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Mini-Review

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Steroid Sulfation in Adrenal Tumors

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Abbreviations: ACC, adrenocortical carcinoma; ACTH, adrenocorticotrophic hormone; E2S2, estradiol disulfate; LC-MS/MS, liquid chromatography (coupled to) mass spectrometry; OATP, organic anion transporting polypeptide.

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Abstract

Context: The adrenal cortex produces specific steroid hormones including steroid sulfates such as dehydroepiandrosterone sulfate (DHEAS), the most abundant steroid hormone in the human circulation. Steroid sulfation involves a multistep enzyme machinery that may be impaired by inborn errors of steroid metabolism. Emerging data suggest a role of steroid sulfates in the pathophysiology of adrenal tumors and as potential biomarkers.
Evidence Acquisition: Selective literature search using "steroid," "sulfat*," "adrenal," "transport," "mass spectrometry" and related terms in different combinations.
Evidence Synthesis: A recent study highlighted the tissue abundance of estrogen sulfates to be of prognostic impact in adrenocortical carcinoma tissue samples using matrix-assisted laser desorption ionization mass spectrometry imaging. General mechanisms of sulfate uptake, activation, and transfer to substrate steroids are reasonably well understood. Key aspects of this pathway, however, have not been investigated in detail in the adrenal; these include the regulation of substrate specificity and the secretion of sulfated steroids. Both for the adrenal and targeted peripheral tissues, steroid sulfates may have relevant biological actions beyond their cognate nuclear receptors after desulfation. Impaired steroid sulfation such as low DHEAS in Cushing adenomas is of diagnostic utility, but more comprehensive studies are lacking. In bioanalytics, the requirement of deconjugation for gas-chromatography/mass-spectrometry has

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precluded the study of steroid sulfates for a long time. This limitation may be overcome by liquid chromatography/tandem mass spectrometry.
Conclusions: A role of steroid sulfation in the pathophysiology of adrenal tumors has been suggested and a diagnostic utility of steroid sulfates as biomarkers is likely. Recent analytical developments may target sulfated steroids specifically.

Key Words: androgens, estrogens, sex hormones, DHEAS, DHEA-S, steroid disulfate, bis-sulfate, sulfation, sulfurylation, sulfonation

Healthy adrenal glands are steroid factories. The synthesis of mineralocorticoids, glucocorticoids, androgens, and precursors such as dehydroepiandrosterone (DHEA) and androstenedione occurs in a spatially coordinated manner (1-3). The sulfated form of DHEA (DHEAS) is the major product of the adrenal zona reticularis (4) and cellular response to adrenocorticotropic hormone (ACTH) is the major driver of its synthesis. DHEAS is by far the most abundant steroid hormone in the circulation (4), but other steroid sulfates are found at significant concentrations in the human circulation as well (4), such as estrogen sulfates. Although steroid sulfates constitute a significant proportion of the steroid metabolome (5), their tissue-specific synthesis and physiological importance remain to be ascertained. Because estradiol contains 2 hydroxyl groups and shows a certain degree of pseudosymmetry, this steroid can be sulfated twice, giving rise to estradiol disulfate (E2S2) (6,7). Taken together, steroid sulfation represents an additional mechanism to regulate the action of steroid hormones (8).

Sulfation pathways have been implicated not only in the pathophysiology of clinical conditions such as polycystic ovary syndrome, but also steroid-hormone-dependent cancers of breast, prostate, and colon (8). The potential involvement of sulfation pathways in adrenal diseases has not yet been established; especially their role in adrenal tumors is only beginning to emerge (9). This review gathers the current evidence linking sulfated steroid metabolites and adrenal tumors with a focus on adrenocortical carcinoma (ACC).

Mechanisms of Steroid Sulfation and Desulfation

Sulfation of steroids—together with glucuronidation—was long regarded as a mere phase II biotransformation reaction to increase water solubility of steroids to enable subsequent renal excretion (Fig. 1) (4). More recently, the dynamic nature of sulfation-desulfation pathways has come into the focus of biomedical research (8). Serum concentrations of many sulfated steroids are usually several-fold higher than the concentrations of the respective unconjugated steroids. This holds true for these steroids: DHEA, androsterone, cholesterol, pregnenolone, and estrone, among others (4).

Sulfation pathways require activation of inorganic sulfate to the ubiquitous sulfate donor 3'-phospho-adenosine-5'-phosphosulfate (PAPS), a process that is catalyzed by the PAPS synthase enzymes PAPSS1 and PAPSS2 (6,10). These 2 enzymes differ in their expression patterns and protein stability (11,12). Patients with impaired function of either PAPS synthase PAPSS1 or PAPSS2 generally present very differently, suggesting that the 2 PAPSS enzymes supply different sets of sulfation pathways with PAPS (6). While patients with mutations in the *PAPSS1* gene have not been described yet, several cases of compound heterozygous or homozygous mutations in the *PAPSS2* gene are known (13-15). These patients suffer from varying degrees of bone and cartilage malformation; they also show a specific steroid phenotype. Due to lacking PAPS androgens are not sulfated, resulting in apparent deficiency of SULT2A1, the sulfotransferase that mainly sulfates the androgen precursor DHEA.

Cytoplasmic PAPS-dependent sulfotransferases are promiscuous (16). They have a wide range of low molecular weight substrates that can be sulfated (17), including steroid hormones and cholesterol (18). SULT2A1 is the sulfotransferase mainly involved in steroid sulfation. It is strongly expressed in the zona reticularis of the adrenal cortex and largely sulfates DHEA (19-21). Other sulfotransferases such as SULT1E1 and SULT2B1 have been implicated in steroid sulfation as well (22). Although SULT1E1 has a high substrate preference for phenolic estrogen OH groups, evidence suggests that estrogens can also be sulfated by SULT1A1 and SULT2A1 (23). However, there are no reports of adrenal SULT1E1 expression suggesting that estrogen sulfation occurs in other tissues, in particular the liver where SULT1E1 activity has been confirmed in humans (24,25). The primary substrate of SULT2B1 is DHEA. There is no evidence of SULT2B1 adrenal expression, and thus this enzyme most likely sulfates DHEA and related steroids in other organs, in particular the colon and liver (26); possibly protecting those tissues from the mitogenic effects of androgens. Vice versa, the ER membrane-localized steroid sulfatase (STS) is a dedicated

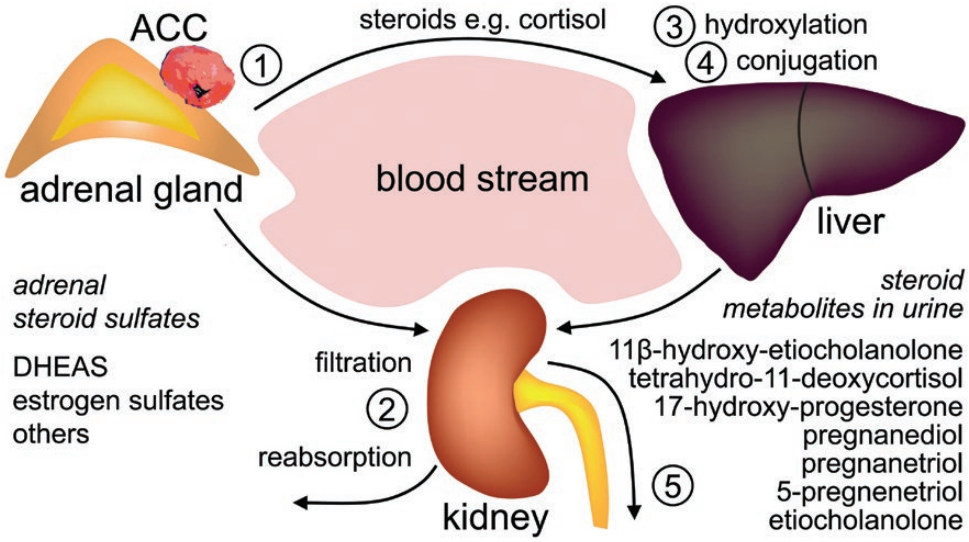

Figure 1. Synthesis and fate of sulfated steroids. Steroid sulfates such as DHEAS are synthesized in the adrenal (1) and other steroidogenic tissues and released into the blood stream where they can be quantified. Examples of such steroid sulfates are indicated at the left-hand side of this schematic. DHEAS and most likely other steroid sulfates are filtrated and reabsorbed in the kidney (2). Several adrenal steroids undergo phase I (mainly oxidation) (3) and phase II biotransformation (sulfation, glucuronidation) (4) and are filtrated and excreted in the kidney (5). In the urine they can be analyzed, usually after deconjugation. Examples of analytes that are detected routinely after hydrolysis of steroid sulfates and glucuronides are listed at the right-hand side of this schematic.

enzyme that can bind intracellular steroid sulfates and reactivate them by hydrolyzing the sulfate ester (8).

Whether or not a tissue produces steroid sulfates depends on the relative expression of dedicated sulfotransferases and STS, the auxiliary PAPS synthase enzymes for sulfate activation, and the sulfatase-modifying factor proteins for STS activation, as well as membrane transporter proteins (6) to mediate the efflux of sulfated steroids (Fig. 2). When both sulfating and desulfating enzymes are expressed in the same cell, this might represent a futile circle and lead to some degree of energy dissipation. Tight control of the enzymatic activities is therefore required.

The Adrenal and Its Role in Steroid Sulfation and Desulfation

The zona reticularis of the adrenal cortex produces androgens and large amounts of the sulfated androgen DHEAS in particular. For efficient sulfation to occur, this tissue expresses the general sulfation machinery that is also present in the liver. Compared to other zones within the adrenal, zona reticularis exhibits a highly selective expression of both the steroid sulfotransferase SULT2A1 as well as the supporting PAPS synthase enzyme PAPSS2 (27). These 2 proteins physically interact with each other (20) and thus provide the mechanistic basis for the highly efficient synthesis of large amounts of DHEAS. The human adrenal gland also secretes other steroid sulfates (28) including sulfated glucocorticoids (29).

Adrenal-specific expression of SULT2A1 is regulated by the transcription factors steroidogenic factor 1 and GATA binding protein 6 (30). Adrenal expression of most sulfation-related genes (6) can be observed at RNA expression level. From a recent data set of different types of adrenal tumors (31), we derived expression information for such genes (Fig. 3). Interestingly, it is primarily the sulfate-activating enzyme PAPSS1 that shows significantly higher expression in ACC compared to benign adrenal adenomas, either cortisol-producing adenomas (CPA) or endocrine-inactive types. Notably, SULT2A1 is most highly expressed in CPA associated with overt Cushing's syndrome. Although circulating DHEAS is low in CPA, due to the suppression of ACTH, this finding may suggest that sulfation (with SULT2A1 as a proxy for it) is of relevance for the function of these tumors and/or autonomous cortisol secretion, with the actual sulfated metabolites still to be discovered.

Beyond regulated messenger RNA expression, the STS sulfatase enzyme is a prime example of a protein with an additional level of regulation—a specific posttranslational modification is required for full activity of this enzyme. It has been known for a long time that the human adrenal expresses the gene *STS* and has STS activity (32), enabling the desulfation of estrone sulfate and DHEAS (33). However, more recent studies have shown limited immuno-reactivity for STS in human fetal and adult adrenal tissue, suggesting a more complex picture with regards to desulfation action (34). Interestingly, patients with X-linked ichthyosis who have a germ-line deletion or inactivating mutation of the

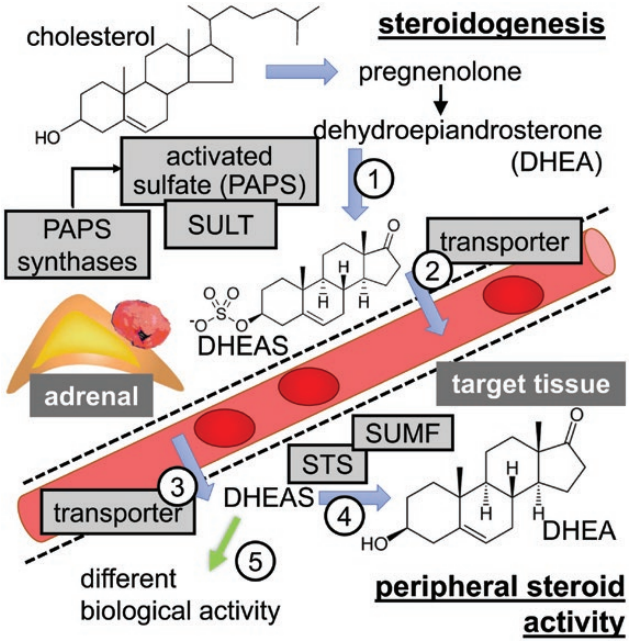

Figure 2. The adrenal as a sulfated steroid factory. The synthesis and secretion of sulfated steroid hormones in the adrenal gland is shown with DHEAS as an example; other steroids that are sulfated in the adrenal include androstenediol and cholesterol. Steroid sulfation occurs by activation of a sulfate via PAPS, with subsequent sulfate transfer by sulfotransferases (1) and secretion via transporter systems (2) into the circulation. Uptake into target cells requires trans-membrane transport (3) and STS-mediated hydrolysis, with the aid of sulfatase-modifying factor proteins (4). DHEA may then be converted to downstream sex hormones before binding to a receptor. Alternatively, the sulfated steroid could bind to molecular targets directly (5).

STS gene, have reduced serum DHEA and testosterone concentrations and elevated urinary DHEAS in comparison to unaffected controls (35). This suggests an importance of STS in adrenal DHEAS desulfation. The same study however also showed that these patients have apparently normal pubertal development, and their 24-h urinary total androgen excretion was similar to control groups (35). An increase in 5α-reductase activity was postulated as a compensatory mechanism for lower serum androgen levels, suggesting a mechanism by which the impact of global STS deficiency is limited for human androgen signaling (35). This would imply that STS action is not an important reaction within the adrenal gland—perhaps it is even redundant there. Instead, adrenal steroid sulfation and subsequent sulfate hydrolysis in peripheral tissue seem to be the key steps to desulfating steroids.

Together, these studies converge at a view that the adrenal is clearly a "sulfating organ" (4) with enzymes of both the sulfation and desulfation machinery, where adrenal sulfation expedites the circulatory transit and subsequent action or excretion of steroids. To our knowledge, the actual sulfosteroid metabolome of healthy and diseased adrenal glands still needs to be mapped out. Endeavors that use different mass spectrometry (MS) imaging approaches of healthy and cancerous adrenals have recently been reviewed (36). Alongside the abundant DHEAS, pregnenolone sulfate and cholesterol sulfate have been identified as adrenal steroid sulfate species (36). A targeted metabolomics study led by Adina Turcu added pregnenolone sulfate and 5-androstenediol-3-sulfate to the list of products of the healthy adrenal (37). Estrogen sulfates are certainly not products of the healthy adrenal, as these glands are known to not express aromatase and the estrogen sulfotransferase SULT1E1. Estrogen sulfates were however detected in ACC samples (22). Thus, it appears that aromatase may be expressed in some ACCs. Overall, the sulfosteroid metabolome of adrenal tumors requires further studies.

Free (nonsulfated) steroid hormones are traditionally believed to cross biological membranes by diffusion, a view that has been challenged recently (38,39). Sulfation further enhances polarity of steroids and renders them membrane-impermeable. Hence, transmembrane flux of steroid sulfates requires dedicated transporters, of which few details are known yet (40,41). A schematic representation of sulfation pathways, including cellular excretion and uptake events, is shown in Figure 4. The sodium-dependent organic anion transporter SLC10A6 is a secondary active uptake transporter that accepts different steroid monosulfates, but not E2S2 as a substrate; it is active in the direction of uptake into target tissues (40). In contrast, the organic anion transporter 4 (OAT4; also known as SLC22A11) is a bidirectional organic anion/dicarboxylate exchanger, with estrone sulfate and DHEAS as substrates (42). This transporter also exhibits affinity for the steroid disulfate E2S2 (43). OAT4 mediates apical secretion of xenobiotics and endogenous compounds such as steroid sulfates in the kidney and uptake in the placenta but OAT4 messenger RNA expression has also been detected in the ACC cell line H295R along with OAT3 (44).

Finally, the heterodimeric organic solute transporters (OSTα/OSTβ) SLC51A and SLC51B appear to be physiologically relevant for the transmembrane transport of sulfated steroids (45,46). While the predominant role of this transport system is in bile acid secretion in the liver, it has been recognized that estrone sulfate is a substrate and adrenal expression has been demonstrated (47).

The actual physiological role of all these transporters and, notably, their relative contribution to the export of steroid mono- and disulfates from steroidogenic tissue is yet to be discovered, alongside potential additional transporters required for steroid sulfate export. It is important to acknowledge that many transporters such as OAT4 may function in both directions depending on the relative

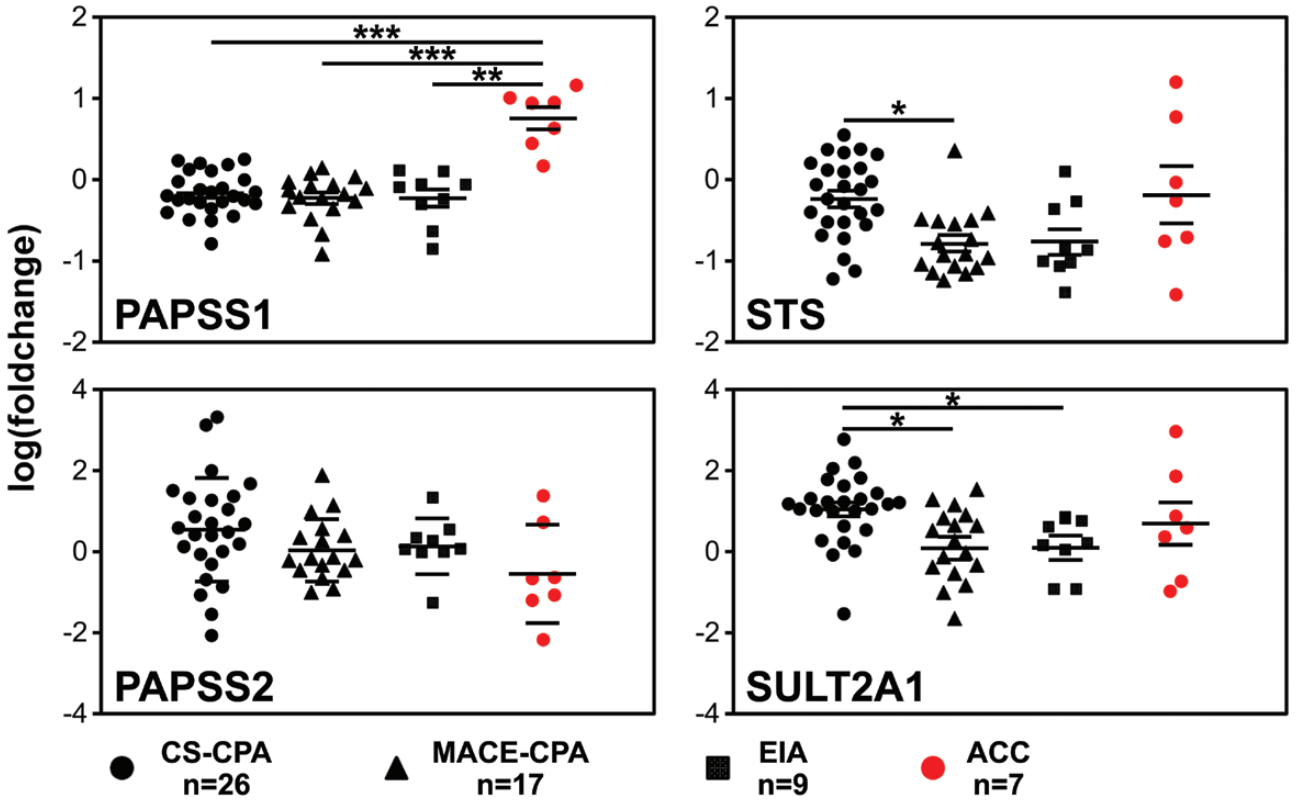

Figure 3. ACC-specific expression of parts of the sulfation machinery. Sulfation-related enzymes are expressed at the RNA level to a different extent in different adrenal tumors. The increased PAPSS1 expression in ACC compared to all others suggests a relevance of sulfation specifically in ACC despite the small number of samples assessed. *Y*-axes show the logarithmic fold change—log(foldchange)—of the messenger RNA expression using a pool of normal adrenal glands as reference. The scatter plots show the individual values, and the mean +/– SEM. *P*-values are from non-parametric analysis of variance (Kruskal-Wallis test) followed by Dunn's multiple comparison test. This data set was derived from a 2020 RNA-seq dataset of bulk tumor samples; expression levels of selected genes were plotted (31). Abbreviations: CS-CPA, cortisol-producing adenomas with overt Cushing's syndrome (previously known as CPA); EIA, endocrine inactive adenoma; ACC, adrenocortical carcinoma; MACE-CPA, cortisol-producing adenomas with mild autonomous cortisol excess (previously known as sCPA).

concentration of substrates. In summary, expression of steroid sulfate transporters in target tissues is a condition for the action of those sulfated steroids.

Biological Actions of Sulfated Steroids

While sex hormones are the best understood steroid sulfates, there is also evidence for sulfation of other steroids such as glucocorticoids (48). Their experimental assessment is difficult, both with a genetic approach and pharmacologic enzyme inhibition, as a direct consequence of the promiscuity of most gene products involved in sulfation pathways. Feedback loops may be in place; for example, cholesterol sulfate was reported as a naturally occurring inhibitor of steroidogenesis (18). Another complication is that several steroid sulfates might have nongenomic effects outside the adrenal, for example, as neurosteroids (49,50). DHEAS and pregnenolone sulfate have been shown to have effects on the *N*-methyl-D-aspartic acid receptors and sigma receptors, although more work is needed here (51,52). Furthermore, the fetal adrenal gland responds differently to sulfated estradiol and unconjugated estradiol (53,54). In comparison to unconjugated estradiol, E2 sulfate (E2S) has a neuroendocrine action to increase the secretion of cortisol, estradiol, and E2S. As a neurosteroid, E2S also inhibits fetal ACTH secretion, influencing the hypothalamic-pituitary-adrenal axis in a strikingly different way compared to free estradiol (54).

The actual role of DHEAS, the most abundant steroid hormone metabolite in the human circulation, is a subject of ongoing discussions. While DHEA may be an important androgen in the intestine or the brain (55,56), DHEAS may also have direct effects by binding to other targets such as the pentose-phosphate pathway enzyme glucose-6-phosphate dehydrogenase (57,58). Whatever the binding properties of DHEAS are, this may be of physiological significance due to the high abundance of DHEAS in circulation (4).

Steroid disulfates are even less well understood (7). The STS inhibitor STX140 has structural similarities to steroid

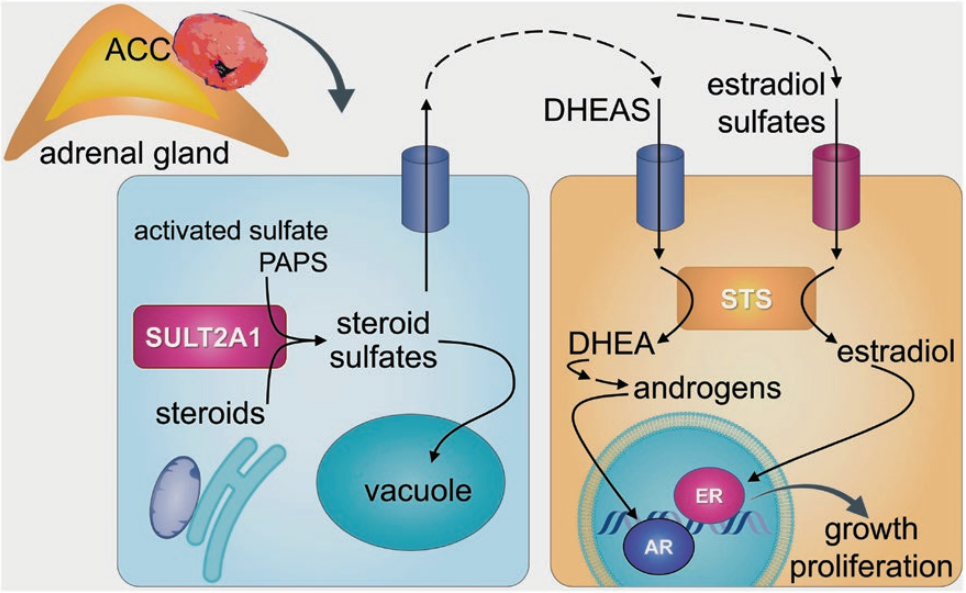

Figure 4. Key processes and potential paracrine action of steroid hormone sulfates in adrenal tumors. The secretion of steroid hormone sulfates and their uptake into adjacent tumoral and immune cells may stimulate tumor growth. Note that other cell types, such as immune cells, that are present in the tumor may also be target sites of steroid sulfates. There is also the scenario where systemic sulfated steroid hormones are taken up by adrenal tumors.

disulfates—with 2 sulfamate groups, it very much resembles E2S2. STX140 was recently reported as an inhibitor of the enzyme carbonic anhydrase IX and found to confer both proapoptotic and antiangiogenic effects, showing promise to treat triple-negative breast cancer (59). This suggests that E2S2 might well bind to biological structures other than the estrogen receptor. To our knowledge, the physiological role of E2S2 is still unknown, both in the adrenal gland and in other organs. With the exception of oxysterol disulfates that can act as antagonist at their cognate receptor (60,61), it is generally assumed that the more water-soluble sulfated metabolites are less active at the receptors that are usually activated by their nonsulfated counterparts.

Analytics of Steroids and Sulfated Steroid Metabolites: Potential Biomarkers of Disease

Sulfated metabolites have been reported as potential biomarkers for a variety of clinical conditions (8) and have been applied for doping control (62,63). Perturbed steroid sulfate concentrations have been used to indicate steroidogenesis deficiencies during pregnancy such as STS deficiency, Antley-Bixler syndrome, or P450 oxidoreductase deficiency, which causes low estriol (64-66). A set of 10 metabolites, including 5 sulfated steroids, was successfully used to detect advanced liver fibrosis with great precision (67). These examples show that the previously understudied steroid sulfates have potential for exploitation as diagnostic markers.

Physiological sulfation of steroids has been known for a long time (8,68-70). In biological matrices, steroid sulfates were first described as conjugated molecules in human urine in 1961 (71); steroid disulfates, only 1 year later (72). Radioimmunoassay has been the standard analytical method for deconjugated steroids in the past and is still used today for quantification of compounds such as DHEAS in serum/plasma. Early conjugate profiles of fetal and neonatal adrenal steroids were also determined by gas chromatography as well as GC-MS (73). A huge increase in sensitivity came at the price of a time-consuming and complex sample workup. The polar nature of steroid glucuronides and sulfates, as well as their thermal instability, precluded the use of GC-MS and required deconjugation and derivatization to be compatible with this analytical approach (74). Various deconjugation techniques beyond acidic hydrolysis have been evaluated in the following decades, in particular enzymatic hydrolysis and hydrolysis catalyzed in 1,4-dioxane (75-78).

Although the first attempt to directly measure steroid conjugates with MS was made in the 1980s, using a fast atom bombardment ionization technique (75), the interest in analyzing intact conjugates only (re-) grew in recent years (79). Using liquid chromatography with tandem MS (LC-MS/MS), the analysis of intact conjugates has become possible, and the first LC-MS/MS method for steroid sulfates in urine was published in 1997 (80). Direct analysis of steroid conjugates became more popular and combined methods for the

quantification of both conjugated and free steroids were developed (79,81). Analytical improvements were moreover made for steroid disulfates (82) and applied in the clinical field of prenatal diagnosis (5).

Although GC-MS remains a preeminent discovery tool in clinical steroid investigations with its strengths of excellent resolution and sensitivity (83), LC-MS/MS is increasingly replacing GC-MS as a routine analytical method and is used more frequently in research and routine analytics (84). Ultra-high-performance supercritical-fluid chromatography coupled to tandem MS has been developed recently, which combines the resolution of GC with the high-throughput capabilities of high-resolution liquid chromatography (85,86). LC-MS/MS-based open scan methods are about to complement this analytical toolbox with regards to conjugated metabolites. Sulfated plant flavonoids have been identified and quantified by neutral loss scan MS (87), and a precursor ion scan method suggests that many sulfated species exist in bovine serum still to be investigated in more detail (88). These emerging techniques will enable new discoveries by untargeted profiling, such as mapping out the entire human sulfometabolome of the adrenal gland.

Matrix-assisted laser desorption/ionization MS imaging is a relatively recent addition to the arsenal of MS-based analytical methods and allows the spatially resolved detection of a broad range of molecules depending on the experimental setup. These include not only small molecules (such as of energy metabolism, proteins, sugars) but also lipids that may be detected in tissue specimens (89,90). Its application to the adrenal will be discussed in more detail in the following discussion.

Adrenal Tumors, Sulfated Steroids and the Special Case of ACC

From a general perspective, adrenocortical adenomas may remain endocrine inactive or produce adrenal steroids excessively that lead to specific clinical syndromes, such as Cushing's syndrome with a presence of cortisol excess or Conn's syndrome caused by aldosterone excess. DHEAS and other adrenal androgens are usually low in adrenal Cushing's syndrome (91,92) due to suppression of ACTH secretion in the pituitary gland. In ACTH-dependent Cushing's syndrome, on the other hand, ACTH stimulates adrenal androgen secretion and thus the levels of DHEAS (and other androgens like androstenedione) are mostly normal or elevated.

In malignant ACC, severe hyperandrogenism is frequent, and high levels of DHEAS and androstenedione (9) are important diagnostic elements (93,94). While combined hypersecretion of cortisol and adrenal androgens like DHEAS is most frequent (95,96), 5% to 20% of ACC produce androgens alone (97). An excess of estrogens, mineralocorticoids or other steroid combinations is diagnosed in less than 5% of patients.

Even in the absence of clinically apparent hormone overproduction, some cases of ACC do produce excess steroid precursors as shown by the urinary excretion of tetrahydro-11-deoxycortisol, pregnanediol, pregnanetriol, and other steroid metabolites (95,98). This characteristic can be used for diagnostic purposes. Different groups have used multisteroid profiling by LC-MS/MS of urine samples to differentiate benign from malignant adrenocortical tumors (98-101). Steroid hormone panel analyses have since been conducted on serum/plasma samples to differentiate benign from malignant adrenocortical tumors (102-104). Very recently, a large collaborative study from the European Network for the Study of Adrenal Tumors demonstrated that a combination of MS-based urinary steroid metabolite profiling, machine-learning, and cross-sectional imaging can significantly improve the detection of ACC (105).

Over the last few years, our understanding of the molecular pathogenesis of ACC has greatly expanded via integrated and comprehensive genomic analyses (106-109). However, this has not been accompanied with significant changes in terms of prognostic biomarkers or treatment options (110,111). Some molecular alterations have been proposed to have relevant prognostic value, such as BUB1-PINK1 expression levels in the tumor and promoter region methylation patterns (107,108,112,113). Recent progress highlighted molecular alterations that might be pharmacologically targetable and may open up new therapeutic opportunities (114). Thanks to these recent advances, new targeted treatment options are on the horizon and have recently been reviewed (115,116). Also, more individualized approaches toward personalized care of patients with ACC have been explored (112,117). It is hoped that these findings will soon been applied within the clinical routine.

With the aim to uncover metabolic processes in ACC that may be relevant for diagnostic and therapeutic purposes, researchers from Würzburg and Munich made use of matrix-assisted laser desorption/ionization MS imaging of ACC tissue samples (22). By using overall survival as a surrogate marker of biological behavior, the authors found that the tissue abundance of estrone sulfate and estradiol monosulfate correlated with better prognosis, while the detection of E2S2 was associated with adverse outcome when compared to tumors with undetectable E2S2 (Fig. 5). In addition, it was demonstrated that the expression of a subset of sulfotransferases was correlated with the abundance of sulfated estrogens and similarly associated with more favorable prognosis. Interestingly, the presence of membrane-delimited vacuoles in the E2S2-expressing

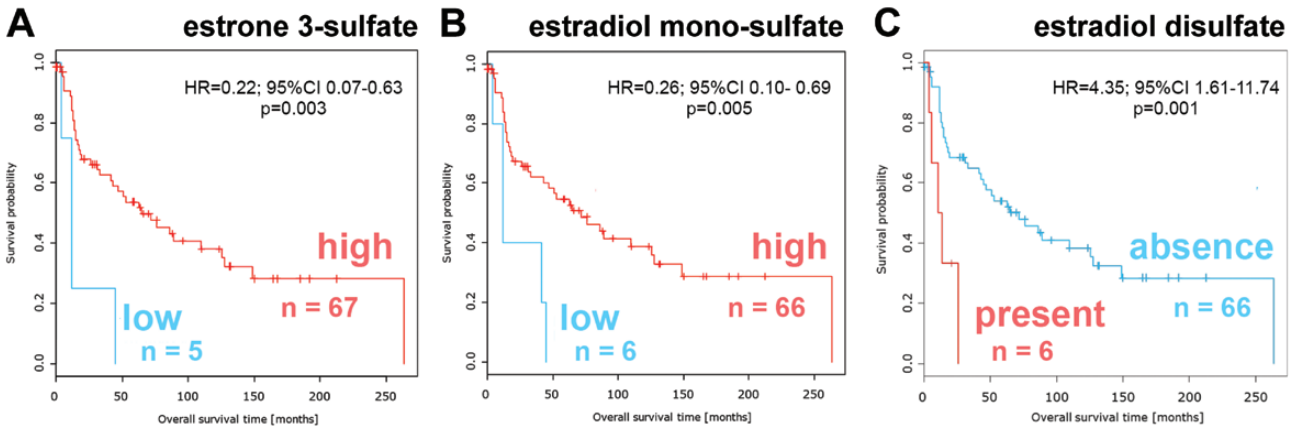

Figure 5. Different estrogen sulfates correlate with overall survival in ACC. Kaplan-Meier plots for a cohort of 72 samples of histologically confirmed ACC with full clinical annotation. Reproduced with permission from (22).

tumors could imply the storage (instead of secretion) of this uncommon steroid disulfate. One would expect that E2S2 would undergo hydrolysis by STS to result in E2S. While it has been shown that inactivating mutations in STS were not present in the initial cohort of tumors with detectable E2S2, 1 tissue sample from the Cancer Genome Atlas did harbor such a mutation. This sample with a damaging STS mutation had E2S2 present and had a particularly low sulfotransferase expression—in line with the concept that the presence of STS activity would protect from E2S2 accumulation.

While opening new windows into the biology of steroid sulfation in the adrenal, this study has the shortcoming of using clinically more readily available formalin-fixed paraffin-embedded tissue. During the procedure of tissue preparation, most lipophilic compounds are lost, which precluded the analysis of nonsulfated steroid hormones. It therefore remains unclear whether these tumors exhibit a generally increased steroidogenesis or an actual sulfation phenotype that remains to be discovered in more detail. In addition, it is yet unclear to what extent these steroid sulfates are secreted into the blood stream and hence can be detected in liquid biomaterials as tumor markers. Because the authors found associations with certain metabolic pathways, the presence of sulfated steroids may be a marker of specific metabolic adaptions rather than a cause of biological aggressiveness. On the other hand, steroid sulfates may act in a paracrine manner and activate steroid hormone receptors in surrounding cells as schematically drawn in Figure 4. Overall, these recent findings fuel the interest in steroid sulfation in adrenal tumors and particularly shows promise in the use of sulfates as diagnostic markers.

New Developments and Open Questions

The link between ACC and steroid sulfation opens up new research avenues, as the presence and diagnostic utility of steroid hormone sulfates is going to be investigated in more detail. Firstly, as cytoplasmic sulfotransferases show overlapping substrate profiles (17), the relative contribution of different sulfotransferases to actual steroid sulfation needs to be better understood. Secondly, the striking observation of E2S2 accumulation in ACC as a prognostic factor (22) raises the question of the metabolic underpinnings of steroid sulfation and their relationship with ACC cell survival and/or proliferation. Finally, at present, which transporters mediate export of steroid hormone monosulfates (and potentially disulfates) in the adrenal, and to what extent paracrine effects exist, is ill-defined.

Research around steroid sulfation may profit from recent biochemical and chemico-biological studies: activity probes specific for individual sulfotransferases have first been described by Falany and colleagues (118-120). Specific substrates for the estrogen sulfotransferase SULT1E1 have been described since (121) and most recently been used to image SULT1E1 activity in the brains of human volunteers (122). Other probes target different sulfotransferase isoforms specifically, such as SULT2A1 (123). These new approaches may result in unprecedented imaging opportunities of sulfotransferase activity, when applied to cellular and/or animal models.

The field will also benefit from improved performance of LC-MS/MS and, in particular, from better access to analytical standards for sulfated steroids. Some steroid disulfates have been synthesized in 2014 (124), and synthetic access to sulfated compounds in general has recently improved (125), now also including bis-glucuronides and mixed sulfoglucuronide steroid species (126). It is expected that this will allow us to identify and quantify more conjugate species in relevant clinical samples using targeted approaches.

A question about dynamic sulfation and desulfation processes remains open, however. Strict spatial separation and/or enzymatic control seems to be required for sulfation pathways to avoid a vicious cycle of the unnecessary dissipation

of energy by simultaneous sulfation and desulfation events (4). An attractive way of thinking of this in the future could be a proofing mechanism, where sulfation is reverted and possibly "corrected." The paradigm for such a process could be the sulfation and desulfation of 24-hydroxycholesterol (60) where abundant sulfation and selective desulfation or "trimming" offer access to sulfated species not biologically accessible otherwise.

Conclusion

Despite the presumed relevance of steroid sulfates and their diversity, little is still known about key aspects of their physiology. There is recent evidence that steroid sulfation plays a pathophysiological role in adrenal tumors, particularly ACC. The growing number of analytical tools and methods are suitable to improve our understanding of steroid sulfation. This may shed light on sulfation-related metabolic reprogramming, which could be of further relevance in conditions beyond adrenal tumors in general and ACC in particular (22).

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