.W., Manstein, D.J., Papenbrock, J. and Fedorov, R., 2017. Structural and biochemical studies of sulphotransferase 18 from Arabidopsis thaliana explain its substrate specificity and reaction mechanism, Sci Rep. 7, 4160.
[20] Carugo, O., 2018. How large B-factors can be in protein crystal structures, BMC Bioinformatics. 19, 61.
[21] Cook, I., Wang, T., Falany, C.N. and Leyh, T.S., 2013. High accuracy in silico sulfotransferase models, J Biol Chem. 288, 34494-501.
[22] Falany, J.L. and Falany, C.N., 2007. Interactions of the human cytosolic sulfotransferases and steroid sulfatase in the metabolism of tibolone and raloxifene, J Steroid Biochem Mol Biol. 107, 202-10.
[23] Mueller, J.W., Idkowiak, J., Gesteira, T.F., Vallet, C., Hardman, R., van den Boom, J., Dhir, V., Knauer, S.K., Rosta, E. and Arlt, W., 2018. Human DHEA sulfation requires direct interaction between PAPS synthase 2 and DHEA sulfotransferase SULT2A1, J Biol Chem. 293, 9724-9735.
[24] Brylski, O., Ebbinghaus, S. and Mueller, J.W., 2019. Melting down protein stability: PAPS synthase 2 in patients and in a cellular environment, Frontiers in Molecular Biosciences. 6, 31.
[25] Wang, L.Q. and James, M.O., 2005. Sulfotransferase 2A1 forms estradiol-17-sulfate and celecoxib switches the dominant product from estradiol-3-sulfate to estradiol-17-sulfate, J Steroid Biochem Mol Biol. 96, 367-74.
[26] Ambadapadi, S., Wang, P.L., Palii, S.P. and James, M.O., 2017. Celecoxib affects estrogen sulfonation catalyzed by several human hepatic sulfotransferases, but does not stimulate 17-sulfonation in rat liver, J Steroid Biochem Mol Biol. 172, 46-54.
[27] Cohen, I.V., Cirulli, E.T., Mitchell, M.W., Jonsson, T.J., Yu, J., Shah, N., Spector, T.D., Guo, L., Venter, J.C. and Telenti, A., 2018. Acetaminophen (Paracetamol) Use Modifies the Sulfation of Sex Hormones, EBioMedicine. 28, 316-323.
[28] Gamage, N.U., Tsvetanov, S., Duggleby, R.G., McManus, M.E. and Martin, J.L., 2005. The structure of human SULT1A1 crystallized with estradiol. An insight into active site plasticity and substrate inhibition with multi-ring substrates, J Biol Chem. 280, 41482-6.
[29] Nakano, H., Ogura, K., Takahashi, E., Harada, T., Nishiyama, T., Muro, K., Hiratsuka, A., Kadota, S. and Watabe, T., 2004. Regioselective monosulfation and disulfation of the phytoestrogens daidzein and genistein by human liver sulfotransferases, Drug Metab Pharmacokinet. 19, 216-26.
[30] Uhlen, M., Fagerberg, L., Hallstrom, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, A., Kampf, C., Sjostedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C.A., Odeberg, J., Djureinovic, D., Takanen, J.O., Hober, S., Alm, T., Edqvist, P.H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J.M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., von Heijne, G., Nielsen, J. and Ponten, F., 2015. Proteomics. Tissue-based map of the human proteome, Science. 347, 1260419.

[31] Adang, L.A., Schlotawa, L., Groeschel, S., Kehrer, C., Harzer, K., Staretz-Chacham, O., Silva, T.O., Schwartz, I.V.D., Gartner, J., De Castro, M., Costin, C., Montgomery, E.F., Dierks, T., Radhakrishnan, K. and Ahrens-Nicklas, R.C., 2020. Natural history of multiple sulfatase deficiency: Retrospective phenotyping and functional variant analysis to characterize an ultra-rare disease, J Inherit Metab Dis.

[32] Staretz-Chacham, O., Schlotawa, L., Wormser, O., Golan-Tripto, I., Birk, O.S., Ferreira, C.R., Dierks, T. and Radhakrishnan, K., 2020. A homozygous missense variant of SUMF1 in the Bedouin population extends the clinical spectrum in ultrarare neonatal multiple sulfatase deficiency, Mol Genet Genomic Med. 8, e1167.

[33] Van Eldere, J., Robben, J., De Pauw, G., Merckx, R. and Eyssen, H., 1988. Isolation and identification of intestinal steroid-desulfating bacteria from rats and humans, Appl Environ Microbiol. 54, 2112-7.

[34] Okamoto, N., Viswanatha, R., Bittar, R., Li, Z., Haga-Yamanaka, S., Perrimon, N. and Yamanaka, N., 2018. A Membrane Transporter Is Required for Steroid Hormone Uptake in Drosophila, Dev Cell. 47, 294-305 e7.

[35] McManus, J.M., Bohn, K., Alyamani, M., Chung, Y.M., Klein, E.A. and Sharifi, N., 2019. Rapid and structure-specific cellular uptake of selected steroids, PLoS One. 14, e0224081.

[36] Doring, B., Lutteke, T., Geyer, J. and Petzinger, E., 2012. The SLC10 carrier family: transport functions and molecular structure, Curr Top Membr. 70, 105-68.

[37] Grosser, G., Bennien, J., Sanchez-Guijo, A., Bakhaus, K., Doring, B., Hartmann, M., Wudy, S.A. and Geyer, J., 2018. Transport of steroid 3-sulfates and steroid 17-sulfates by the sodium-dependent organic anion transporter SOAT (SLC10A6), J Steroid Biochem Mol Biol. 179, 20-25.

[38] Zhou, F., Illsley, N.P. and You, G., 2006. Functional characterization of a human organic anion transporter hOAT4 in placental BeWo cells, Eur J Pharm Sci. 27, 518-23.

[39] Li, N., Hong, W., Huang, H., Lu, H., Lin, G. and Hong, M., 2012. Identification of amino acids essential for estrone-3-sulfate transport within transmembrane domain 2 of organic anion transporting polypeptide 1B1, PLoS One. 7, e36647.

[40] Iliev, D.I., Braun, R., Sanchez-Guijo, A., Hartmann, M., Wudy, S.A., Heckmann, D., Bruchelt, G., Rosner, A., Grosser, G., Geyer, J. and Binder, G., 2020. Very High Dehydroepiandrosterone Sulfate (DHEAS) in Serum of an Overweight Female Adolescent Without a Tumor, Front Endocrinol (Lausanne). 11, 240.

[41] Sepe, V., Ummarino, R., D'Auria, M.V., Lauro, G., Bifulco, G., D'Amore, C., Renga, B., Fiorucci, S. and Zampella, A., 2012. Modification in the side chain of solomonsterol A: discovery of cholestan disulfate as a potent pregnane-X-receptor agonist, Org Biomol Chem. 10, 6350-62.

[42] Ren, S., Kim, J.K., Kakiyama, G., Rodriguez-Agudo, D., Pandak, W.M., Min, H.K. and Ning, Y., 2014. Identification of novel regulatory cholesterol metabolite, 5-cholesten, 3beta,25-diol, disulfate, PLoS One. 9, e103621.

[43] Dalla Valle, L., Toffolo, V., Nardi, A., Fiore, C., Bernante, P., Di Liddo, R., Parnigotto, P.P. and Colombo, L., 2006. Tissue-specific transcriptional initiation and activity of steroid sulfatase complementing dehydroepiandrosterone sulfate uptake and intracrine steroid activations in human adipose tissue, J Endocrinol. 190, 129-39.

[44] Li, C.Y., Renaud, H.J., Klaassen, C.D. and Cui, J.Y., 2016. Age-Specific Regulation of Drug-Processing Genes in Mouse Liver by Ligands of Xenobiotic-Sensing Transcription Factors, Drug Metab Dispos. 44, 1038-49.

[45] Andring, J.T., Dohle, W., Tu, C., Potter, B.V.L. and McKenna, R., 2019. 3,17beta-Bis-sulfamoyloxy-2-methoxyestra-1,3,5(10)-triene and Nonsteroidal Sulfamate Derivatives Inhibit Carbonic Anhydrase IX: Structure-Activity Optimization for Isoform Selectivity, J Med Chem. 62, 2202-2212.

[46] Potter, B.V.L., 2018. SULFATION PATHWAYS: Steroid sulphatase inhibition via aryl sulphamates: clinical progress, mechanism and future prospects, J Mol Endocrinol. 61, T233-T252.
[47] Thomas, M.P. and Potter, B.V., 2015. Discovery and Development of the Aryl O-Sulfamate Pharmacophore for Oncology and Women's Health, J Med Chem. 58, 7634-58.
[48] Meyer-Losic, F., Newman, S.P., Day, J.M., Reed, M.J., Kasprzyk, P.G., Purohit, A. and Foster, P.A., 2013. STX140, but not paclitaxel, inhibits mammary tumour initiation and progression in C3(1)/SV40 T/t-antigen transgenic mice, PLoS One. 8, e80305.
[49] El-Gamal, M.I., Semreen, M.H., Foster, P.A. and Potter, B.V., 2016. Design, synthesis, and biological evaluation of new arylamide derivatives possessing sulfonate or sulfamate moieties as steroid sulfatase enzyme inhibitors, Bioorg Med Chem. 24, 2762-7.
[50] Jayawickrama, G.S., Nematollahi, A., Sun, G., Gorrell, M.D. and Church, W.B., 2017. Inhibition of human kynurenine aminotransferase isozymes by estrogen and its derivatives, Sci Rep. 7, 17559.
[51] Pozo, O.J., Marcos, J., Khymenets, O., Pranata, A., Fitzgerald, C.C., McLeod, M.D. and Shackleton, C., 2018. SULFATION PATHWAYS: Alternate steroid sulfation pathways targeted by LC-MS/MS analysis of disulfates: application to prenatal diagnosis of steroid synthesis disorders, J Mol Endocrinol. 61, M1-M12.
[52] Shackleton, C., Marcos, J., Arlt, W. and Hauffa, B.P., 2004. Prenatal diagnosis of P450 oxidoreductase deficiency (ORD): a disorder causing low pregnancy estriol, maternal and fetal virilization, and the Antley-Bixler syndrome phenotype, Am J Med Genet A. 129A, 105-12.
[53] Shackleton, C., Marcos, J., Malunowicz, E.M., Szarras-Czapnik, M., Jira, P., Taylor, N.F., Murphy, N., Crushell, E., Gottschalk, M., Hauffa, B., Cragun, D.L., Hopkin, R.J., Adachi, M. and Arlt, W., 2004. Biochemical diagnosis of Antley-Bixler syndrome by steroid analysis, Am J Med Genet A. 128A, 223-31.
[54] Sovio, U., Goulding, N., McBride, N., Cook, E., Gaccioli, F., Charnock-Jones, D.S., Lawlor, D.A. and Smith, G.C.S., 2020. A maternal serum metabolite ratio predicts fetal growth restriction at term, Nat Med. 26, 348-353.
[55] Ross, K.M., Baer, R.J., Ryckman, K., Feuer, S.K., Bandoli, G., Chambers, C., Flowers, E., Liang, L., Oltman, S., Dunkel Schetter, C. and Jelliffe-Pawlowski, L., 2019. Second trimester inflammatory and metabolic markers in women delivering preterm with and without preeclampsia, J Perinatol. 39, 314-320.
[56] Reisch, N., Taylor, A.E., Nogueira, E.F., Asby, D.J., Dhir, V., Berry, A., Krone, N., Auchus, R.J., Shackleton, C.H.L., Hanley, N.A. and Arlt, W., 2019. Alternative pathway androgen biosynthesis and human fetal female virilization, Proc Natl Acad Sci U S A. 116, 22294-22299.
[57] Oikarinen, A., Kaar, M.L. and Ruokonen, A., 1980. A congenital ichthyosiform syndrome with deafness and elevated serum steroid disulphate levels, Acta Derm Venereol. 60, 503-7.
[58] Laatikainen, T.J., Peltonen, J.I. and Nylander, P.L., 1974. Effect of maternal intrahepatic cholestasis on fetal steroid metabolism, J Clin Invest. 53, 1709-15.
[59] Caussy, C., Ajmera, V.H., Puri, P., Hsu, C.L., Bassirian, S., Mgdsyan, M., Singh, S., Faulkner, C., Valasek, M.A., Rizo, E., Richards, L., Brenner, D.A., Sirlin, C.B., Sanyal, A.J. and Loomba, R., 2019. Serum metabolites detect the presence of advanced fibrosis in derivation and validation cohorts of patients with non-alcoholic fatty liver disease, Gut. 68, 1884-1892.
[60] Playdon, M.C., Ziegler, R.G., Sampson, J.N., Stolzenberg-Solomon, R., Thompson, H.J., Irwin, M.L., Mayne, S.T., Hoover, R.N. and Moore, S.C., 2017. Nutritional metabolomics and breast cancer risk in a prospective study, Am J Clin Nutr. 106, 637-649.
[61] Utsumi, T., Yoshimura, N., Takeuchi, S., Ando, J., Maruta, M., Maeda, K. and Harada, N., 1999. Steroid sulfatase expression is an independent predictor of recurrence in human breast cancer, Cancer Res. 59, 377-81.

[62] Huang, J., Weinstein, S.J., Moore, S.C., Derkach, A., Hua, X., Mondul, A.M., Sampson, J.N. and Albanes, D., 2019. Pre-diagnostic Serum Metabolomic Profiling of Prostate Cancer Survival, J Gerontol A Biol Sci Med Sci. 74, 853-859.
[63] Huang, J., Mondul, A.M., Weinstein, S.J., Derkach, A., Moore, S.C., Sampson, J.N. and Albanes, D., 2019. Prospective serum metabolomic profiling of lethal prostate cancer, Int J Cancer. 145, 3231-3243.
[64] Vehof, J., Hysi, P.G. and Hammond, C.J., 2017. A Metabolome-Wide Study of Dry Eye Disease Reveals Serum Androgens as Biomarkers, Ophthalmology. 124, 505-511.
[65] Zheng, Y., Yu, B., Alexander, D., Steffen, L.M. and Boerwinkle, E., 2014. Human metabolome associates with dietary intake habits among African Americans in the atherosclerosis risk in communities study, Am J Epidemiol. 179, 1424-33.
[66] Yu, B., Heiss, G., Alexander, D., Grams, M.E. and Boerwinkle, E., 2016. Associations Between the Serum Metabolome and All-Cause Mortality Among African Americans in the Atherosclerosis Risk in Communities (ARIC) Study, Am J Epidemiol. 183, 650-6.
[67] Zheng, Y., Yu, B., Alexander, D., Mosley, T.H., Heiss, G., Nettleton, J.A. and Boerwinkle, E., 2013. Metabolomics and incident hypertension among blacks: the atherosclerosis risk in communities study, Hypertension. 62, 398-403.
[68] Huang, J., Weinstein, S.J., Moore, S.C., Derkach, A., Hua, X., Liao, L.M., Gu, F., Mondul, A.M., Sampson, J.N. and Albanes, D., 2018. Serum Metabolomic Profiling of All-Cause Mortality: A Prospective Analysis in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study Cohort, Am J Epidemiol. 187, 1721-1732.
[69] Zheng, Y., Yu, B., Alexander, D., Steffen, L.M., Nettleton, J.A. and Boerwinkle, E., 2014. Metabolomic patterns and alcohol consumption in African Americans in the Atherosclerosis Risk in Communities Study, Am J Clin Nutr. 99, 1470-8.
[70] Guertin, K.A., Moore, S.C., Sampson, J.N., Huang, W.Y., Xiao, Q., Stolzenberg-Solomon, R.Z., Sinha, R. and Cross, A.J., 2014. Metabolomics in nutritional epidemiology: identifying metabolites associated with diet and quantifying their potential to uncover diet-disease relations in populations, Am J Clin Nutr. 100, 208-17.
[71] Pallister, T., Jennings, A., Mohney, R.P., Yarand, D., Mangino, M., Cassidy, A., MacGregor, A., Spector, T.D. and Menni, C., 2016. Characterizing Blood Metabolomics Profiles Associated with Self-Reported Food Intakes in Female Twins, PLoS One. 11, e0158568.
[72] Maiti, S. and Chen, G., 2015. Ethanol up-regulates phenol sulfotransferase (SULT1A1) and hydroxysteroid sulfotransferase (SULT2A1) in rat liver and intestine, Arch Physiol Biochem. 121, 68-74.
[73] Feofanova, E.V., Yu, B., Metcalf, G.A., Liu, X., Muzny, D., Below, J.E., Wagenknecht, L.E., Gibbs, R.A., Morrison, A.C. and Boerwinkle, E., 2018. Sequence-Based Analysis of Lipid-Related Metabolites in a Multiethnic Study, Genetics. 209, 607-616.
[74] Dorgan, J.F., Jung, S., Dallal, C.M., Zhan, M., Stennett, C.A., Zhang, Y., Eckert, R.L., Snetselaar, L.G. and Van Horn, L., 2020. Alcohol consumption and serum metabolite concentrations in young women, Cancer Causes Control. 31, 113-126.
[75] Horishita, T., Yanagihara, N., Ueno, S., Sudo, Y., Uezono, Y., Okura, D., Minami, T., Kawasaki, T. and Sata, T., 2014. Neurosteroids allopregnanolone sulfate and pregnanolone sulfate have diverse effect on the alpha subunit of the neuronal voltage-gated sodium channels Nav1.2, Nav1.6, Nav1.7, and Nav1.8 expressed in xenopus oocytes, Anesthesiology. 121, 620-31.
[76] Davies, W., 2018. SULFATION PATHWAYS: The steroid sulfate axis and its relationship to maternal behaviour and mental health, J Mol Endocrinol. 61, T199-T210.
[77] Ratner, M.H., Kumaresan, V. and Farb, D.H., 2019. Neurosteroid Actions in Memory and Neurologic/Neuropsychiatric Disorders, Front Endocrinol (Lausanne). 10, 169.
[78] Bressler, J., Yu, B., Mosley, T.H., Knopman, D.S., Gottesman, R.F., Alonso, A., Sharrett, A.R., Wruck, L.M. and Boerwinkle, E., 2017. Metabolomics and cognition in African American adults in midlife: the atherosclerosis risk in communities study, Transl Psychiatry. 7, e1173.

[79] Saha, S., Chant, D., Welham, J. and McGrath, J., 2005. A systematic review of the prevalence of schizophrenia, PLoS Med. 2, e141.
[80] Jayawickrama, G.S., Sadig, R.R., Sun, G., Nematollahi, A., Nadvi, N.A., Hanrahan, J.R., Gorrell, M.D. and Church, W.B., 2015. Kynurenine Aminotransferases and the Prospects of Inhibitors for the Treatment of Schizophrenia, Curr Med Chem. 22, 2902-18.
[81] Jayawickrama, G.S., Nematollahi, A., Sun, G. and Church, W.B., 2018. Improvement of kynurenine aminotransferase-II inhibitors guided by mimicking sulfate esters, PLoS One. 13, e0196404.
[82] Thomas, J.L., Duax, W.L., Addlagatta, A., Brandt, S., Fuller, R.R. and Norris, W., 2003. Structure/function relationships responsible for coenzyme specificity and the isomerase activity of human type 1 3 beta-hydroxysteroid dehydrogenase/isomerase, J Biol Chem. 278, 35483-90.
[83] Gill, D.M., Male, L. and Jones, A.M., 2019. Sulfation made simple: a strategy for synthesising sulfated molecules, Chem Commun (Camb). 55, 4319-4322.
[84] Waller, C.C. and McLeod, M.D., 2014. A simple method for the small scale synthesis and solid-phase extraction purification of steroid sulfates, Steroids. 92, 74-80.
[85] Pranata, A., Fitzgerald, C.C., Khymenets, O., Westley, E., Anderson, N.J., Ma, P., Pozo, O.J. and McLeod, M.D., 2019. Synthesis of steroid bisglucuronide and sulfate glucuronide reference materials: Unearthing neglected treasures of steroid metabolism, Steroids. 143, 25-40.
[86] Kleinenkuhnen, N., Buchel, F., Gerlich, S.C., Kopriva, S. and Metzger, S., 2019. A Novel Method for Identification and Quantification of Sulfated Flavonoids in Plants by Neutral Loss Scan Mass Spectrometry, Front Plant Sci. 10, 885.
[87] Bergwerf, H., 2015. MolView: an attempt to get the cloud into chemistry classrooms, DivCHED CCCE: Committee on Computers in Chemical Education. pp. Paper 9.
[88] Pan, P.W.D., A.; Amaya, M.; Edwards, A. M., 2009. 3F3Y, Crystal structure of human cytosolic sulfotransferase SULT2A1 in complex with PAP and lithocholic acid. The Protein Data Base.
[89] Krieger, E. and Vriend, G., 2014. YASARA View - molecular graphics for all devices - from smartphones to workstations, Bioinformatics. 30, 2981-2.
[90] Arcos, M. and Lieberman, S., 1967. 5-Pregnene-3-beta,20-alpha-diol-3-sulfate-20-(2'-acetamido-2'-deoxy-alpha-D-gluco side) and 5-pregnene-3-beta,20-alpha-diol-3,20-disulfate. Two novel urinary conjugates, Biochemistry. 6, 2032-9.
[91] Shackleton, C.H., Kelly, R.W., Adhikary, P.M., Brooks, C.J., Harkness, R.A., Sykes, P.J. and Mitchell, F.L., 1968. The identification and measurement of a new steroid 16 beta-hydroxydehydroepiandrosterone in infant urine, Steroids. 12, 705-16.
[92] Shackleton, C.H., Livingstone, J.R. and Mitchell, F.L., 1968. The conjugated 17-hydroxy epimers of delta5-androstene-3beta-17-diol in infant and adult urine and umbilical cord plasma, Steroids. 11, 299-311.
[93] Janne, O., Vihko, R., Sjovall, J. and Sjovall, K., 1969. Determination of steroid mono- and disulfates in human plasma, Clin Chim Acta. 23, 405-12.
[94] Eriksson, H., Gustafsson, J.A. and Sjovall, J., 1970. Excretion of steroid hormones in adults. C19 and C21 steroids in faeces from pregnant women, Eur J Biochem. 12, 520-6.
[95] Laatikainen, T., Peltonen, J. and Nylander, P., 1973. Determination of estriol, estriol sulfate, progesterone and neutral steroid mono- and disulfates in umbilical cord blood plasma, Steroids. 21, 347-59.
[96] Claus, S.P., 2014. Development of personalized functional foods needs metabolic profiling, Curr Opin Clin Nutr Metab Care. 17, 567-73.
[97] Zheng, Y., Yu, B., Alexander, D., Couper, D.J. and Boerwinkle, E., 2014. Medium-term variability of the human serum metabolome in the Atherosclerosis Risk in Communities (ARIC) study, OMICS. 18, 364-73.

[98] Yazdani, A., Yazdani, A., Elsea, S.H., Schaid, D.J., Kosorok, M.R., Dangol, G. and Samiei, A., 2019. Genome analysis and pleiotropy assessment using causal networks with loss of function mutation and metabolomics, BMC Genomics. 20, 395.

Figure legends

Figure 1: Structural views on a steroid disulfate.

3β,17β-Androstanediol disulfate is depicted here as a paradigm for steroid disulfates, both in 2D (A) and in 3D representations (B). The 3D structure is shown from two different perspectives to demonstrate the pseudosymmetry of certain steroids. All chemical structure 2D and 3D representations of steroid molecules in this article were generated using MolView (Bergwerf, 2015).

Figure 2: Human sulfotransferase SULT2A1.

The crystal structure 3F3Y of human SULT2A1 is shown, complexed with the PAP nucleotide and lithocholic acid (Pan, 2009). The complex is colored for its B factor, also known as temperature factor. Please note the prevalence of higher B factors (red tones) around the substrate at the left - high B factors are commonly observed in areas of the protein with high flexibility. Lower B factors (blue tones) are observed around the nucleotide cofactor at the right - low B factors are usually observed in protein regions with low flexibility. Protein structures were visualized using the PDB browser YASARA (Krieger and Vriend, 2014).

Figure 3: Visual summary of putative biological functions of steroid disulfates.
Different potential biological functions of steroid disulfates are displayed. Steroids or steroid mono-sulfates are taken up into the cell (UPTAKE) and then converted into a disulfated steroid by SULT enzymes. The biological function of the formed disulfate depends on whether steroid disulfates can still cross membranes via a transporter and hence leave the cell (EFFLUX), influence intracellular signaling (ACTION) or accumulate as terminal waste products (TRAPPED). There is still also the possibility that the disulfate can be desulfated by STS back into a normally functioning mono-sulfate.

References within the schematic are: **1** (Zhou et al., 2006); **2** (Andring et al., 2019,Meyer-Losic et al., 2013); **3** (Cook et al., 2009,Sepe et al., 2012,Ren et al., 2014); **4** (Sun et al., 2019); **5** (Grosser et al., 2018); and **6** (Cook et al., 2009). The graphical depiction of membranes is based on a study by O'Reilly and coworkers (O'Reilly, House and Tomlinson, 2014).

Figure 4: Structural panel of several steroid disulfates.

Various steroid disulfates and the steroid-related drug STX140, all show a high degree of structural similarity. **A**, androstan-diol disulfate. **B**, 5-androsten-diol disulfate. **C**, estradiol disulfate. **D**, the bis-sulfamate STX140 drug (aka 3,17β-bis-sulfamoyloxy-2-methoxyestra1,3,5(10)-triene). **E**, 5α-pregnan-3β,20α-diol disulfate. **F**, 5-pregnen-3β,20α-diol disulfate. **G**, 21-hydroxy-pregnenolone disulfate. **H**, 24-hydroxy-cholesterol-3,24 disulfate.

Figure 5: 4-En-3-ol steroids are not compatible with the sequential mechanism of 3β-hydroxysteroid dehydrogenases.

3β-Hydroxysteroid dehydrogenases are bifunctional enzymes. They oxidize the 3-hydroxy group to a carbonyl functionality and then isomerize a double bond from the 5-en to the 4-en position (Thomas et al., 2003), shown in **A**. This mechanism would never allow for the occurrence of 4-en-3-ol steroids (**B**). One such compound, purported in some metabolomics studies, is 4-androsten-diol disulfate (**C**). We have listed it in our table as androsten-diol, but maintained the original name in the reported-as column.

Tables

Table 1: Historic discoveries of steroid disulfates

Year	Disulfates discovered	Reference
1962	3β,21-dihydroxy-5-pregnene-20-one disulfate	(Pasqualini and Jayle, 1962)
1967	5-pregnene-3β,20α-diol-3,20-disulfate	(Arcos and Lieberman, 1967)
1968	16β-hydroxy-dehydroepiandrosterone disulfate	(Shackleton, Kelly, Adhikary et al., 1968)
1968	17-androstenediol disulfate	(Shackleton, Livingstone and Mitchell, 1968)
1969	5-androstene-3β,17α-diol disulfate 5-androstene-3β,17β-diol disulfate 5-pregnene-3β,20α-diol disulfate	(Janne, Vihko, Sjovall et al., 1969)
1970	5α-androstan-3α,17α-diol disulfate	(Eriksson, Gustafsson and Sjovall, 1970)
1973 & 1974	5-androstene-3β,17α-diol disulfate 5-androstene-3β,17β-diol disulfate 16β-hydroxy-dehydroepiandrosterone disulfate 3β,17β-dihydroxy-5-androsten-16-one disulfate 5-androstene-3β,16β,17α-triol disulfate 5-pregnene-3β,20α-diol disulfate 5α-pregnane-3α,20α-diol disulfate 5α-pregnane-3β,20α-diol disulfate 5β-pregnane-3α,20α-diol disulfate 21-hydroxypregnenolone disulfate 5α-pregnane-3α,20α,21-triol disulfate	(Laatikainen et al., 1974,Laatikainen, Peltonen and Nylander, 1973)

Table 2: Overview of disulfated steroids recently reported

Metabolite	Link	Condition	Reported as	Reference
androsten-3β,17α-diol disulfate	Largely raised levels in ichthyosiform syndrome and deafness	Ichthyosiform syndrome and deafness	5-androstene-3 beta,17 alpha-diol disulfate	(Oikarinen et al., 1980)
androsten-3β,17α-diol disulfate	Response ratio between 6 disulfates in urine sample was markedly increased in steroid sulfatase deficiency (STSD)	STSD	androst-5-ene-3β,17α-diol disulfate	(Pozo et al., 2018)
androsten-3β,17β-diol disulfate	Sex hormone derivatives and food intake biomarkers (specifically nuts and peanut butter)	Food intake biomarker	4-androsten-3β,17β-diol disulfate	(Claus, 2014)
androsten-3β,17β-diol disulfate	Associated with cardiovascular disease mortality	cardiovascular disease mortality	4-androsten-3β,17β-diol disulfate	(Huang et al., 2018)
androsten-3β,17β-diol disulfate	Largely raised levels in ichthyosiform syndrome and deafness	Ichthyosiform syndrome and deafness	5-androstene-3 beta,17 beta-diol disulfate	(Oikarinen et al., 1980)
androsten-3β,17β-diol disulfate	Significant positive correlation with total alcohol intake	Alcohol consumption	4-Androsten-3β,17β-diol disulfate 1	(Guertin et al., 2014)
androsten-3β,17β-diol disulfate	Positively associated with alcohol consumption	Alcohol consumption	4-androsten-3β and 17β-diol disulfate 1	(Zheng et al., 2014)
androsten-3β,17β-diol disulfate	Inversely associated with intakes of sugar-rich food and beverages (specifically nuts and peanut butter)	Food intake biomarker	4-Androsten-3β,17β-diol disulfate 1	(Zheng et al., 2014)

androsten-3β,17β-diol disulfate	Had higher intraclass correlation coefficient (ICC) in females than that in males	Biologic variability of the human serum metabolome	4-Androsten-3beta,17beta-diol disulfate 2	(Zheng, Yu, Alexander et al., 2014)
androsten-3β,17β-diol disulfate	Associated with SULT2A1 (rs2547231 and rs296396) as elevated levels of metabolites were associated with higher reported alcohol intake	SULT2A1 associations and alcohol consumption	4-androsten-3beta,17beta-diol disulfate 1	(Pallister et al., 2016)
androsten-3β,17β-diol disulfate	Negative correlation with dryness and irritation symptoms of the eye	Dryness and irritation symptoms of the eye	Different results for 4-androsten-3beta,17beta-diol disulfate 1 and 4-androsten-3beta,17beta-diol disulfate 2	(Vehof et al., 2017)
androsten-3β,17β-diol disulfate	Significantly associated with estrogen-receptor-positive (ER+) breast cancer, alcohol-related	ER+ breast cancer (alcohol related)	4-Androsten-3β,17β-diol disulfate (1)	(Playdon et al., 2017)
androsten-3β,17β-diol disulfate	Significantly associated with ER+ breast cancer, alcohol-related	ER+ breast cancer (alcohol related)	4-androsten-3β,17β-diol-disulfate (2)	(Playdon et al., 2017)
androsten-3β,17β-diol disulfate	Increased risk of incident hospitalized dementia in African Americans. Was also found to be significantly associated with individuals of European ancestry	Dementia	4-androsten-3 beta, 17 beta-diol disulfate 1	(Bressler et al., 2017)
androsten-3β,17β-diol disulfate	Response ratio between 6 disulfates in urine sample was markedly increased in STSD	STSD	androst-5-ene-3β,17β-diol disulfate	(Pozo et al., 2018)

androsten-3β,17β-diol disulfate	Positively associated with prostate cancer survival	Prostate cancer	4-androsten-3beta,17beta-diol disulfate	(Huang et al., 2019)
androsten-3β,17β-diol disulfate	In the European American causal network the steroid was influenced by LTK, DSE, and PLAC4	-	androsten-3-beta-17-beta-diol-disulfate 1	(Yazdani, Yazdani, Elsea et al., 2019)
androsten-3β,17β-diol disulfate	Lower levels were associated with lethal disease	Prostate cancer	Androstenediol (3beta,17beta) disulfate (2)	(Huang et al., 2019)
5α-androstan-3α,17α-diol disulfate	Negatively associated with term fetal growth restriction	Fetal growth restriction	5α-androstan-3α,17α-diol disulfate	(Sovio et al., 2020)
5α-androstan-3α,17β-diol disulfate	Significantly associated with ER+ breast cancer, alcohol-related	ER+ breast cancer (alcohol related)	5α-Androstan-3α,17β-diol disulfate	(Playdon et al., 2017)
5α-androstan-3β disulfate	Detects the presence of advanced fibrosis	Fibrosis	5alpha-androstan-3beta disulfate	(Caussy et al., 2019)
5α-androstan-3β,17β-diol disulfate	Nominal significant predictor of incident hypertension	Hypertension	5α-androstan-3β,17β-diol disulfate	(Zheng et al., 2013)
5α-androstan-3β,17β-diol disulfate	Significant positive correlation with total alcohol intake	Alcohol consumption	5-α-Androstan-3β,17β-diol disulfate	(Guertin et al., 2014)
5α-androstan-3β,17β-diol disulfate	Positively associated with alcohol consumption	Alcohol consumption	5α-Androstan-3β,17β-diol disulfate	(Zheng et al., 2014)
5α-androstan-3β,17β-diol disulfate	Inversely associated with intakes of sugar-rich food and beverages (specifically nuts and peanut butter)	Food intake biomarker	5α-Androstan-3β,17β-diol disulfate	(Zheng et al., 2014)

5α-androstan-3β,17β-diol disulfate	Had higher ICCs in females than that in males	Biologic variability of the human serum metabolome	5alpha-Androstan-3beta,17beta-diol disulfate	(Zheng et al., 2014)
5α-androstan-3β,17β-diol disulfate	Sex hormone derivatives and food intake biomarkers (specifically nuts and peanut butter)	Food intake biomarker	5α-androstan-3β,17β-diol disulfate	(Claus, 2014)
5α-androstan-3β,17β-diol disulfate	Associated with SULT2A1 (rs2547231 and rs296396) as elevated levels of metabolites were associated with higher reported alcohol intake	SULT2A1 associations and alcohol consumption	5-alpha-androstan-3beta,17beta-diol disulfate	(Pallister et al., 2016)
5α-androstan-3β,17β-diol disulfate	Significantly associated with ER+ breast cancer, alcohol-related	ER+ breast cancer (alcohol related)	5α-Androstan-3β,17β-diol disulfate	(Playdon et al., 2017)
5α-androstan-3β,17β-diol disulfate	Increased risk of incident hospitalized dementia in African Americans	Dementia	5 alpha-androstan-3 beta, 17 beta-diol disulfate	(Bressler et al., 2017)
5α-androstan-3β,17β-diol disulfate	Significant sulfated steroids with trans-ethnic consistency - CYP3A43 decreased levels	-	5α-androstan-3β,17β-diol disulfate	(Feofanova et al., 2018)
5α-androstan-3β,17β-diol disulfate	Positively associated with prostate cancer survival	Prostate cancer	5alpha-androstan-3beta,17beta-diol disulfate	(Huang et al., 2019)
24-hydroxycholesterol-3,24 disulfate	Liver-x-receptor agonist/activator, cholesterol homeostasis	Liver-x-receptor agonist	24-OHChol-3, 24-disulfate	(Cook et al., 2009)

16α-hydroxydehydroepiandrosterone disulfate	Response ratio between 6 disulfates in urine sample was markedly increased in STSD	STSD	16α-hydroxydehydroepiandrosterone disulfate	(Pozo et al., 2018)
estradiol-3,17β disulfate	Raised levels gave a poorer prognosis of adrenocortical carcinoma	Adrenocortical carcinoma	estradiol-17β 3,17-disulfate	(Sun et al., 2019)
21-hydroxypregnenolone disulfate	Had higher ICCs in males	Biologic variability of the human serum metabolome	21-Hydroxypregnenolone disulfate	(Zheng et al., 2014)
21-hydroxypregnenolone disulfate	Response ratio between 6 disulfates in urine sample was markedly increased in STSD	STSD	21-hydroxypregnenolone disulfate	(Pozo et al., 2018)
3β,21-dihydroxy-5α-pregnan-20-one disulfate	Dominant 'feto-placental' maternal urinary steroids in pregnancies with P450 oxido-reductase deficiency (PORD)	PORD	3β,21-dihydroxy-5α-pregnan-20-one disulfate	(Pozo et al., 2018)
5-pregnen-3α,20α-diol disulfate	Response ratio between 6 disulfates in urine sample was markedly increased in STSD	STSD	pregn-5-ene-3α,20S-diol disulfate	(Pozo et al., 2018)
5-pregnen-3α,20α-diol disulfate	Dominant 'feto-placental' maternal urinary steroids in PORD pregnancies	PORD	pregn-5-ene-3α,20S-diol disulfate	(Pozo et al., 2018)
5-pregnen-3β,17α,20α-triol 3,20 disulfate	Response ratio between 6 disulfates in urine sample was markedly increased in STSD	STSD	pregn-5-ene-3β,17α,20S-triol 3,20disulfate	(Pozo et al., 2018)

5-pregnene-3β,20α-diol disulfate	Largely raised levels in ichthyosiform syndrome and deafness	Ichthyosiform syndrome and deafness	5-pregnene-3 beta,20 alpha-diol disulfate	(Oikarinen et al., 1980)
5α-pregnan-3β,20α-diol disulfate	Had lower ICCs in males than that in females	Biologic variability of the human serum metabolome	5alpha-Pregnan-3beta,20alpha-diol disulfate	(Zheng et al., 2014)
5α-pregnan-3β,20α-diol disulfate	Dominant 'feto-placental' maternal urinary steroids in PORD pregnancies	PORD	5α-pregnane-3β,20S-diol disulfate	(Pozo et al., 2018)
5α-pregnan-3β,20α-diol disulfate	Raised levels predicted increased odds of pre-eclampsia	Pre-eclampsia	5-α-pregnan-3β,20α-diol disulfate	(Ross et al., 2019)
5α-pregnane-3α,20α-diol disulfate	Elevated levels in cord plasma following maternal intrahepatic cholestasis	Maternal cholestasis	5alpha-pregnane-3alpha,20alpha-diol disulfate	(Laatikainen et al., 1974)
5β-pregnane-3α,20α-diol disulfate	Elevated levels in cord plasma following maternal intrahepatic cholestasis	Maternal cholestasis	5beta-pregnane-3alpha,20alpha-diol disulfate	(Laatikainen et al., 1974)
dihydroxy-pregnen-diol disulfate	Observed in all samples of Smith-Lemli-Opitz syndrome (SLOS), also known as 7-dehydrosterol reductase deficiency	SLOS	DHPD-diS	(Pozo et al., 2018)
dihydroxy-pregnen-triol disulfate	Observed in all SLOS samples	SLOS	DHPT-diS	(Pozo et al., 2018)

pregnen-diol disulfate	Had higher ICCs in males	Biologic variability of the human serum metabolome	Pregnen-diol disulfate	(Zheng et al., 2014)
pregnen-diol disulfate	Independent association with all-cause mortality among African Americans	All-cause mortality	pregnendiol disulfate	(Yu et al., 2016)
pregnen-diol disulfate	Significantly associated with ER+ breast cancer, alcohol-related	ER+ breast cancer (alcohol related)	Pregnen-diol disulfate	(Playdon et al., 2017)
pregnen-diol disulfate	Increased risk of incident hospitalized dementia in African Americans	Dementia	pregnen-diol disulfate	(Bressler et al., 2017)
pregnen-diol disulfate	Decreased levels are associated with rare variants downstream of SULT2A1	SULT2A1 variants	pregnen-diol disulfate	(Feofanova et al., 2018)
pregnen-diol disulfate	Positively associated with prostate cancer survival	Prostate cancer	pregnen-diol disulfate	(Huang et al., 2019)

- Steroid disulfates are a special case of sulfated metabolites
- Steroid pseudosymmetry and sulfotransferase promiscuity allow their biosynthesis
- Transport and biological functions of steroid disulfates are still not fully established
- Monosulfated and disulfated steroids are associated with several diseases

Figure 1

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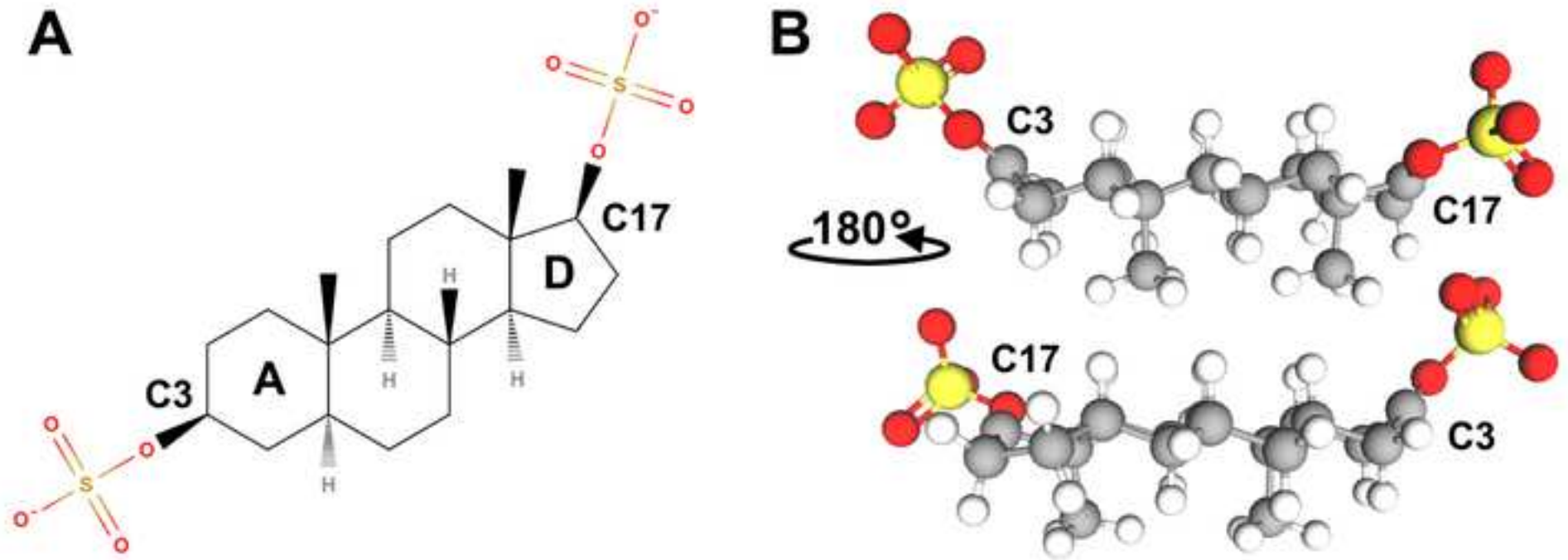

Figure 2

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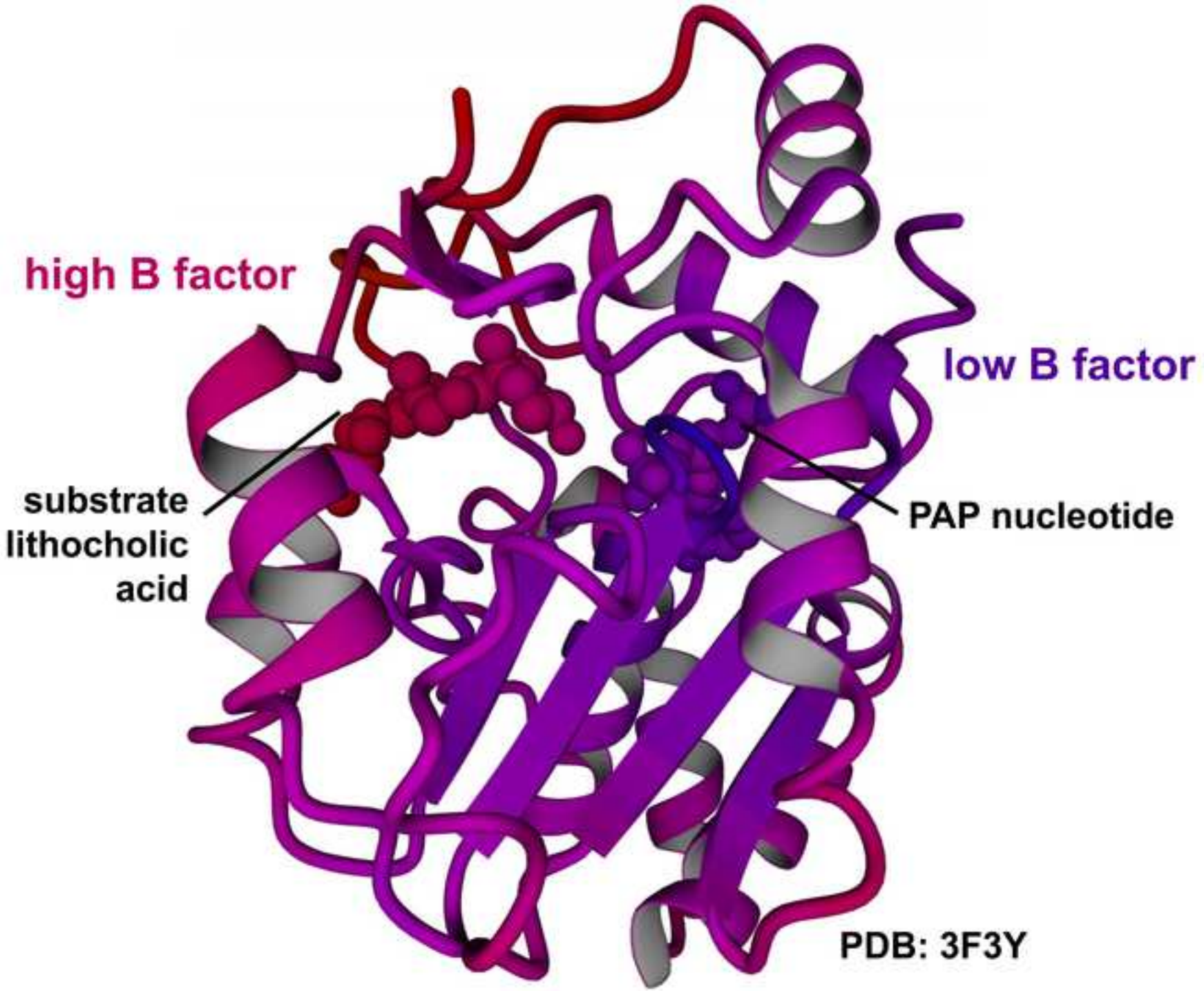

Figure 3

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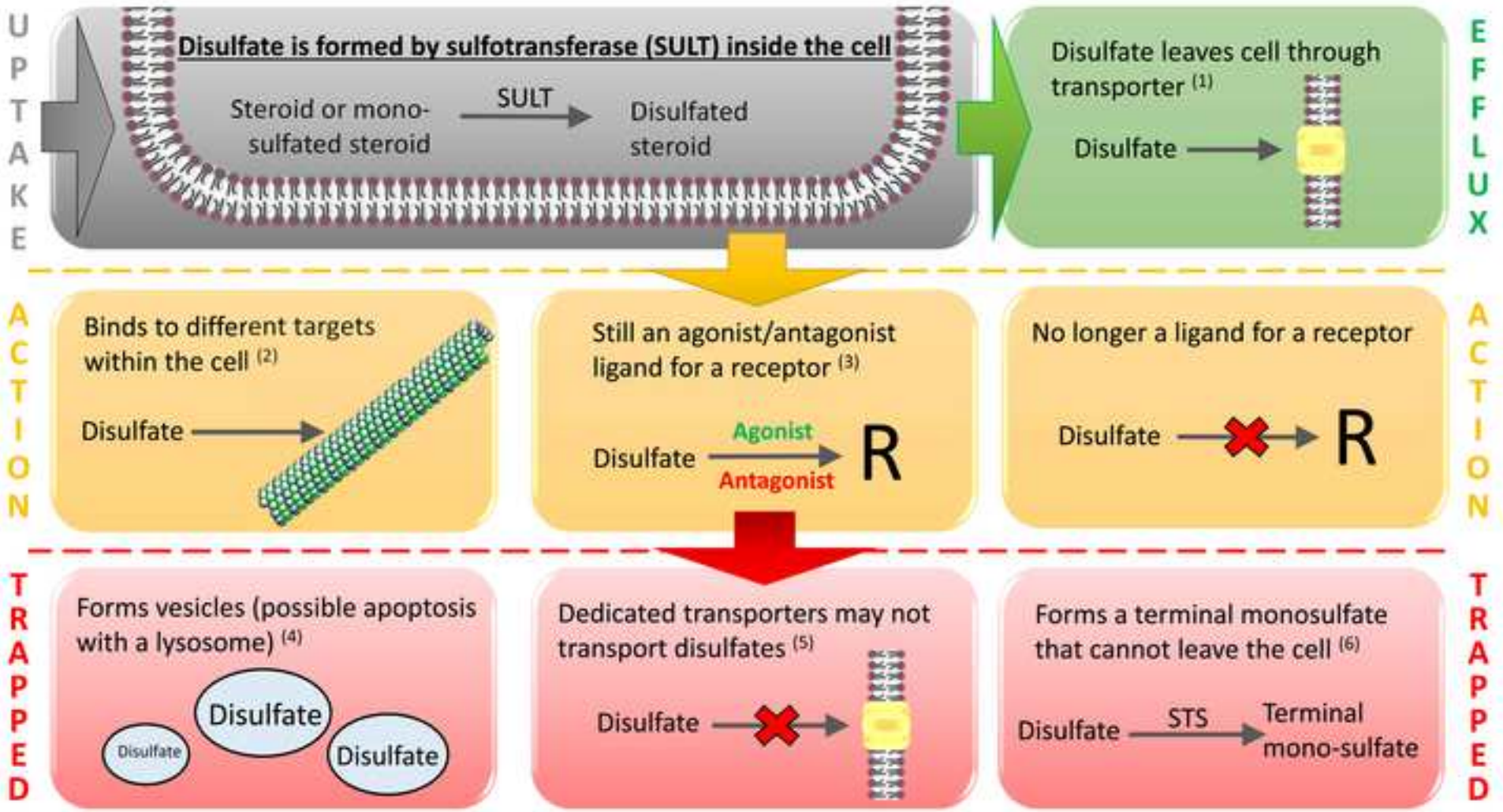

Figure 4

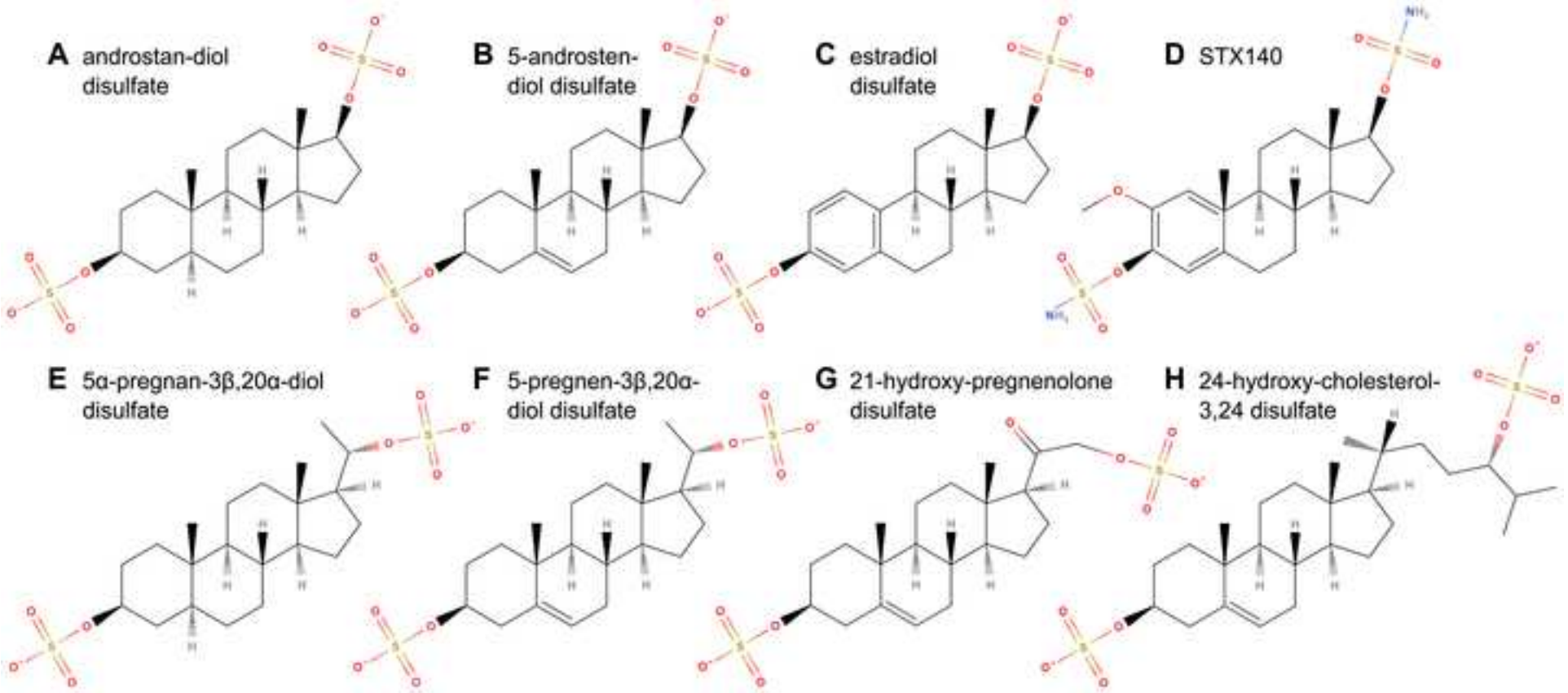

Figure 5