Aus der Medizinischen Klinik und Poliklinik I
der Universität Würzburg
Direktor: Professor Dr. med G. Ertl

Expression of the DHEA/DHEAS-Shuttle in cell lines and foetal tissue of human liver, adrenal and cartilage

Inaugural-Dissertation

zur Erlangung der Doktorwürde
der medizinischen Fakultät
der
Julius-Maximilians-Universität Würzburg

vorgelegt von
Florian Schlereth
aus Oberthulba

Würzburg, Oktober 2013

Referent: Prof. Dr. med. B. Allolio

Koreferent: Prof. Dr. J. Wischhusen

Dekan: Prof. Dr. med. M. Frosch

Tag der mündlichen Prüfung: 13. August 2014

Der Promovend ist Arzt.

Table of Contents

1 Introduction

1.1 Adrenal gland and steroidogenesis pathway

1.1.1 Anatomy and histology of the adrenal gland

The human adrenal gland is a paired, pyramidal shaped organ positioned at the upper pole of the kidneys (Figure 1-1). Each of them is roughly 3 cm wide, 5 cm long and 1 cm thick. It weighs approximately 4 g [1] in the adult and consists of two functionally and morphologically distinct tissues of different embryological origin within a single capsule. About 10% of the adrenal weight is medulla which produces the catecholamine hormones epinephrine and norepinephrine and the cortex consisting of three zones with specific functions and enzyme expressions [2] (Figure 1-2). The outer zona glomerulosa (ZG) cells of the cortex produce the mineralocorticoid aldosterone which plays a major role in controlling the blood's water and electrolyte levels and therefore the body's blood pressure [3]. Cells in the zona fasciculata (ZF) secrete cortisol which is a key player in the regulation of blood glucose levels and crucial for response to stress [4]. The inner zona reticularis (ZR) produces the adrenal androgens Dehydroepiandrosterone (DHEA), its sulphonated conjugate DHEAS and androstenedione. Although they do not bind the androgen receptor (AR), they are called androgens because they are converted peripherally to testosterone [5]. In addition, Nakamuro et al confirmed that testosterone is also secreted directly by the human adrenal [6].

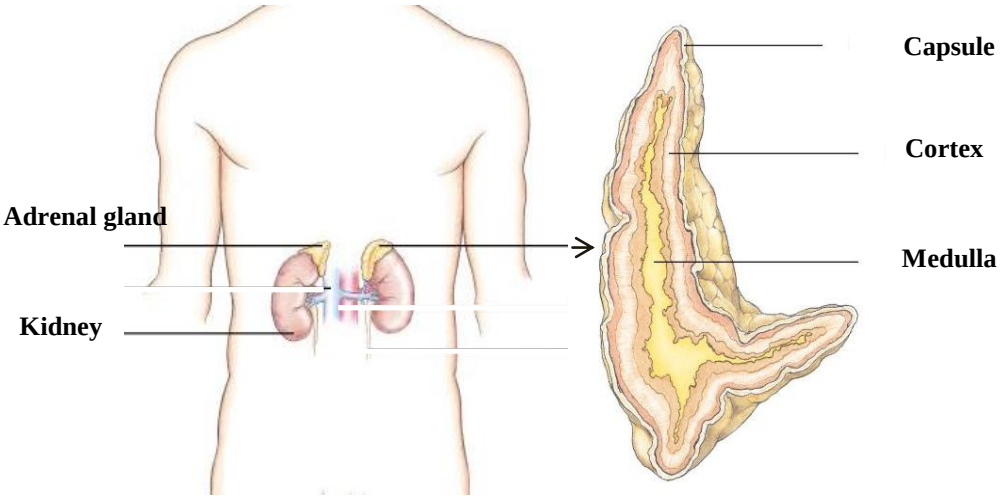


Figure 1-1 Anatomy of the human adrenal gland

Taken and adapted from http://www.eesom.com/go/Hormonsystem/Nebenniere

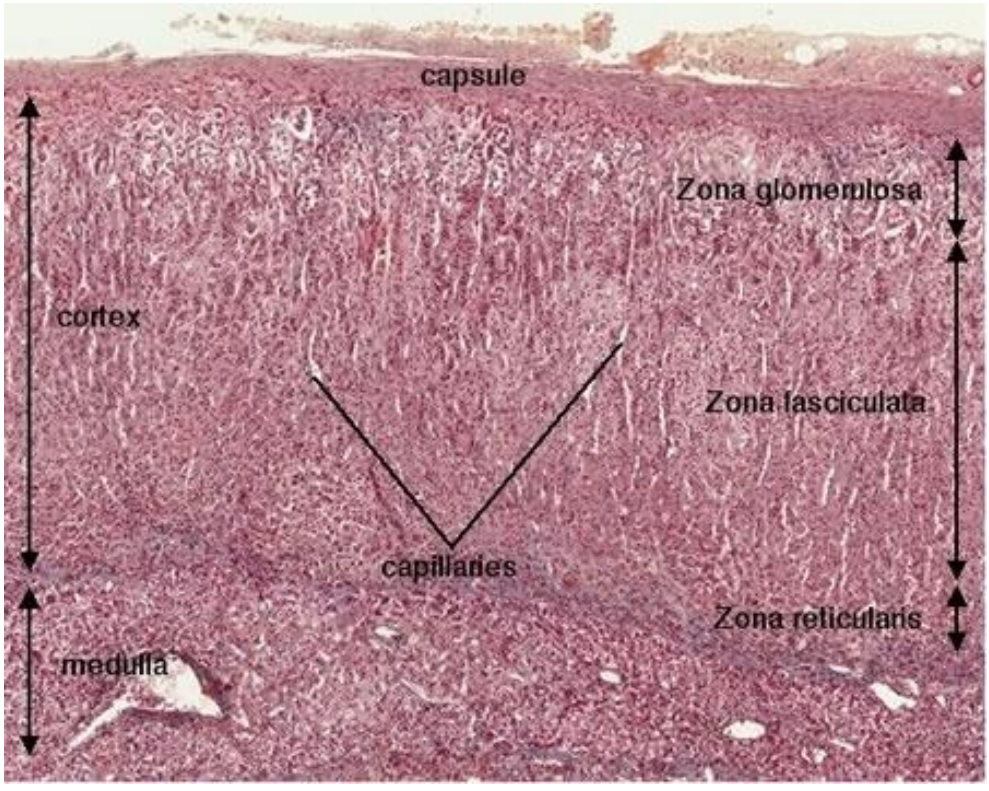


Figure 1-2 Histology slide of the adrenal gland in low magnification

Adrenal medulla and cortex with its zona glomerulosa, zona fasciculata and zona reticularis (Mallory-Azan stain) taken from http://missinglink.ucsf.edu/lm/IDS_106_Endocrine/Assets/Histology_Images/461Adrenallp.JPG

1.1.2 **Development of the adrenal gland**

Whilst the adrenal medulla originates from neuroectoderm, the cortex evolves from mesodermal tissue. The first phase of adrenal development is "condensation" of the coelomic epithelium at around 3-4 weeks gestation [7]. Then, the human adrenal is first identifiable at around 4-5 weeks of human development. After 8 weeks, the cortex consists of a smaller outer definitive zone, which is steroidogenically inactive until late gestation and a larger inner foetal zone active throughout gestation. A transitional zone between those two zones exists and produces cortisol towards the end of foetal development [8-10]. The neuroendocrine cells that will form the medulla start moving into the adrenal at about two months of gestation [2]. (Figure 1-3)

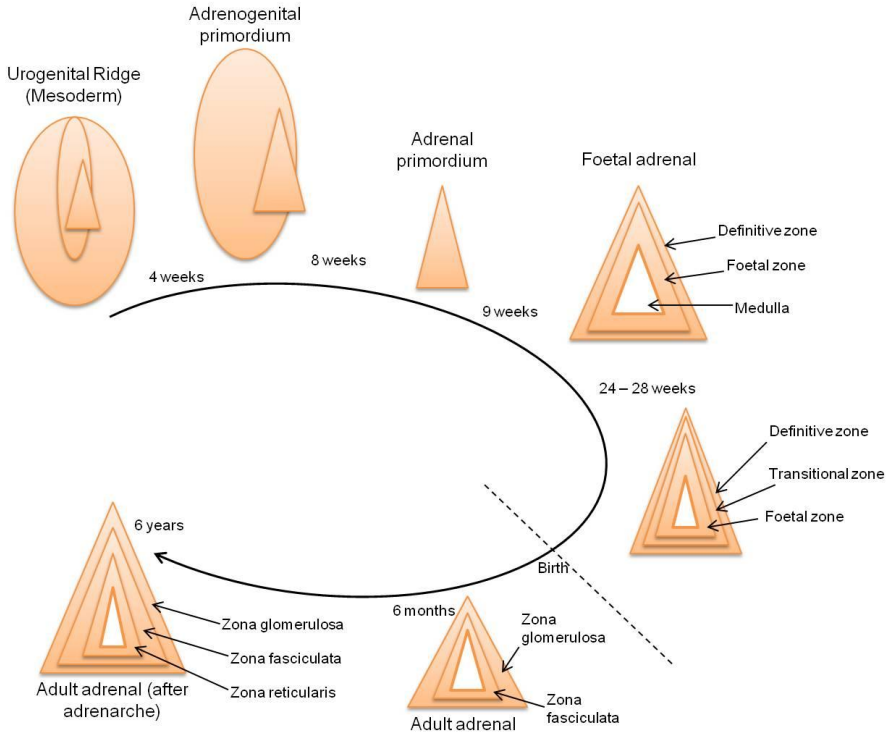


Figure 1-3 Adrenal development

The human adrenal cortex develops from mesoderm together with the kidneys and the gonads (urogenital ridge). Until around 9 weeks of gestation, the foetal adrenal has separated from the other "anlagen" and the cortex differentiates into a definitive zone and a foetal zone. An additional transitional zone appears after 24 weeks' gestation. After birth, the foetal adrenals involve and the adult adrenals form. By 6 months of age, the adult adrenal cortex consists of the mineralocoritcoid-producing zona glomerulosa (ZG) and the glucocorticoid-producing zona fasciculata (ZF). In an event called "adrenarche" around 6 years of age, the zona reticularis (ZR) forms and starts to produce adrenal androgens. Taken and adapted from [10]

Whilst the foetal adrenal secrets large amounts of the adrenal androgens DHEA and DHEAS, the secretion rapidly decreases after birth due to an involution of the foetal zone and remains low for the first years of life [10-11].

With the beginning of adrenarche around the age of 6 years, the ZR develops and adrenal androgen production rises again. It reaches a high in early adulthood and slowly decreases with age (Figure 1-4) accompanied by shrinkage of the ZR thickness [12-14].

For a long time, Adrenarche has been thought to be a relatively sudden onset of hormone secretion. However, this might be attributed to the low sensitivity of the immune assay used to detect DHEAS. Secretion of adrenal androgens determined by urinary excretion of metabolites can be detected at as early as 3 years of age applying gas chromatograpy / mass spectrometry (GC/MS), a highly sensitive method [15-16].

Initiation and development of adrenarche as well as its significance for prepubertal development are not well understood. There are several hypotheses about the reason for the above-described course of androgen secretion. It is thought to play an important role in changes of metabolism during transition from childhood to adulthood [17], especially since early onset of androgen excess in premature adrenarche has been linked to metabolic disease [16]. In addition, Campbell suggests that the known neuro-modulatory effects of DHEAS may protect metabolically active parts of the developing brain during adolescence helping to extend the development of the prefrontal cortex [18].

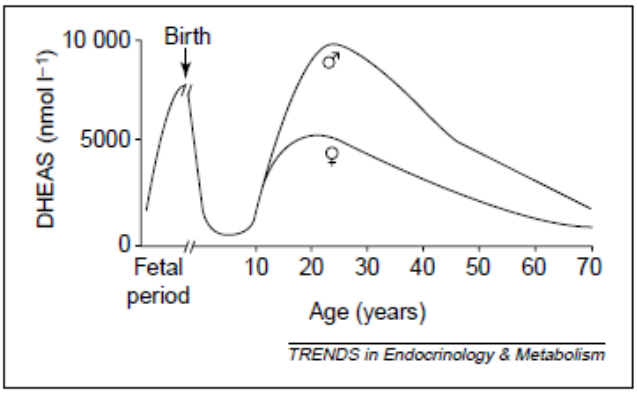


Figure 1-4 Variation in circulating DHEAS levels throughout human life

During pregnancy, foetal levels of DHEAS climb progressively to a peak at birth, which is followed by a rapid fall. At approximately 6 years of age, DHEAS levels rise again (adrenarche), reach a peak during early adulthood, and decline thereafter. The levels of circulating DHEAS during adult life exhibit a gender difference, with higher levels found in men than in women. Figure taken from [14]

1.1.3 **Steroidogenesis**

All human steroid hormones derive from cholesterol [2] (Figure 1-5), which cortical adrenal cells acquire from three principal sources. It can be synthesised *de novo* from acetate in the endoplasmic reticulum [19] or mobilised by hormone sensitive lipase from intracellular cholesterol esters stored in lipid droplets. However, steroidogenic cells gain about 80% of the cholesterol for steroid hormone formation by endocytosis of circulating lipoprotein particles from the plasma [2, 20-21]. Cholesterol side-chain cleavage enzyme (CYP11A1 or $P450_{scc}$) converts cholesterol to pregnenolone in the mitochondrion. This is the first and rate-limiting step in steroidogenesis [2]. Whilst the amount of this enzyme controls long-term steroidogenic capacity, the access of cholesterol to mitochondria via the steroidogenic acute regulatory protein (STAR) determines acute regulation, where steroids are released within minutes of a stimulus [5]. The short-lived STAR facilitates the rapid influx of cholesterol from the outer mitochondrial membrane to the inner membrane [5, 21]. Pregnenolone is then further converted in the smooth endoplasmic reticulum by 17α-hydroxylase/17,20 lyase (CYP17A1), a unique enzyme with two differentially regulated activities at a single active site [22]. In the ZG, the absence of CYP17A1 directs towards the synthesis of mineralocorticoids by 3β-hydroxysteroid dehydrogenase type II (HSD3B2). In the ZF and ZR, the 17α-hydroxylase activity of CYP17A1 allows conversion of pregnenolone to 17α-hydroxypregnenolone. In the ZF, HSD3B2 activity in absence of 17,20 lyase activity of CYP17A1 leads to production of glucocorticoids. In contrast, low expression of HSD3B2 in the ZR combined with the 17,20 lyase activity of CYP17A1, which is enhanced by cytochrome b_5, drives steroidogenesis towards DHEA synthesis. Hence, the differential expression and activity of CYP17A1 in the adrenal zones is the qualitative regulator of steroid synthesis. The adrenals secrete DHEA predominantly as DHEAS, the form sulphonated by the cytosolic enzyme DHEA sulphotransferase (SULT2A1) [5, 14, 22-23]. (Figure 1-6)

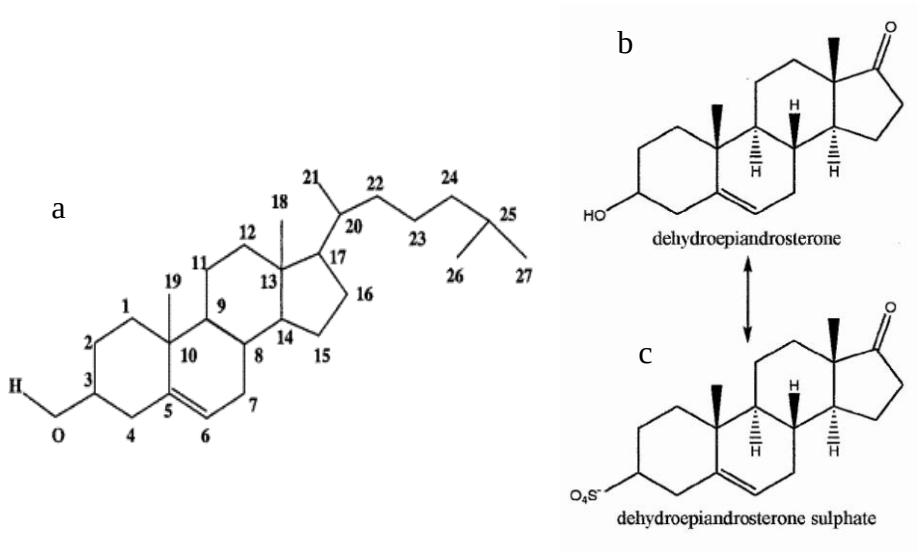


Figure 1-5 Molecular structure of (a) cholesterol, (b) DHEA and (c) DHEAS

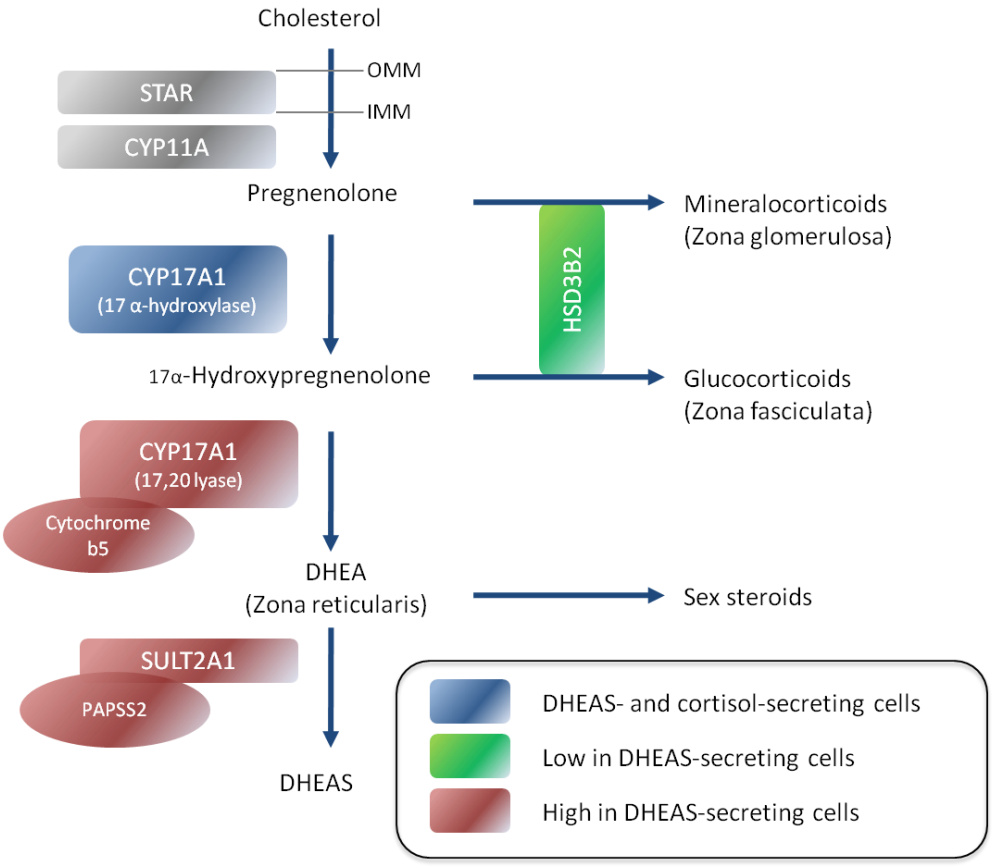


Figure 1-6 Steroidogenic pathway for the production of DEHA and DHEAS in the human adrenal

The absence of HSD3B2 in the ZR leads to the synthesis of DHEAS from cholesterol in the presence of STAR, CYP11A1, CYP17, Cytochrome b5 and SULT2A1. IMM/OMM: inner/outer mitochondrial membrane. Figure taken and adapted from [14]

1.2 DHEA and DHEAS

The adrenal androgens DHEA and its sulphate ester DHEAS (Figure 1-5) are the most abundant hormones in human circulation [24]. In adult men and women, serum DHEAS levels are 100 to 500 times higher than those of testosterone are and 1000 to 10000 times higher than those of estradiol [25-26]. DHEA represents the crucial precursor of sex steroid biosynthesis and is mainly released as DHEAS by the adrenal zona reticularis. It is found in some, but not all mammals and thus represents a recent evolutionary development [27]

Generally, hormones can act in endocrine, paracrine, autocrine or intracrine ways. DHEA exerts its action mainly by conversion to sex steroid in the target cells and therefore represents an example of intracrinology (Figure 1-7). Intracrine activity represents an economical system, which requires minimal amounts of hormone to exert maximal function, since it is not diluted in systemic circulation and the time delay is minimal [28].

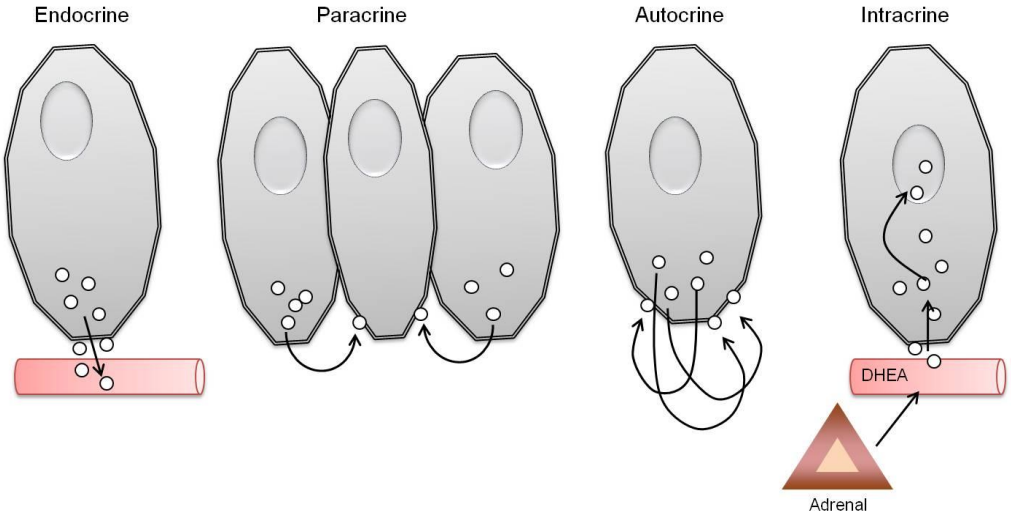


Figure 1-7 Schematic representation of endocrine, paracrine, autocrine and intracrine hormone action

The classic way of hormone action is endocrine where hormones are synthesised by a specialised gland and released into circulation to act at their target cells via intracellular or membrane-bound receptors. Additionally, secreted hormones can exert their action on neighbouring cells (paracrine) or on themselves (autocrine). Intracrine action describes the activity of hormones within the synthesising cell without prior release. Hormones are synthesised in peripheral tissue from hormone precursors such as DHEA. Taken and adapted from [29]

1.2.1.1 Downstream metabolism

Only desulphated DHEA, but not DHEAS, can be converted towards sex steroids [30]. However, hydrolysis of DHEAS to DHEA by steroid sulphatase (STS) may be restricted to only certain peripheral tissues including brain, placenta, prostate and mammary gland [31-35]. In most tissues, sulphonation by SULT2A1 overweighs, limiting the availability of DHEA for downstream conversion [36-37]. Therefore, SULT2A1 represents a crucial regulator of DHEA action.

Downstream conversion of DHEA leads to active sex steroids, i.e. androgens (C19 steroids) and oestrogens (C18 steroids), which act via the androgen (AR) and oestrogen receptor (ER), respectively. 3β-hydroxysteroid dehydrogenase 2 (HSD3B2) converts DHEA to androstenedione, which then can be transformed to either oestrone (E1) by P450 aromatase (CYP19) or to testosterone by 17β-hydroxysteroid dehydrogenases (HSD17B) [29, 38]. Aromatase also converts testosterone to 17β-oestradiol (E2) and there is interconversion between E1 and E2 by HSD17B. 5α-reductases (SRD5A) lead to the conversion of the much more potent androgen 5α-dihydrotestosterone (DHT) from testosterone and 5α-androstanedione from androstenedione, respectively [29, 38-39]. 3α-hydroxysteroid dehydrogenase (AKR1C) inactivates those to androsterone or 5α-androstanediol [38]. Once more, there is interconversion between the latter steroids by HSD17B. The final inactive androgen metabolites are 5-androstanediol glucorunide (ADG) and androsterone glucorunide (ATG), which are released into circulation and therefore reflect tissue-specific action of DHEA better than circulating sex steroid themselves [29, 40-41]. (Figure 1-8)

The widespread expression of HSD3B2, HSD17B, SRD5A and CYP19 in various target tissues like liver, skin, prostate, bone breast and brain results in almost ubiquitous peripheral generation of sex steroids from DHEA [38, 42]. Importantly, pharmacogenetic studies of DHEA administration in humans with low circulating levels of DHEAS revealed that administration of DHEA leads to sexually dimorphic conversion [38]: in women it significantly increases circulating androgens [43], whereas it increases circulating oestrogens in men [44].

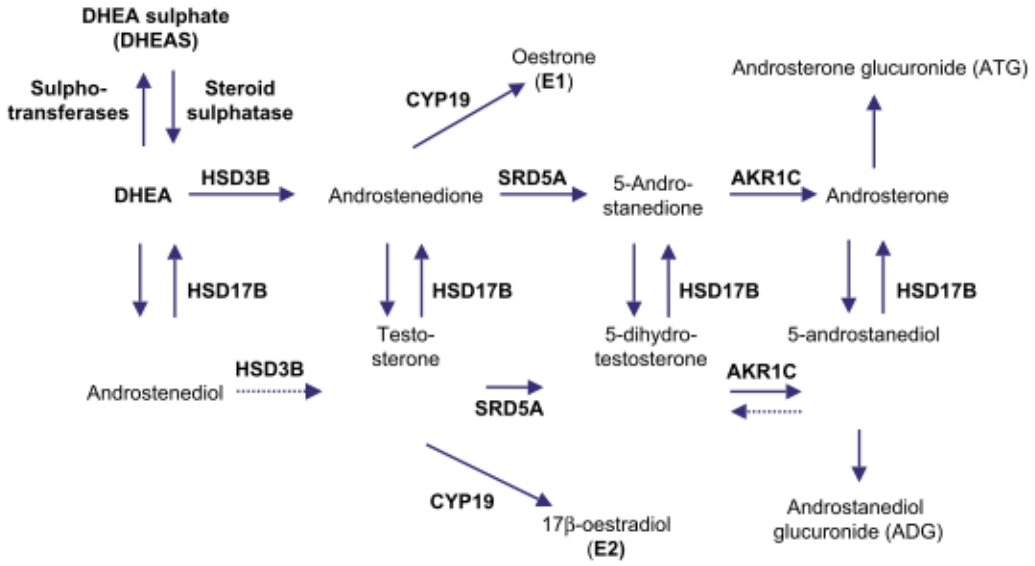


Figure 1-8 Conversion of DHEA to inactive DHEAS and sex steroids by various enzymes (gene names) HSD3B: 3β-hydroxysteroid dehydrogenase isozymes. HSD17B: 17β-hydroxysteroid dehydrogenase isozymes. SRD5A: 5α-reductase isozymes. AKR1C: 3α-hydroxysteroid dehydrogenase isozymes. CYP19: P450 aromatase. Figure taken from [38]

Furthermore, in some tissues like peripheral blood mononuclear cells (PBMC) other metabolites of DHEA can potentially exert immune modulatory actions [45-46]. This comprises conversion to androstenetriol via androstenediol [46] and conversion to 7α-hydroxy-DHEA by CYP7B1 in human brain, skin and liver [47]. Muller et al found that 7α-hydroxy-DHEA is converted to 7β-hydroxy-DHEA with 7-oxo-DHEA as an intermediate (Figure 1-9) by 11β-hydroxysteroid dehydrogenase 1 (HSD11B1), which is also the essential enzyme for reduction of cortisone to the active cortisol in glucocorticoid target cells. This interference with HSD11B1 may explain the anti-glucocorticoid effects of these DHEA metabolites [48] (also see chapter 1.2.2.3). In addition, isoforms 4 and 5 of CYP3A in adult and isoform 7 in foetal/neonatal liver microsomes convert DHEA to 16α-hydroxy-DHEA, which is less active and easier to eliminate [49-50].

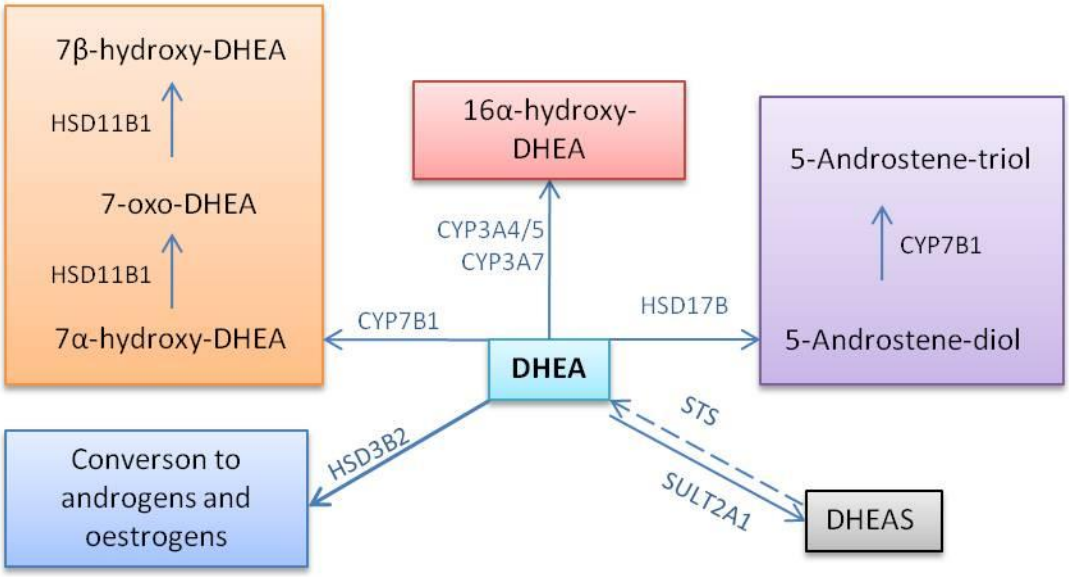


Figure 1-9 Schematic representation of the possible downstream conversion pathways of DHEA
DHEA serves as a major precursor for the generation of downstream metabolites of potentially distinct immune modulatory properties as well as for sex steroid synthesis. [38, 45]

1.2.1.2 Direct effects of DHEA

Apart from its intracrine effects, DHEA has been thought to have other direct physiological functions [51-52]. This includes the ability to alter cell membrane properties [53], to interact with ion channels [54] and to modify intracellular enzyme activity [55]. This is thought to be mediated by intracellular [56-57] or G-protein coupled plasma membrane receptors [58-62].

Although evidence of receptor-dependent mechanisms is accumulating, identification of a genetic sequence of such a receptor has not been successful yet. These studies found high-affinity binding sites of DHEA in several cell types of the vascular and the immune system. In murine T-lymphocytes, the affinities of DHT and cortisol to these binding sites were approximately 10- to 100-fold lower than DHEA [56] and in human T-lymphocytes the affinities of DHT, DHEA and dexamethasone were approximately 3- to 10-fold lower [57], respectively. Liu and Dillon found that in bovine aortic endothelial cells (BAEC) DHEA stimulates endothelial nitric oxide synthase (eNOS) via a G-protein coupled receptor and the MAPK/$Erk_{1/2}$ signalling pathway. DHEA bound with high affinity (K_d = 48.8 pM) at physiological concentrations of DHEA (0 - 10 nM) in a saturable manner and was not inhibited by other sex steroids (DHEAS, E2,

testosterone, 17α-hydroxy pregnenolone and androstenedione). In addition, inhibitors of oestrogen, androgen, progesterone and glucocorticoid receptors failed to inhibit the effect of DHEA on eNOS. [60, 63-64]

DHEA has been extracted from the brain of several species including humans, and it is therefore also called a neurosteroid [65]. In the brain of rodents, DHEA has been found independent of its peripheral origin [66], and expression of steroidogenic enzymes including *Cyp17a1* in the cerebral cortex of neonatal rats suggests its *de novo* synthesis [67]. Additionally, one report showed mRNA expression of steroidogenic enzymes in adult rat brain; however, distribution varied between brain regions [68]. In contrast, MacKenzie et al were unable to detect mRNA expression of *CYP17A1* in human adult tissue samples of the hippocampus and cerebellum in a more recent study, ruling out *de novo* synthesis in these tissues [69]. Further research is required, to investigate other CYP17-independent pathways as suggested by Cascio et al [70].

Extensive research has elucidated the mechanisms by which DHEA and DHEAS act in the brain. Apart from the function as a hormone precursor, DHEA showed to modulate actions of the $GABA_A$ (γ-amino butyric acid type A), NMDA (N-methyl-D-aspartate) and σ_1 (sigma subtype 1) receptors [71]. GABA is the major inhibitory neurotransmitter of the central nervous system (CNS) of vertebrates, used by as many as 40% of all neurons [72]. Both, DHEA and DHEAS, act as non-competitive, allosteric antagonists at the $GABA_A$ receptor [72]. Activation of the GABA receptor complex opens a chloride ion channel resulting in repolarisation of the plasma membrane and in inhibition of further neuronal firing [73]. Therefore, DHEA and DHEAS acts in an excitatory fashion in the CNS.

NMDA receptors are a subfamily of the excitatory L-glutamate family of neurotransmitter receptors [74]. It regulates a non-selective cation channel with a high permeability for calcium at the postsynaptic membrane [74-75]. DHEA and DHEAS stimulation potentiated NMDA-evoked catecholamine release [76] and firing rate [77] in rat hippocampal neurons. On the other hand, DHEA and DHEAS reduce NMDA-induced cytotoxicity [71]. Kurata et al suggested that DHEA inhibits NMDA-induced nitric oxide production and that DHEAS exerts its neuroprotective effect at least partly via σ_1-receptor in primary culture of rat hippocampal neurons [78]. The σ_1-receptor has

recently been identified as a unique ligand-regulated molecular chaperone in the endoplasmic reticulum inhibiting voltage-gated ion channels and potentiating ligand-gated ion channels [79-80]. Furthermore, DHEAS modulates the release of a number of neurotransmitters like glutamate [81], norepinephrine [76] and acetylcholine [71, 82-83].

1.2.2 Biological role of DHEA

With the discovery of DHEA, its age dependent decline was linked to the increasing incidence of disease with age. This facilitated speculation that DHEA might be a remedy for a variety of medical conditions. The negative correlation with age also triggered its advertisement as an anti-aging drug in the USA, where it is available without prescription as a food supplement [11]. However, it also led to a growing number of epidemiological and clinical studies to investigate the role of DHEA in human health and illness. The following chapters will summarise the available data about the biological role of DHEA. They demonstrate its involvement in many diseases, yet conflicting data indicate that further research is needed to really understand the physiology and pathophysiology of DHEA.

1.2.2.1 DHEA and ageing

Recently, Enomoto et al concluded from their 27-year follow-up study in a community-based cohort of 940 men and women aged 21 to 88 that high serum DHEAS levels were a predictor of longevity in men, independent of age, blood pressure and plasma glucose [84]. However, the results of most studies investigating the effects of DHEA replacement in the elderly were rather disappointing. By administering an oral dose of 50 mg/die DHEA to healthy elderly subjects over a certain period, levels of DHEA and DHEAS were generally raised to those of young subjects.

Several studies investigated effects on quality of life, sexual function, cardiovascular system, bone, skin, physical exercise tolerance, body composition and insulin sensitivity. The DHEAge study (280 healthy men and women, 60-79 years old) found a positive effect on bone turnover only in women, on skin (sebum production, skin hydration, skin pigmentation, epidermal thickness), but no effect on vascular properties in men older than 70 years. Sexual interest, activity and satisfaction improved in this study population [85].

Furthermore, in another randomized double-blind and placebo controlled trial involving 87 elderly men and 57 elderly women with low levels of DHEAS, Nair et al found no significant effect of DHEA (men and women) or low-dose testosterone (men) replacement on body composition, physical performance, insulin sensitivity or quality of life [86]. Data for effects on bone mineral density after one year of DHEA administration in the Dehydroepiandrosterone And WellNess (DAWN) study showed no benefits in men and only a positive effect on lumbar spine BMD, but not on hip, femoral neck and total body BMD in women [87]. Cognitive function did not differ after 12 months treatment, but depression scores decreased and Satisfaction with Life Scale scores increased for women. However, there were no differences between the DHEA and placebo groups over time on these measures or the SF-36, Life Satisfaction Index-Z scale, or sexual function scales [88].

1.2.2.2 DHEA and metabolism

In vitro and *in vivo* studies in rodents suggest that DHEA is able to modulate metabolic parameters and body composition directly, independently of downstream conversion to active sex steroids.

DHEA administration in young rodents blocks or retards fat gain and reduces weight gain [89-90]. Other studies showed a reduction of weight, percentage body fat, adipocyte number and serum triglyceride levels in adult mice [91-92] without affecting food or water intake [92-93].

In vitro studies on murine pre-adipocytes found direct effects of DHEA on proliferation, differentiation and lipid accumulation [94-96]. Furthermore, DHEA increased insulin stimulated glucose uptake in murine adipocytes *in vitro* [97].

In obese and insulin resistant C57BL/KsJ mice, DHEA administration did not prevent obesity, but it reduced hyperglycaemia and hyperinsulinaemia [98]. In skeletal muscle, DHEA and testosterone/DHT, which muscle cells are able to synthesize from DHEA, increased expression of GLUT-4 and accelerated its translocation to the plasma membrane. Furthermore, the activities of phosphofructokinase and hexokinase, the main glycolytic enzymes, were enhanced by testosterone and DHEA, respectively [99]. In other experiments by Yamashita, DHEA significantly reduced expression and activity of the gluconeogenic enzyme glucose-6-phosphatase (G6Pase) in the human hepatic cell

line HepG2 [100]. Similarly, Aoki found that DHEA administration suppressed elevated levels of G6Pase in the diabetic C57BL/KsJ mice and significantly decreased blood glucose levels [101]. This strongly suggests that DHEA might modulate hepatic gluconeogenesis.

In a cross-sectional cohort analysis, DHEAS was negatively correlated to insulin resistance in patients with PCOS. However, it is not clear, whether this is a direct effect of adrenal androgens like DHEA or simply reflects the high circulating levels of insulin [102].

Again, results of clinical intervention studies are not consistent. DHEA treatment for 6 months in elderly men and women reduced visceral and abdominal subcutaneous fat and increased insulin sensitivity [103].

Other studies showed variable changes in men [104-105] or no changes at all in body composition in healthy men with an age-related decline of DHEA secretion [106]. Furthermore, a study in 112 elderly subjects with relative DHEA deficiency, DHEA replacement over two years did not improve insulin secretion, or the pattern of postprandial glucose metabolism [107].

1.2.2.3 DHEA and the immune system

Studies in neutrophils suggest that as well as enhancing immune function directly, DHEA and DHEAS influence immunity indirectly by counteracting the immunosuppressive effects of glucocorticoids [108]. The mechanisms behind this are unclear, but it is suggested that AR and glucocorticoid receptor (GR) recognise the same hormone response element and therefore compete for the same regulated target genes. Furthermore, DHEA-derived oestrogen significantly reduced the transcription of GR in neuronal cells [109]. Moreover, DHEAS inhibited the inflammatory transcription factor NF-κb reducing inflammation in endothelial cells [110]. Interestingly, downstream metabolites like 7α-hydroxy-DHEA compete with cortisone as a substrate for HSD11B1, which is necessary to activate cortisone to cortisol [111].

Whilst levels of DHEA and DHEAS decline with age, levels of IL-6 increase [112]. DHEA treatment of mononuclear cells *in vitro* reduces IL-6 production, which is important for leukocyte differentiation and activation and for regulating the

immunological switch from innate to acquired immune system [113-114]. Interestingly, the anti-inflammatory effects of glucocorticoids are also mediated via inhibition of IL-6 production [115].

DHEAS but not DHEA increased superoxide generation in neutrophils that were stimulated by bacterial formylated peptide formyl methionyl leucyl phenylalanine [116]. Importantly, steroid receptor antagonists did not reverse this effect, ruling out mediation by downstream metabolites of DHEA. Moreover, Radford et al showed an activation of the NADPH oxidase complex mediated by activation of the proteinkinase C (PKC) pathway. It is surprising that DHEAS but not DHEA activated rat brain PKC directly in the physiological range of 1-10 μM and it was shown that activation was restricted to (recombinant human) PKC-βI and PKC-βII in a dose dependent manner [116]. This provides evidence that PKC-β acts as an intracellular receptor for DHEAS in neutrophils and therefore supports the assumptions that DHEA and DHEAS have important roles in addition to their function as sex steroid precursor.

DHEA showed protective effects in lethal bacterial and viral infections in rodents and various cell models [117-120]. However, DHEA was much less potent than androstenediol and androstenetriol [46]. Interestingly, DHEAS was found significantly reduced in sepsis patients whilst DHEA was increased compared to healthy controls [121], likely because SULT2A1 is down regulated as shown in experiments with rodents [122]. This mechanism might balance the increased cortisol release that is seen in sepsis. Generally, an increased cortisol-DHEA ratio was found to be associated with increased mortality in severe illness [121].

It is remarkable that the incidence of autoimmune diseases is higher in females than in males. In both sexes, glucocorticoids, DHEA and androgens are inadequately low in patients when compared to healthy controls [123-124]. As hormonally active androgens are anti-inflammatory, whereas oestrogens are pro-inflammatory, the local metabolism of androgens and oestrogens may determine suppression or promotion of inflammation [125].

In mild to moderate systemic lupus erythematosus (SLE), treatment with 200 mg DHEA per day resulted in significant reduction of serum levels of IL-10, which correlates with disease activity and is suggested to play an important role in the pathogenesis of SLE

[126]. This finding may explain why DHEA can stabilize or even improve disease activity [127], significantly reduce lupus flares [128] and have glucocorticoid sparing effects [129].

Impaired production of cytokines by T-helper type 1 lymphocytes (Th1), such as interleukin-2 (IL-2) or interferon-gamma (IFN-γ), and T-helper type 2 lymphocyte (Th2) dominated response to allergens are hallmarks of atopic diseases, including atopic dermatitis [130]. DHEA increases Th1 cytokines including IL-2 and IFN-γ [131] and decreased the production of Th2-associated cytokines, such as IL-4 and IL-5, in atopic eczema/dermatitis syndrome (AEDS) patients [132].

DHEA administration in mice during allergic sensitization lowered Th2 cytokine production and significantly suppressed eosinophilic inflammation and IgE antibody production after provocation, suggesting an immune modulating effect of DHEA upon allergic response [133]. Moreover, it suppressed progression of allergic airway inflammation in a Dermatophagoides farinae-induced asthma model [134]. However, a small study with only 13 female patients with severe AEDS found no difference in serum concentrations of DHEAS or testosterone compared to weight- and age-matched healthy controls. Furthermore, no correlation between serum concentrations of DHEAS and total IgE was found [135].

1.2.2.4 DHEA and proliferation and cancer

Several studies associated low levels of DHEA and DHEAS with carcinogenesis. Animal and cell culture studies showed an anti-proliferative effect of DHEA, possibly mediated by a G1/S phase cell cycle arrest [136]. DHEA and DHEAS inhibited proliferation of myeloma [137], cultured T lymphocytes [138] and human vascular endothelial cells [139]. In experiments on bovine chromaffin cells, DHEA decreased the proliferative effect induced by growth factors. Surprisingly, DHEAS enhanced proliferation in those cells from adult but not from young animals [140].

In the oestrogen-dependent breast cancer cell line MCF-7, DHEA in physiological concentrations showed proliferative effects, which is not surprising. However, supraphysiological concentrations were antiproliferative exerting this effect independently of AR and ER [141].

1.2.2.5 DHEA and the nervous system

Results from studies in rodents demonstrated that DHEA and DHEAS administration can enhance memory in several test paradigms. It showed antiamnestic as well as memory enhancing properties in a foot shock avoidance paradigm [142], in a working memory task [143] and in the morris watermaze [144].

In a recent community-based, cross-sectional study of 295 women, aged 21-77 years, higher DHEAS levels were independently associated with executive function, concentration and working memory [145]. The data of the InCHIANTI study with 1034 elderly residents aged ≥65 years showed a significant and positive association between DHEAS and cognitive function, assessed by MMSE test. Low DHEAS levels predicted an accelerated decline in MMSE score during the 3-years follow-up period [146].

Interestingly, also depressive symptomatology has been linked to reduced androgen levels. In 18 male and 43 female patients with dysthymic disorder, Markianos et al found significant lower DHEAS levels than in healthy sex- and age-matched controls [147].

Furthermore, alterations in DHEA, DHEAS and androstenedione in schizophrenia patients were associated with emotional distress, anxiety and dysphoric mood. DHEA and androstenedione levels were increased, but levels of DHEAS decreased [148]. In addition, cognitive impairment, a core phenomenon of schizophrenia, was associated with low levels of DHEAS and a reduced DHEAS/cortisol ratio [149]. In a double blind placebo controlled randomized study with 30 patients exhibiting prominent negative schizophrenia symptoms, treatment with 100 mg/die DHEA in addition to regular antipsychotic medication significantly improved negative symptoms, as well as depressive and anxiety symptoms [150].

Adrenal insufficiency is a valuable model to investigate the effects of DHEA and DHEAS. Whereas the glucocorticoid hydrocortisone and if required the mineralocorticoid aldosterone are substituted routinely, low serum levels of DHEAS are usually not treated. However, there are a few trials where DHEA was given in adrenal insufficiency, to elucidate its role in general and sexual well-being as well as metabolic parameters.

Arlt et al showed that DHEA treatment in women with adrenal insufficiency improves well-being and sexuality. In this double-blind study, 24 women with adrenal insufficiency received 50 mg DHEA or placebo orally each morning for four months interrupted by a one-month washout period. This raised the initially low serum concentrations of DHEA, DHEAS, androstenedione, and testosterone into the normal range. DHEA significantly improved overall well-being as well as scores for depression and anxiety, measured by validated questionnaires. DHEA significantly increased the frequency of sexual thoughts, sexual interest and satisfaction with both mental and physical aspects of sexuality [151].

However, in a double-blind randomized placebo-controlled trial with 106 subjects (44 males and 62 females), long-term DHEA replacement of 50 mg/die for 12 months in primary adrenal insufficiency improved significantly only in one subset (role emotional in Short Form-36) of two questionnaires (SF-36, General Health Questionnaire-30) [152].

Another very recent double blind, randomised, placebo controlled, crossover study investigated the effects of DHEA substitution (50 mg/die for 6 months) on body composition, fuel metabolism, and inflammatory markers in 10 female patients with adrenal failure. Bone markers, bone mineral content, markers of tissue inflammation (adiponectin, IL-6, IL-10, MCP-1, and TNF-α) in fat and muscle tissue were unaffected, as was indirect calorimetry and maximal oxygen uptake. However, lean body mass (LBM) slightly increased compared to placebo with no alterations in total or abdominal fat mass. [153].

1.2.2.6 DHEA and the cardiovascular system

Several prospective studies have examined the relationship between DHEAS levels and cardiovascular disease (CVD), but results have been conflicting. Low DHEAS levels in men were connected to increased risk of cardiovascular events [154]. Although higher DHEAS levels correlate with several major CVD risk factors [155], they were unrelated to the risk of fatal CVD in postmenopausal women in the Rancho Bernardo Study [156]. In contrast, another study found low DHEAS levels to predict ischemic heart disease (IHD) in women [157]. On the other side, data from the Helsinki Heart Study associated higher DHEAS levels with cardiac events [158] and the Honolulu Heart Program found

no significant relation for DHEAS with fatal and non-fatal myocardial infarction [159]. It is also noteworthy that populations, which have the lowest coronary heart disease rates and greatest longevity such as the Japanese, reportedly have low mean DHEAS levels [160].

In several studies on animal models and in tissue culture DHEA and DHEAS influenced the vascular system and atherosclerosis. DHEA decreased pulmonary artery hypertension in hypertensive rats and prevented cardiac right ventricle hypertrophy [161]. DHEA also reduced vascular remodeling after injury in human carotid muscle cells and saphenous vein graft via a G-protein coupled membrane receptor [162] and DHEAS treatment reduced neointima formation after injury in a rabbit balloon carotid injury model [163]. However, in another study DHEA increased macrophage foam cell formation in human umbilical vein cells via AR, a potentially pro-atherogenic effect [164].

Short-term DHEA supplementation for only 12 weeks in a randomized cross-over trial, did not significantly affect measures on arterial stiffness or endothelial function in patients with Addison's disease and panhypopituitarism [165].

1.2.2.7 DHEA and the bone

Sex steroids play an important role in maintenance of skeletal health and some studies suggest a beneficial effect of DHEA. *In vitro* and mouse studies showed, that DHEA and DHEAS stimulate cell proliferation in human osteoblastic cells via the AR, however independently of conversion by HSD3B2 and SRD5a [166-167]. In a randomized double-blinded, placebo controlled trial, Jankowski et al treated elderly men and women with low serum DHEAS levels with 50 mg/d DHEA and found improved bone mineral density, which seemed to be mediated by serum oestrogens [168-169].

1.2.3 DHEA-DHEAS-Shuttle: Interconversion of DHEA and DHEAS

1.2.3.1 Transport of DHEAS across the cell membrane

Whilst the lipophilic DHEA is thought to cross the plasma membrane by passive diffusion, its hydrophilic sulphate ester DHEAS requires transport proteins (Figure

1-10). The proteins mediating inward transport of DHEAS belong to the organic anion transport protein family (OATP or soluble carrier family SLCO). OATP-A (SLCO1A2) [170], OATP-B (SLCO2B1) [171], OATP-C (SLCO1B1) [172], OATP-8 (SLCO1B3) [173], OATP-D (SLCO3A1), OATP-E (SLCO4A1) [174], and OATP-F (SLCO1C1) [175] are the main transporters of DHEAS into the cell.

The multidrug resistance-associated protein 4 (MRP4), also known as ATP-binding cassette transporter family class C4 (ABCC4), is a member of a large family of trans-membrane proteins involved in active transport of substances out of cells [176-177]. It is widely distributed and highest expression has been found in kidney, lung, prostate, liver, tonsils, bladder [178]. MRP4 is the member of the MRP family that is most relevant for transport of DHEAS [179]. Two isoforms of MRP4 have been found to exist, but the differential function or expression has not been investigated yet. Isoform 1 represents the longer variant (NM_005845.3, GenBank) [180].

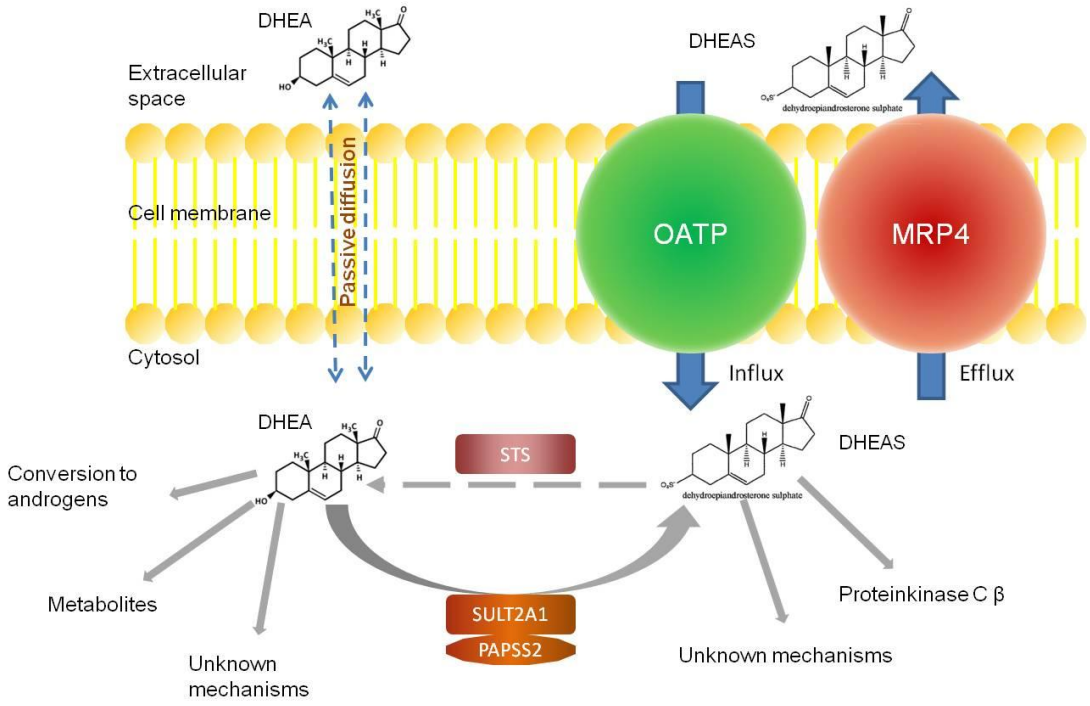


Figure 1-10 Passive diffusion of DHEA and active transport of DHEAS across the cell membrane
Lipophilic DHEA crosses the plasma membrane by passive diffusion. By contrast, the hydrophilic DHEAS needs active transport by OATPs for influx and MRP4 for efflux. Furthermore, an overview of intracellular effects of DHEA and DHEAS is presented.

1.2.3.2 Steroid sulphatase

Steroid sulphatase (STS) is a member of a superfamily of 12 different sulphatases [181]. It is a membrane-bound enzyme, mainly found in the rough endoplasmic reticulum and the Golgi apparatus [182]. It is an almost ubiquitous enzyme [183], but placenta was found to be the richest source of STS [184].

The concept of free interconversion of DHEAS and DHEA was recently challenged by a study of Hammer et al, which confirmed increase in DHEAS after oral administration of DHEA but did not find increased DHEA after administration of DHEAS. This was confirmed in a cell model with HepG2 cells that expressed similar amounts of STS and DHEA sulphotransferase. Therefore, conversion to DHEAS generally limits availability of DHEA for further downstream conversion [36]. However, in some tissues, e.g. breast and prostate, conversion of DHEAS to DHEA is possible. Sulphatase activity in placenta tissue mobilises DHEA and so enables sufficient synthesis of oestrone and oestradiol, making the placenta the main source of maternal oestrogens [185].

1.2.3.3 DHEA sulphotransferase

Cytosolic sulphotransferases (SULTs) are phase II detoxification enzymes that are involved in a variety of biotransformations of structurally diverse endo- and xenobiotics, including many therapeutic agents and endogenous steroids. There are also membrane-bound SULTs which are genetically distinct form cytosolic SULTs and do not have xenobiotic-metabolizing function. SULTs belong to a large superfamily of genes that are divided into subfamilies. The phenol SULTs (SULT1) and the hydroxysteroid SULTs (SULT2) represent the largest and most widely examined families.

Their tissue distribution is widespread, with expression in liver, lung, brain, skin, platelets, breast, kidney and gastrointestinal tissue. SULTs catalyse the transfer of the sulphonyl group from 3’-phosphoadenosine 5’-phosphosulphate (PAPS) to hydroxyl, sulfhydryl, amino or N-oxide groups of various agents. The correct term is sulphonation, although the term sulphation is commonly used [186]. Of the 13 different SULTs, SULT1A1 and SULT2A1 are the predominant members [187-188].

SULT2A1 is the major SULT isoform involved in the formation of steroid sulphate esters and is capable of sulphonating both 3α- and 3β- hydroxysteroids with highest efficiency whilst activity towards estrogens, testosterone is lower. In addition, with its broad substrate specificity human SULT2A1 can sulphonate bile acids [189].

In contrast, SULT2B1 sulphonates pregnenolone but not androsterone, bile acids, testosterone, estrogens or cortisol and neither SULT2B1 subtype is able to sulphonate DHEA efficiently [186].

1.2.3.4 PAPS synthase 1 and 2

3'-phosphoadenosine 5'-phosphosulphate (PAPS) is the universal sulphate donor for all sulphotransferase reactions in mammals [190]. It is synthesised from ATP and SO_4^{2-} in two steps by a single bi-functional cytosolic enzyme, PAPS synthase (PAPSS) [191]. The first step is catalysed by ATP sulphurylase and involves the reaction of inorganic sulphate with ATP to form adenosine 5'-phosphosulphate (APS) and inorganic pyrophosphate (PP_i) which results in formation of a high-energy phosphoric-sulphuric acid anhydride bond that is the chemical basis for sulphate activation. The second step is catalysed by APS kinase and involves the reaction of APS with ATP to form PAPS and ADP. [190] (Figure 1-11)

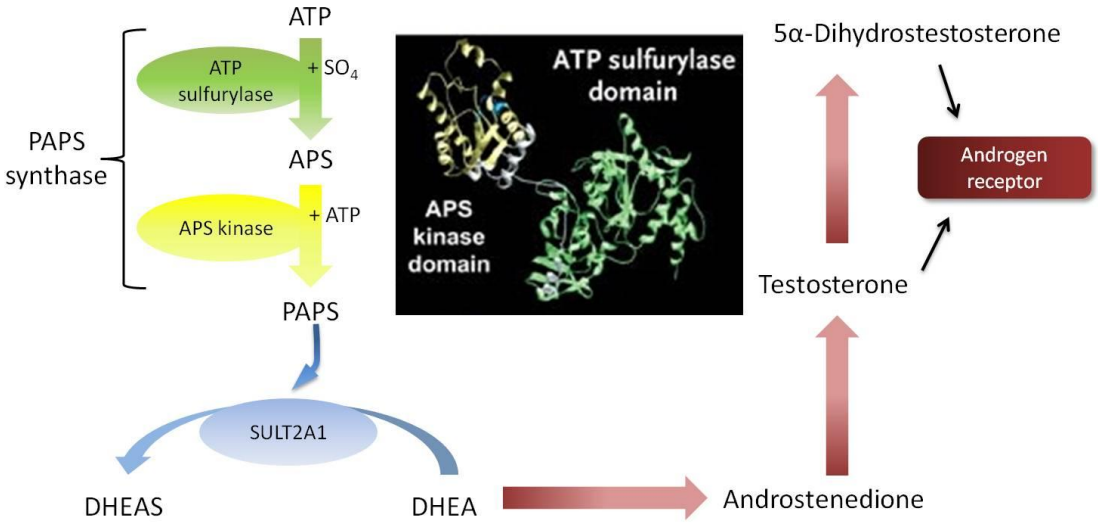


Figure 1-11 Ultrastructural model of PAPS synthase and schematic illustration of domain functions
PAPSS1 and PAPSS2 have two separate catalytic sites. The activity of the ATP sulfurylase site adds SO_4 to ATP. The resulting adenosyl phosphosulfate (APS) is further phosphorylated to PAPS, which is ubiquitously used as a co-factor for sulphonation such as inactivation of DHEA to DHEAS. This prevents downstream conversion of DHEA to sex steroids that activate the androgen receptor. Adapted from [192]

Two isoforms of PAPSS have been identified in humans and mice, PAPSS1 and PAPSS2. Both consist of 12 exons and are 76.5% identical in amino acid sequence [193]. Whilst PAPSS1 is expressed ubiquitously, PAPSS2 is found in CNS, bone marrow, liver and steroidogenic tissue [186].

Interestingly, two splice variants are found for PAPSS2, 2a and 2b, distinguished by the presence or absence of a five-amino-acid segment (G-M-A-L-P) in the ATP sulphurylase domain of the protein. The catalytic activity of the human PAPS synthase 2a splice variant is modestly but significantly lower (~30%) than that of the 2b variant. Furthermore, the catalytic activity of the PAPSS2 variants is 10 to 15-fold higher than that for PAPSS1 [194]. The physiological significance of the two genes encoding for related proteins that carry out identical function is presently unclear [186]. Although considered cytosolic enzymes, Besset et al reported a nuclear localisation of PAPSS1 as well as of PAPSS2 when co-expressed with PAPSS1 [195].

Furthermore, PAPSS2 is significantly involved in chondrogenesis [196]. Rare, inactivating mutations of PAPSS2 are associated with congenital skeletal disorders in both, humans [197] and mice [198-199], despite the presence of PAPSS1. However, information about the expression profile of PAPSS in cartilage is limited [200]. In human adult cartilage, PAPSS1 seems to be the predominant form. Similarly, in mature guinea pigs, PAPSS1 is predominant, but in immature animals, mainly PAPSS2 is expressed [194].

1.3 Aims of this thesis

DHEA plays an important role in endocrine physiology and pathophysiology. Rodent models are only to a certain extent suitable for investigating the regulation of DHEA because rodents' adrenal physiology differs considerably and ethical issues evolve using primates as animal models.

With this thesis, I contributed to different projects. My first goal was to find a human cell line that would be a suitable model for investigations regarding the regulation of DHEA sulphonation. It was important to find a cell line with high expression of SULT2A1 and similar expression of PAPSS 1 and 2. I investigated the expression of

these enzymes in several hepatic and adrenal cell lines because these tissues are the major sites of SULT2A1 activity. Furthermore, my aim was to show the activity of these enzymes and investigate their subcellular localisation in a human cell line model.

In addition, I had the opportunity to look further into the pathophysiology of PAPSS2 malfunction. We investigated several mutations and their effect on PAPSS2 function in a patient with androgen excess and skeletal malformation. For this project, I examined the differential mRNA expression of *SULT2A1*, *PAPSS1* and the two variants of *PAPSS2* in human foetal chondrocytes, adrenal and liver.

2 Material and Methods

2.1 Cell culture

The following chapter will list which cell lines were investigated and give details about culture environment needed to propagate the cells.

2.1.1 **HepG2**

The cell line HepG2 is derived from a hepatocellular carcinoma and therefore is frequently used for studies in metabolism and liver pathophysiology. Cells were propagated in T75 tissue culture flasks at 37°C in a humidified atmosphere consisting of 95% oxygen and 5% carbon dioxide. Full growth medium was DMEM/HAM-F12 (Gibco, UK) with 2 mM L-Glutamine (Gibco) and 10% foetal calf serum.

2.1.2 **WRL68**

WRL68 has been considered as a cell line derived from human embryonic liver tissue for a long time and has been used as a hepatocyte model. However, the European Collection of Cell Cultures (EACC) identified this cell line as a HeLa contaminant and therefore as a human cervix carcinoma cell line [201].
Cells were grown in DMEM/HAM-F12 (Gibco) with 2 mM L-Glutamine (Gibco) and 10% foetal calf serum in T75 tissue culture flasks at 37 °C in a humidified atmosphere consisting of 95% oxygen and 5% carbon dioxide.

2.1.3 **NCI-h295**

NCI-h295 cells were received from Prof. B Allolio (Medizinische Klinik I, Universität Würzburg, Germany). This cell line was derived from a relatively differentiated adrenocortical carcinoma and is known to secrete adrenal hormones [202]. Cells were propagated in T175 tissue culture flasks as suspension cells. Full growth medium was RPMI 1640 (Gibco, UK) plus 2 mM L-glutamine and 10% foetal calf serum (Gibco, UK) and supplemented with insulin, transferrin and selenite (Sigma-Aldrich, UK). For an undetermined reason, this cell line could not be propagated as needed. Cell growth was very slow with doubling times of more than 14 days. A second attempt with another batch of cells failed to improve the doubling time to an acceptable level. Expression analysis of both batches were carried out, however further experiments were not done.

2.1.4 **NCI-h295R**

The two different substrains 1 and 3 of the adrenal cell line NCI-h295R were received from W. E. Rainey (Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center, Dallas, USA). Both strains are derived from adrenal cortical cells [203] with substrain 1 representing properties of mineralocorticoid secreting cells of the ZG and substrain 3 representing properties of ZR cells.

NCI-h295R1 cells were grown in DMEM/HAM'S-F12 plus HEPES (Sigma-Aldrich, UK) supplemented with 2 mM L-glutamine (Gibco, UK) and 5% Nu-Serum I (BD Biosciences, USA).

NCI-h295R3 cells were cultivated in DMEM/HAM'S-F12 plus HEPES supplemented with 2 mM L-glutamine, 10% HyClone cosmic calf serum (Thermo Scientific, USA) and antibiotics (Penicillin, Streptomycin and Gentamicin). Growth medium was renewed every other day and cells were split when reaching a confluence of about 70%.

In addition, some experiments were carried out using a strain of HCI-h295R that had been transfected with a vector for further experiments. This strain is named h295R(T) in this thesis.

2.1.5 **SW13**

A batch of the adrenal cell line SW13 was received from Prof. B. Allolio (Medizinische Klinik I, Universität Würzburg, Germany). This cell line originates from a relatively undifferentiated adrenocortical tumour and produces only small amounts of adrenal hormones. Cells were grown in Leibovitz-L15 (Gibco, UK) medium supplemented with 2 mM L-glutamine and 10% foetal calf serum (FCS) and were kept at 37 °C in absence of carbon dioxide. Growth medium was changed every other day and cells were split when reaching a confluence of about 70%.

2.1.6 **Cos-7**

Cos-7 is a monkey kidney cell line, which is commonly used for transfections. Full growth medium was DMEM/HAM-F12 supplemented with 2 mM L-Glutamine and 10% foetal calf serum (Gibco, UK).

2.2 RNA methods

2.2.1 **Principles of RNA methods**

As a first step, expression of enzymes can be investigated at mRNA (messenger RNA) level. mRNA is complementary to the gene which encodes the correspondent protein. The process of transferring DNA into mRNA is called transcription. Importantly, a few modifications during transcription have to be considered. The nucleotide thymidine is exchanged by uracil and instead of desoxyribose one finds ribose. Furthermore, some parts of the genetic sequence are cut out, splitting a gene into coding (exons) and non-coding (introns) regions. Thirdly, during transcription mRNA is tagged with a poly-A-tail at the 3'-end of the strand (tailing), which is important for terminating transcription, and a cap-structure at the 5'-end of the strand (capping) which is essential for the transport of the mRNA from the nucleus to the cytosolic ribosomes where translation to proteins happens.

In biochemical experiments, it is often preferred to work with DNA because it is more stable than RNA and easier to handle. In 1970, Temin and Baltimore independently discovered RNA-dependent DNA polymerase in certain viruses [204-205]. This enzyme

is applied to transform single stranded mRNA into double stranded DNA, which is then called cDNA (complementary DNA). This method is called reverse transcription (Figure 2-1). Importantly, cDNA contains the information of the mRNA strand and therefore only the coding regions of the genetic DNA.

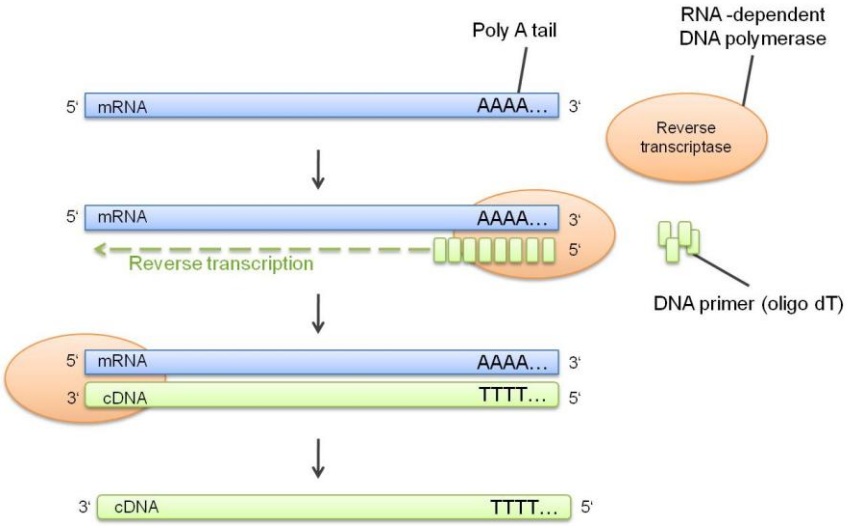


Figure 2-1 Schematic illustration of Reverse Transription

mRNA is transcribed to cDNA by RNA-dependant DNA polymerase (reverse transcriptase). Reaction starts after a DNA primer consisting of several Thymidin nucleotides recognises the universal poly-A tail of mRNA. The resulting complementary DNA represents the DNA code of a protein without introns that are found in the genetic DNA code.

However, mRNA has to be extracted from a mix of all kind of cell components. The here used method was originally developed by Piotr Chomczynski and Nicoletta Sacchi in the late 1980ies [206]. In principle, cell structures are broken up mechanically and chemically by a commercially available reagent (TRI reagent, Sigma-Aldrich UK), which is based on the acid guanidinium thiocyanate-phenol-chloroform mixture, and the so generated phases (aqueous phase, organic phase and interphase) can be separated and cleaned with chloroform. Purified total RNA is then recovered by precipitation with isopropanol [207]. The advantage of this method over e.g. columns is that it provides a better yield of RNA.

2.2.2 **RNA extraction from cell lines**

Cells were seeded equally in 12 well plates (Greiner Bio-one, UK) and grown until 70% confluent. Media was discarded and cells of two wells were completely dissolved in 1

mL TRI reagent (Sigma-Aldrich, UK). The mixture from those two wells was transferred into RNase-free Eppendorf tubes and RNA was extracted according to manufacturer's protocol using a two-step extraction method with chloroform and isopropanol.

After letting the nucleoprotein complexes permit to dissociate for 5 minutes at room temperature, RNA was separated from protein and DNA by adding 200 µL cold chloroform. Tubes were inverted 10 times and after incubation at room temperature for 15 minutes centrifuged for 15 minutes at 4°C at 12,000g in a chilled table-top centrifuge, model 5415R (Cambridge, UK). This centrifugation step separates three layers of which the top phase is RNA, the bottom phase protein and the intermediate phase DNA. 400-600 µL of the RNA phase were carefully transferred into a new tube.

To precipitate the RNA 500 µL n-Isopropanol were added and the tubes inverted 10 times. After incubation for 15 minutes at room temperature, precipitated RNA was centrifuged into a pellet for 15 minutes at 12,000g at 4°C and supernatant discarded. The remaining pellet was washed by vortexing with 1 mL RNase-free 70% ethanol and centrifuged another 5 minutes at 12,000g at 4°C. The supernatant was discarded and the remaining pellet of RNA was air dried for 10-15 minutes at room temperature. The pellet was dissolved in 30 µL RNase free water by vortexing briefly and heating at 55°C for 5 minutes.

Gel electrophoresis was carried out on a 1% agarose gel to test integrity of RNA and RNA concentration photometrically measured at 260/280nm wavelength using a Nanodrop spectrometer (ND-1000, Nanodrop, Wilmington, USA).

2.2.3 **RNA extraction from tissue**

Foetal tissue samples (cartilage, adrenal, liver) from abortions were obtained by Prof. N. Hanley (University of Southampton, UK) according to the ethical and informed-consent guidelines of the Polkinghorne Committee [208]. Tissue was mechanically homogenised in 1 mL TRI reagent (Sigma-Aldrich, UK) and RNA extracted as described in chapter 2.2.

2.2.4 **Reverse transcription with the Promega kit**

Promega Reverse Transcription System A3500 was used for reverse transcription of RNA obtained from cell culture. To generate cDNA, which is complementary to the

isolated mRNA, 1 µg RNA per sample was diluted to a final volume of 10 µL with RNase free water and incubated for 10 minutes at 70°C. A mix of 2 µL RT 10x Buffer, 4 µL 25 mM $MgCl_2$, 2 µL 10 nM dNTP, 1 µL Random Hexamer Primers, 0.5 µL Ribonuclease Inhibitor and 0.6 µL AMV Reverse Transcriptase were added to each sample and incubated at room temperature for another 10 minutes. Then, RNA was reverse transcribed for 60 minutes at 42°C and the reaction stopped by heating the sample to 95°C for 5 minutes. Samples were stored at -20°C until further usage. The success of the reverse transcription was checked by amplification of the ubiquitously existent 18S ribosomal RNA (rRNA) by PCR (see 2.3.1).

2.2.5 Reverse transcription with the Applied Biosystems kit

Reverse transcription (RT) of RNA from foetal tissue was carried out with a different kit from Applied Biosystems, UK (Cat no N808-0234), which promises a better yield of cDNA. For one reaction of 20 µL volume, 2 µL 10x RT buffer, 4.4 µL 25mM $MgCl_2$, 4.0 µL dNTPs (10 mM each), 1.0 µL random hexamer primers, 0.4 µL RNase inhibitor, and 1.2 µL Multiscribe Reverse transcriptase (50 U/µL) were mixed with 1.0 µg RNA in 7.0 µL RNase free water, keeping all ingredients on ice at all times. Reactions were carried out in a thermal PCR cycler (Applied Biosystems, GeneAmp PCR system 2700, California, USA) at 25°C for 10 minutes, 37^{o}C for 60 minutes, 48^{o}C for 30 minutes, 95^{o}C for 5 minutes. Success of RT reactions was evaluated by multiplying cDNA of the ubiquitously found 18S rRNA.

2.3 DNA methods

2.3.1 Principle of Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a highly specific and sensitive method to show the existence of DNA in a sample. The targeted DNA can be genomic DNA or cDNA, i.e. reverse transcribed mRNA. Under the right conditions, thermophilic DNA polymerase (taq polymerase, Bioline, UK) can amplify targeted DNA. The double stranded DNA has to be denatured to single strands so that gene specific primers can anneal to their complementary single DNA strand at a primer specific temperature. In a next step, the Polymerase elongates the primer according to the bound DNA. This

process is repeated for several cycles, which leads to exponential amplification of the targeted DNA. (Figure 2-2)

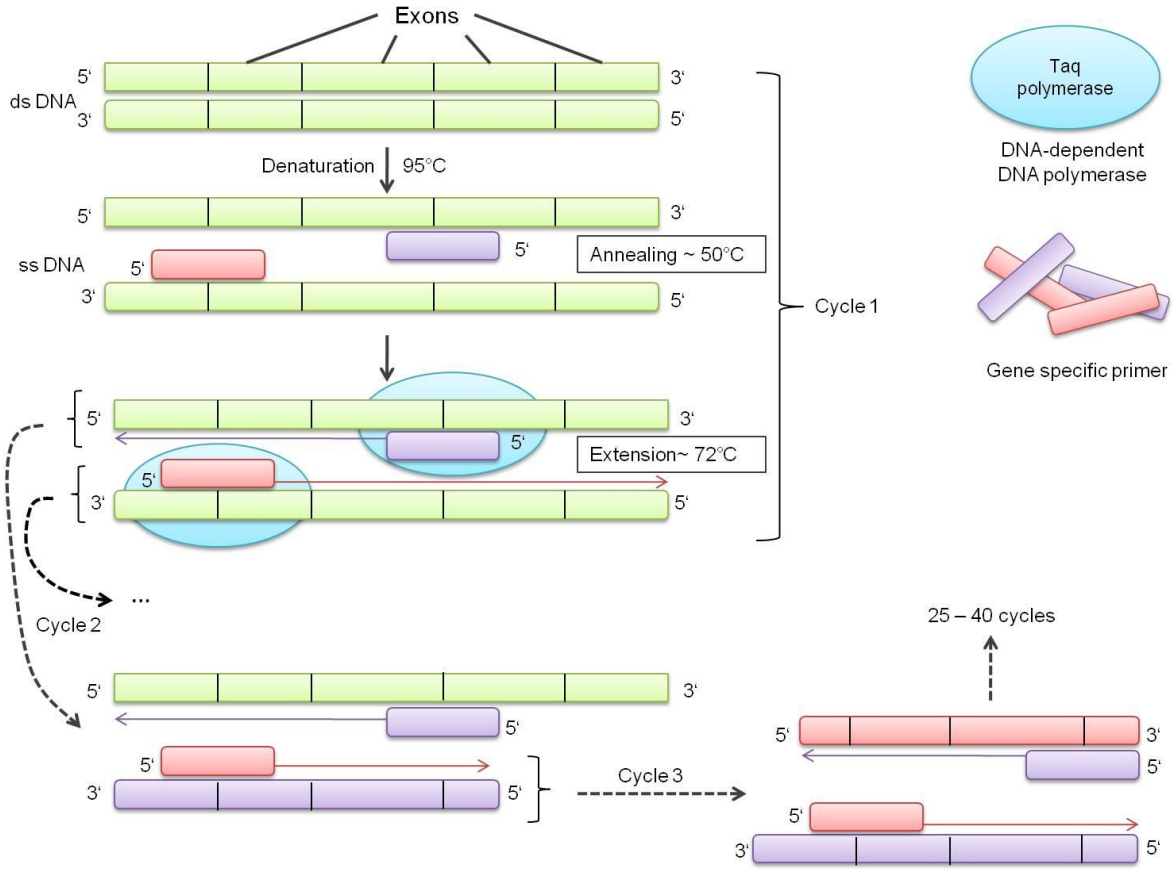


Figure 2-2 Schematic illustration of Polymerase Chain Reaction of cDNA (RT-PCR)

Double stranded cDNA denatures at high temperature (95°C). Gene-specific primers bind to the single stranded DNA at a specific annealing temperature. Primers are designed to span exon-exon boundaries to exclude amplification of genetic DNA in a sample. Afterwards, DNA-dependant DNA polymerase extends the primer from 5' to 3' complementary to the original strand. This is repeated 25-40 times resulting in a doubling of DNA strands in each cycle and therefore exponential amplification.

2.3.2 **Specific information on conventional PCR**

Table 2-1 gives an overview of all gene specific primers and conditions used for RT-PCRs. Primers were designed to bind towards the 3'-end of a gene, with similar melting temperatures and GC contents for forward and reverse primers. To reduce risk of amplification of genomic DNA primers were designed to span introns and include an exon border if possible. Primers were designed using http://frodo.wi.mit.edu/, checked with BLAST (http://ncbi.nlm.nih.gov/blast/) and purchased from Sigma-Aldrich, UK.

All conventional PCR reactions were performed using reagents purchased from Bioline, UK unless stated otherwise. For each PCR, a master mix was prepared consisting of 10xPCR Buffer, $MgCl_2$ (1-1.5 µM), 1 µL DMSO, dNTP (0.5 µM), forward and reverse primer (0.6 µM), Taq DNA polymerase (0.05 U/µL) and a variable volume of nuclease free water. Each reaction consisted of 1 µL cDNA and 24 µL master mix. The samples were incubated in a thermal cycler (Applied Biosystems GeneAmp PCR system 2700, USA) at 95ºC for 5 min and then heated, for an optimised number of cycles, to 95ºC for 30 seconds, the primer specific annealing temperature for 30 sec and 72ºC for 30 sec. After the appropriate number of cycles, samples were kept at 72ºC for 7 minutes and subsequently at 4°C.

10 µL of each PCR sample were separated by gel electrophoresis in TAE buffer (tris-acetate-EDTA) on an ethidium bromide (2.5 µL/mL TAE) stained 1-3% agarose/TAE gel (Geneflow Ltd, UK) or 3% MetaPhor agarose/TAE gel (Lonza, Wakersville USA) for approximately 60 minutes at 100 mV (longer on 3% gels). To identify size of PCR products, an appropriate size marker (Hyperladder I or V, Bioline, UK) was loaded in a separate line on the gel. PCR products were visualised and photographed on an UV transilluminator. Similar DNA load and DNA quality were evaluated by 18S PCR for each DNA sample.

A	**SULT2A1**	**STS**	**PAPSS1**	**PAPSS2**
Forward sequence	caggaagaaccatagagaagatctg	aggacttcccaccgatgagattacctttg	ctgctggcatgcctcatc	cactcccctcaaaggtttc
Reverse sequence	gtcttacacaatgaccccagtc	aaaagggtcaggattagggctgctaggaa	gtggtcccctcttgttactag	cagcgtctcgtaagatagc
Annealing temp	55 °C	50 °C	55 °C	55 °C
Number of cycles	32 cycles	32 cycles	32 cycles	32 cycles
Product size	375 bp	364 bp	361	**a:** 200 bp **b:** 215 bp

B	**MRP4**	**MRP4 ISOFORM 1**	**MRP4 ISOFORM 2**	**18S**
Forward sequence	tcttcggtttggtctcaaca	tgtttcagtggtgtgttcgac	gaggaggaaatgtaaccgagaa	gttggtggagcgatttgtct
Reverse sequence	atgccaaaaagaacggtagc	caaaacaggttcctgaggtatg	atctcgctgtgttgtcctgg	ggcctcactaaaccatccaa
Annealing temp	55 °C	57 °C	57 °C	55 °C
Number of cycles	32 cycles	32 cycles	32 cycles	18 cycles
Product size	371 bp	404 bp	310 bp	397 bp

C	**OATP-A**	**OATP-B**	**OATP-C**	**OATP-D**	**OATP-E**	**OATP-F**	**OATP-8**
Forward sequence	ccacaagatttatatgtggaaaatg	catgggacccaggatagggcca	Gttcaacctgaattgaaatcac	gctgagaacgcaaccggttcc	gccatgccactgcaggtgaaatg	cagaaagacaatgatgtcc	gaataaaacagcagagtcagcatc
Reverse sequence	catatatccaggtatggcagcc	ggcctggccccatcatggtcactg	Gatgtggaattatatgtcctacatgac	gacttgagttcagggctgactgtcc	ttctggtacaccaagcaggagccc	cacatcttttaaatccccatttgaggc	gcaatatagctgaatgacagg
Annealing temp	55 °C	50 °C	56 °C	50 °C	65 °C	55°C	55 °C
Number of cycles	32	32	32	40	32	32	32
Product size	321 bp	718 bp	173 bp	163 bp	291 bp	249 bp	98 bp

D	**SRD5A1**	**SRD5A2**	**HSD3B1**	**HSD3B2**	**HSD17B3**
Forward sequence	cagatccccgttttctaatagg	ctggagaaatcagctacagg	ggaatctgaaaaacggcggc	gatcgtccgcctgttggtg	tcctgaacgcaccggatgaaat
Reverse sequence	aaacgtgaagaaagcaaaagc	gctttccgagatttggggta	ctgagatatagtagaactgtcctcggatg	ctcttcttcgtggccgttctggatgat	tacctgaccttggtgttgagcttcag
Annealing temp	60°C	55°C	56 °C	56 °C	60 °C
No of cycles	40	40	34	34	34
Product size	239 bp	226 bp	257 bp	382 bp	505 bp

Table 2-1 Specific information on PCR conditions including primer sequences, annealing temperatures, number of cycles and expected product sizes

2.3.3 **Principle of quantitative real-time RT-PCR**

The principle of real-time PCR is based on the previously described RT-PCR but additionally using TaqMan probes (Applied Biosystems). These probes are tagged with a fluorescent reporter and a quencher, which suppresses the fluorescent signal of the reporter whilst the probe is unbound. When binding to their complementary cDNA, the quencher is cut off by the 5'-3'-exonuclease activity of taq polymerase while synthesising the double strand and the fluorescent signal emitted. The real-time PCR machine measures the increasing fluorescent signal each cycle. (Figure 2-3)

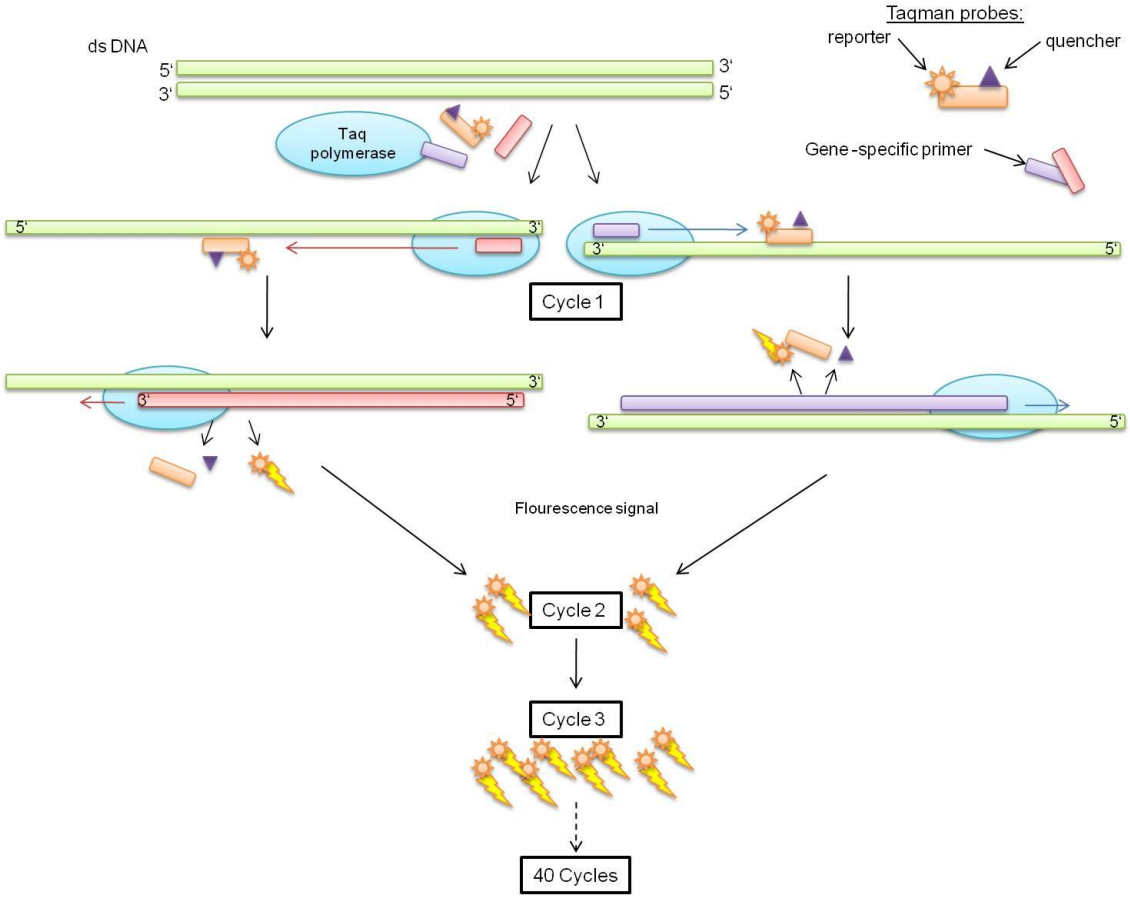


Figure 2-3 Schematic illustration of Real-time PCR method

After denaturation at 95°C, gene-specific primers and Taqman probes (further towards the 5' end) bind to the targeted DNA sequence. Taqman probes consist of a reporter with fluorescence activity and a quencher that suppresses this signal. Taq polymerase synthesises new dsDNA and splits off the reporter and the quencher when reaching the Taqman probe. Without suppression by the quencher, a fluorescence signal is emitted by the reporter and recorded by the real-time PCR cycler.

To quantify gene expression, the ΔC_t method was applied. The C_t value is defined by the cycle number, at which the logarithmic plot crosses a threshold line in the exponential part of the amplification curve. ΔC_t values present the difference between the C_t values of the sample and of the "house-keeping" gene 18S (internal control). Arbitrary units (AU) were calculated with the formula $AU=10000x2^{-\Delta Ct}$. (Figure 2-4)

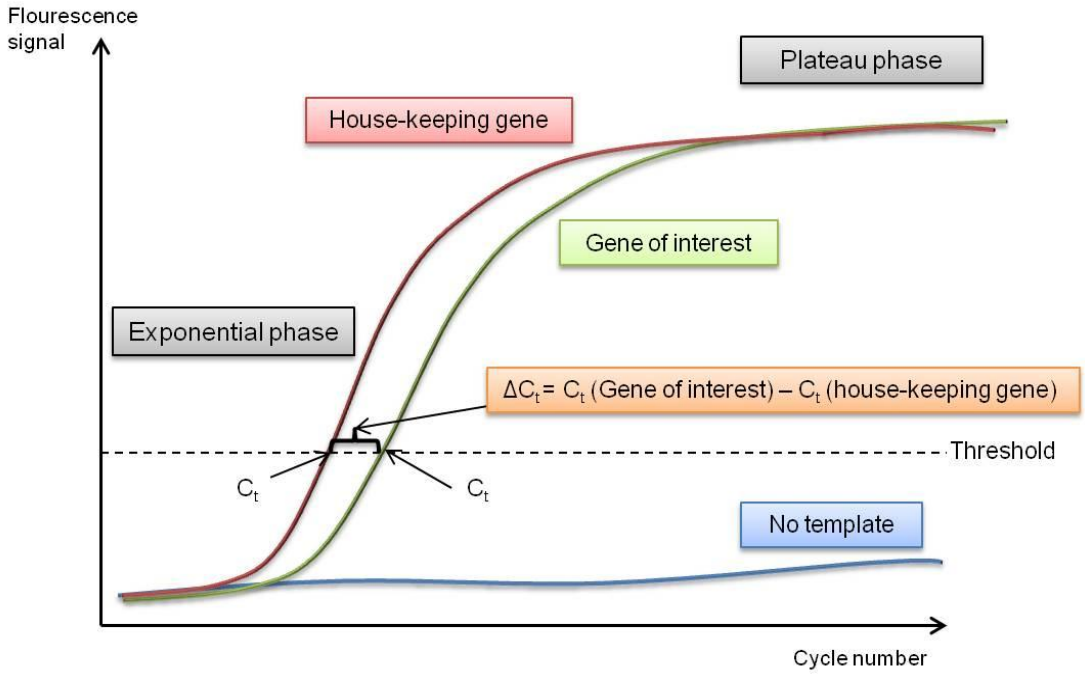


Figure 2-4 Analysis of real-time PCR by the ΔC_t method

Gene expression is quantified by plotting the fluorescence signal versus cycle number. The C_t values is the calculated cycle number at which the plot crosses the threshold line in the exponential phase of the PCR reaction. ΔC_t is calculated by comparing the gene expression of the target gene with a so called house-keeping gene which is expressed ubiquitously expressed in similar amounts.

2.3.4 **Specific information on real-time PCR experiments**

Unless otherwise indicated, reagents were purchased from Applied Biosystems, UK. Information about TaqMan probes *for SULT2A1, STS, PAPSS1* and *PAPSS2* are given in table Table 2-2. Briefly, 19 µL of a mix of nuclease free water, 2x master mix and 1 µL of 20x 'assay on demand' gene expression assay was added to 1 µL cDNA per well on a 96 well plate. Negative controls (nuclease free water) and the "house-keeping" gene 18S were analysed in separate wells. The 20 µL mixes for the 18S reactions consisted of 1 µL cDNA, 10 µL 2x master mix, 0.1 µL of each 18S-Forward, 18S-Reverse and 18S-Probe (each 25 nM final concentration) and nuclease free water.

Reactions were carried out in duplicates using a real-time PCR cycler ABI Prism 7500 (Applied Biosystems, UK) and data was analysed with SDS v1.2 software. Experiments were done in three independent experiment and statistical error presented as standard error of the mean (S.E.M.).

Gene	**Supplier**	**Assay ID**
SULT2A1	Applied Biosystems	Hs00234219_m1
STS	Applied Biosystems	Hs00165853_m1
PAPSS1	Applied Biosystems	Hs00193745_m1
PAPSS2	Applied Biosystems	Hs00190682_m1

Table 2-2 Specific Information about used Assay on Demands (Taqman probes) used for real-time PCR

2.4 Protein methods

Solutions:

- RIPA buffer: 50 mmol/L Tris pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulphonyl fluoride, and protease inhibitor cocktail (Roche, Lewes, U.K.)
- Loading buffer (4X): 50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% 14.7 M β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue.
- Running buffer: 20 X NuPAGE MOPS SDS running buffer (Invitrogen). Diluted 1:20 with dH_20.
- Transfer buffer (10X): 30.3 g Tris base, 144 g glycine diluted in 1 L of dH_20, adjusted using HCl to pH 8.3.
- Transfer buffer (working solution) for 1 L: 100 mL transfer buffer (10X), 200 mL absolute methanol, 700 mL H_20
- Washing solution: 0.1% Tween20 in PBS.
- Blocking solution: 10% non-fat re-suspended dry milk (Marvel), in washing solution.

2.4.1 Protein extraction

Cells were grown in a T75 tissue culture flask until 70% confluent. Media was discarded and cells carefully washed with ice cold sterile 1xPBS. Cells were scraped into 200 µL RIPA buffer, incubated at -80°C for at least 10 minutes and centrifuged at 14000 rpm at 4°C for 10 minutes. The supernatant was transferred to a fresh tube and total protein concentration determined by a commercially available protein assay (Bio-Rad Laboratories, Hercules, CA).

2.4.2 Western Blot

2.4.2.1 Principle of the method

Western blot is a method first published by Burnette in 1981 [209]. It is used to specifically detect a protein and quantify its expression. In this thesis, proteins were denatured and separated by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Briefly, as a first step, proteins are denatured to break up their secondary and tertiary structure. This is achieved by boiling samples as well as chemically. SDS stabilizes the proteins' structure and masks their charge so that gel electrophoresis separates proteins by size only. Small proteins migrate faster than larger ones on the gel. Proteins of known sizes are used to determine sizes of proteins in the sample. After electrophoresis, proteins are transferred i.e. blotted from the gel onto a nitrocellulose membrane by electricity.

Since the membrane is capable of binding all kind of proteins, non-specific binding on the membrane itself must be prevented before applying the primary antibody. This is achieved by blocking the membrane with a protein solution, usually non-fat dry milk or bovine serum albumin (BSA). In a next step, the membrane is incubated with a primary antibody for a certain time, ranging for 1 hour to overnight, at a certain temperature. At 4°C, antibody binding is more specific than at room temperature but the risk of false negatives is also increased. Afterwards, excess antibody is washed off the membrane.

Primary antibody bound to the protein stays on the membrane and is detected by a secondary antibody that is tagged with an enzyme, e.g. horseradish peroxidase, which emits light in the presence of a chemiluminescent reagent. The strength of the signal is proportional to the amount of protein on the membrane and can be recorded by various

methods, e.g. photographic film or digitally on camera. The signal strength can then be quantified by imaging software and expression of a protein between different samples can be compared. The same membrane can be re-probed with a new primary antibody against a house-keeping gene, to evaluate if similar amounts of total protein were analysed. (Figure 2-5)

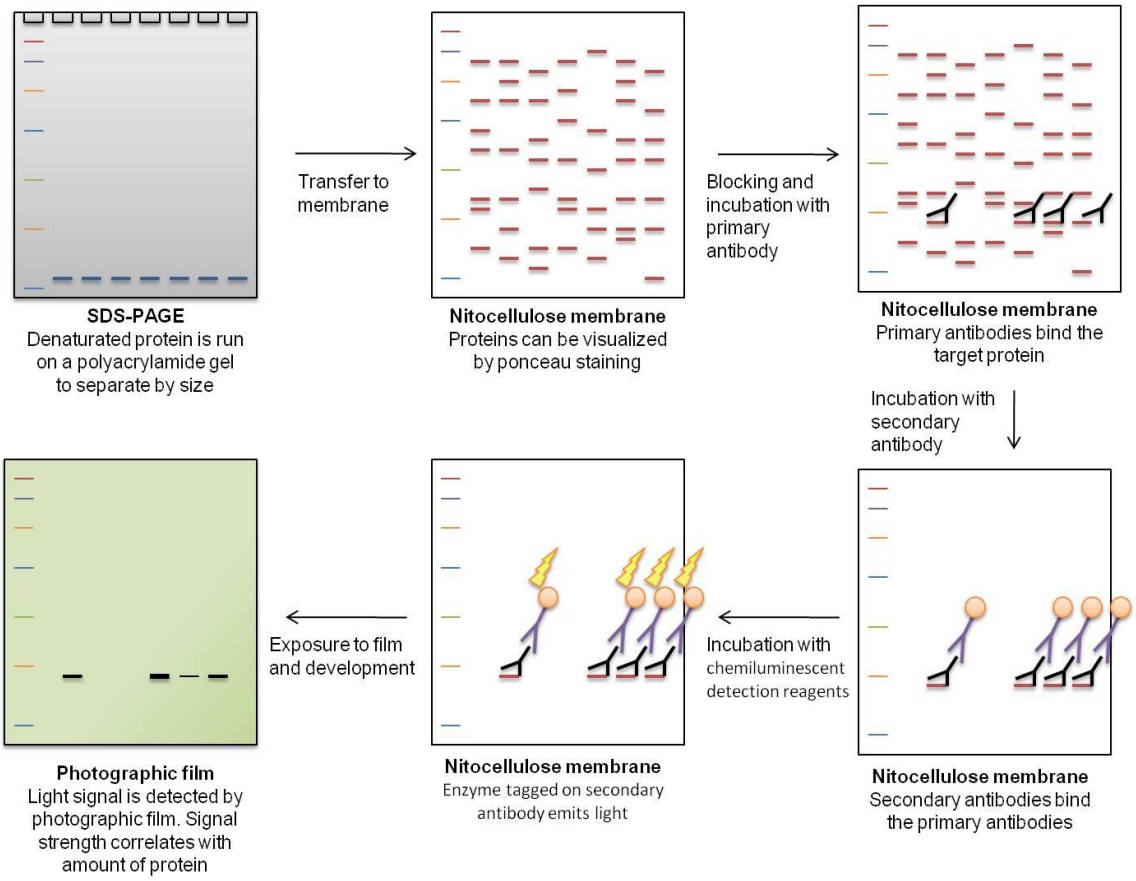


Figure 2-5 Schematic illustration of detecting protein expression by western blotting

A sample of a protein mix from tissue or cell culture is denatured mechanically and chemically and separated by size on a polyacrylamide gel using electrophoresis. SDS stabilises the protein structure and covers their electric charge. In a second step, proteins are transferred from the gel to a nitrocellulose membrane. Non-specific protein binding on the membrane is prevented by incubation with non-fat dry milk. After incubation with a primary antibody against the target protein and a secondary antibody against the primary antibody, protein is detected by a chemiluminescent reaction of an enzyme bound to the secondary antibody. This signal is recorded on a photographic film or digitally by a camera. Signal strength is proportional to the amount of protein on the membrane.

2.4.2.2 Specific information

Protein samples were boiled for 7 minutes in 4xSDS loading buffer. Equal amounts of protein were loaded on a precast 10% SDS-gel (Invitrogen, UK) and electrophoresis carried out for 50 minutes at 200 V in a tank containing 500 mL running buffer. To determine the protein size, a marker (Bioline, UK) was loaded on each gel. Afterwards, proteins were transferred from the gel on a nitrocellulose membrane (GE Healthcare, Amersham, UK) at 30 V for 75 minutes. Successful transfer and similar protein load were visually assessed by colouring proteins with Ponceau Solution (Sigma-Aldrich, UK). The membrane was then blocked by incubating the membrane in 10% non-fat dry milk dissolved in 1xPBS/0.1% Tween20 for 60 minutes. To probe the protein of interest, membranes were incubated with primary antibody in an antibody specific dilution in blocking solution (Table 2-3) over night at 4°C.

Afterwards, membranes were washed 3 times for 15 minutes with 1xPBS/0.1%Tween20 and subsequently incubated with a peroxidase-linked secondary antibody targeted against the primary antibody for 90 minutes. Again, membranes were washed 3 times for 15 minutes with 1xPBS/0.1% Tween20 to wash off excess antibody. Target protein was detected by the secondary antibodies' luminescence reaction with ECL (G.E. Healthcare) which was left on the membrane for 1 min. The luminescent signal was recorded by exposure of the membrane to light sensitive Kodak MXB photographic film (G.R.I, Rayne, UK) for between 2 seconds and 90 minutes. Films were developed using an X4 developer (Xenograph Imaging Systems, Gloucestershire, UK).

For a second incubation with β-actin primary antibody, the membranes were stripped by incubating the sample in stripping buffer at 50˚C for 30 min. The membranes were then washed once and re-probed with an antibody against β-actin to confirm similar yield in the analysed sample.

Antibody	Species raised	Supplier	Dilution
Human SULT2A1	Rabbit	Abcam (ab38416)	1:500
Human PAPSS1	Mouse	Abcam (ab56389)	1:1000
Human PAPSS2	Mouse	Abcam (ab56393)	1:1000
β-actin	Mouse	Abcam (ab49900)	1:25000

Table 2-3 Information on primary antibodies used for Western blot

2.5 Functional enzyme assays

2.5.1 Principle of the method

Enzyme assays are widely used to determine enzyme activity, kinetics and function. In this thesis, SULT activity in the adrenal cell line NCI-h295R strain 3 was investigated. Therefore, a number of cells were exposed to tritiated (^{3}H-labeled) DHEA for a certain time. Medium also contained an optimised concentration of unlabeled DHEA to offer SULT2A1 sufficient substrate for the reaction over a longer time. In addition, effects of certain substances on enzyme function can be tested while activity is in its linear range, i.e. there is enough substrate and the catalytic site is not saturated yet.

The next steps are crucial to recover and measure remaining and sulphonated ^{3}H-DHEA(S). This way specific activity can be calculated. Steroids are recovered from cell medium and purified. To quantify conversion of DHEA, samples are separated by thin layer chromatography (TLC) where a solvent moves in a stationary phase (e.g. silica gel) driven by capillary action. Thereby, the solvent and compounds in a sample on the stationary phase run together. However, different steroids move with different speed on a silica gel (stationary phase) due to different properties such as solubility in the solvent and attraction to the stationary phase. By running reference samples on the same plate, one can determine steroids by their distance travelled on the plate. Conversion to different products can then be quantified by measuring radioactive counts over each lane on the plate. (Figure 2-6)

2.5.2 Used solutions and materials

- 500 nM DHEA (in 100% ethanol)
- Experimental medium 1: Serum and antibiotic free medium with 500 nM DHEA
- Experimental medium 2: Serum and antibiotic free medium with 1 µM DHEAS
- Experimental medium 3: Serum and antibiotic free medium with 500 nM DHEA and 1 µM trilostane (in DMSO)
- 0.01 M reference steroids: DHEA, DHEAS, testosterone, dihydrotestosterone, androstenediol, androstenetriol, androstenedione, androstanedione, androsterone, oestrone, oestradiol, 16α-DHEA, 7α-DHEA (Sigma, UK and Steraloids, USA)

- ^{3}H-DHEA stock solution: specific activity 145 Ci/mmol (GE Healthcare, UK)
- ^{3}H-DHEA working solution: diluted stock solution with 1000 cpm/µL
- ^{3}H-DHEAS stock solution (GE Healthcare, UK)
- ^{3}H-DHEAS working solution: diluted stock solution with 1000 cpm/µL
- TLC:
    - mobile phase 1: hexane/hexanol (75:25)
    - mobile phase 2: chloroform/methanol/acetone/acetic acid/dH_2O (8:2:4:2:1)
- Lieberman-Burchard reagent (2 mL acetic acid, 8 mL sulphuric acid, 24 mL ethanol)

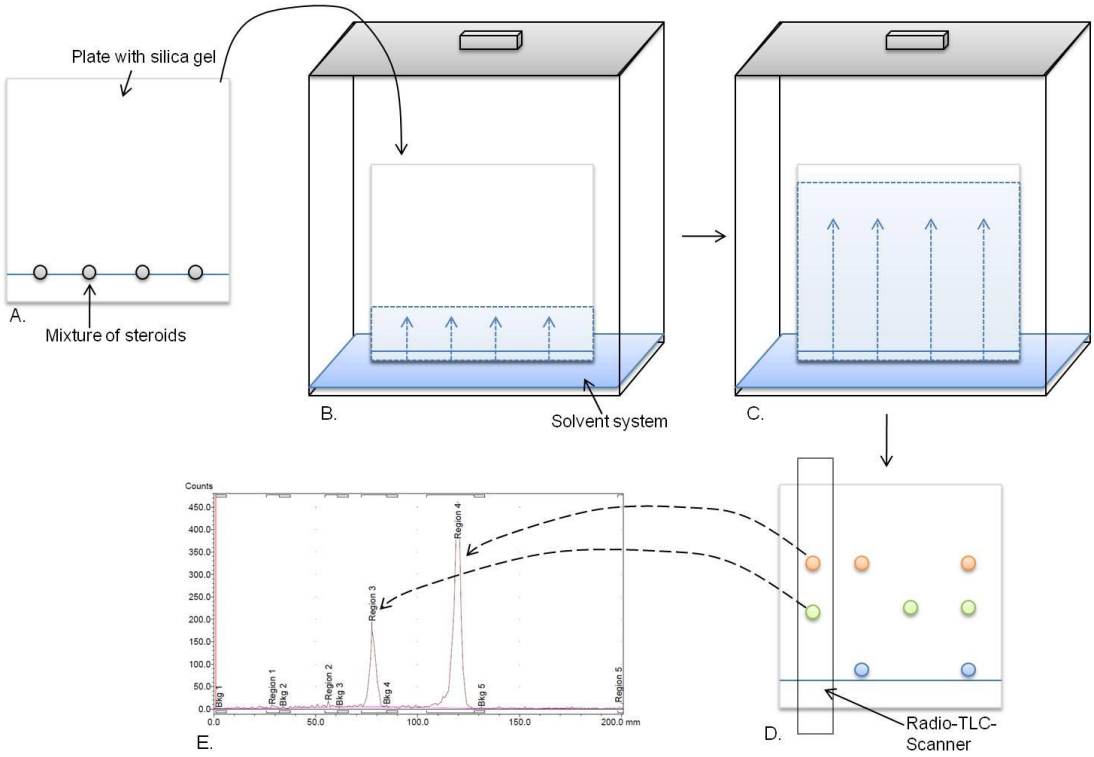


Figure 2-6 Principle of Thin Layer Chromatography (TLC) to detect steroid metabolites converted from ^{3}H DHEA Samples with steroids dissolved in dichloromethane are spotted on a silica gel TLC plate as a stationary phase (B) and run in a closed chamber with a specific solvent system (mobile phase). Adsorption and capillary effects take the different steroids up the plate by different speed rates depending on steroid properties (B,C). Reference steroids in higher concentrations are also run on the plate and can be visualised in different colours (D) by Lieberman Burchard reagent. Then radioactive counts on each lane are quantified using a TLC-Scanner (D, E).

2.5.3 Incubations of cell lines with ^{3}H-DHEA and ^{3}H-DHEAS

Cells were equally seeded and grown until 70% confluence in a 12 well plate. Prior to experiments, cells were washed with 1xPBS and serum free medium. 999 µL experimental medium 1 with 500 nM DHEA plus 1 µL ^{3}H-DHEA working solution were added to each well and cells incubated for 30/60/90/120 minutes. Alternatively, for STS activity assay, 999 µL experimental medium 1 with 1 µM DHEAS plus 1 µL ^{3}H-DHEAS working solution was added to each well and cells incubated for 60/120 minutes and 24 hours.

2.5.4 Treatment of cells with trilostane

Trilostane (4-alpha-5-epoxy-17 beta-hydroxy-3-oxo-5-alpha-androstan-2-carbonitrile) is a competitive inhibitor of HSD3B activity [210]. Preceding experiments with NCI-h295R strain 3 revealed conversion to other metabolites of DHEA pointing towards activity of HSD3B. Hence, cells were incubated in 999 µL experimental medium 3 with 500 nM DHEA and 1 µM trilostane for 1 hour before activity assay to prevent conversion of by HSD3B. To start the SULT2A1 activity assay, 1 µL ^{3}H-DHEA was added carefully to each well and cells left for 30/60/90/120 minutes at 37°C. All experiments were carried out in duplicates and results were confirmed in three independent experiments. Cell free incubations were used as negative controls and to check sufficient recovery of steroids.

2.5.5 Steroid extraction from cell culture medium

After incubation of cells, media was transferred to glass vials and steroids were extracted with 5mL dichloromethane per 1 mL medium by vortexing for 1 minute. Samples were centrifuged at 500g for 10 minutes, the steroid-containing organic solvent phase was separated and the aqueous upper phase and cell/protein-containing intermediate phase were sucked off. The remaining organic solvent phase was evaporated at 55°C under nitrogen flow, re-diluted in 80 µL dichloromethane and stored at 4°C.

2.5.6 **Thin layer chromatography (TLC)**

Samples were spotted on silica-gel coated TLC-plates and run in two solvent systems, in mobile phase 1 (hexane/hexanol, 75:25) for 180 minutes and followed by mobile phase 2 (chloroform/methanol/acetone/acetic acid/water, 8:2:4:2:1) for 10 min to separate substrate and conversion products. Conversion was quantified on a Bioscan 2000 image analyzer (Lablogic, Sheffield, UK) and steroids were identified by co-migration with unlabelled reference steroids, which were visualised by spraying with Liebermann-Burchard reagent and subsequent incubation at 115°C for 15 min. (Figure 2-7)

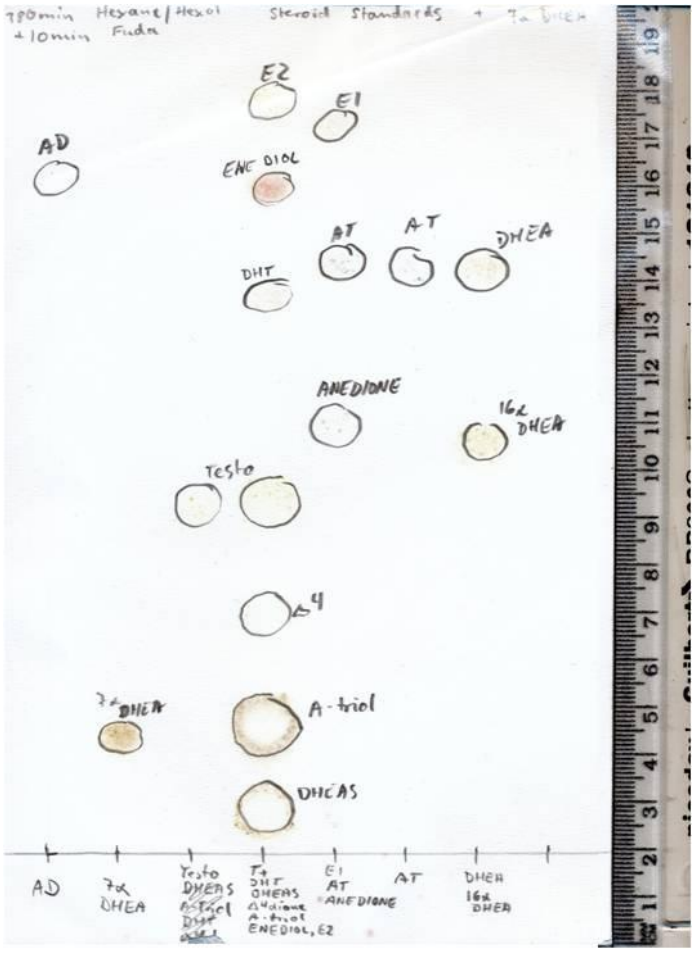


Figure 2-7 TLC plate with reference steroids

The plates were run in mobile phase 1 for 180 minutes and mobile phase 2 for 10 minutes. Steroids were visualised with Liebermann-Burchard reagent. Most steroids are clearly separated (DHEAS, testosterone (Testo) dihydrotestosterone (DHT), androstenedione (Δ4), androstanedione (ANE-DIONE), oestrone (E1), oestradiol (E2), 16α-DHEA). Possible conversion products of DHEA, which could not be clearly distinguished, are 7α-DHEA from androstenetriol (A-triol), androsterone (AT) from DHEA, androstanediol (AD) from androstenediol (ENE-DIOL). However, these steroids are not direct products but require several steps.

2.6 Immunocytochemistry

2.6.1 Principle of the method

Immunocytochemistry (ICC) is a technique that enables to analyse protein expression. In contrast to Western Blot, it is possible to acquire additional information about subcellular localisation of the target protein in cultured cells and its changes during experiments. In principle, cells are grown on a microscope slide or cover slip and then prepared in several steps for protein visualisation under a microscope. Depending on the requirements for the experiments, preparations can vary widely. Cells need to be fixed, e.g. by methanol, ethanol or acetate, and cell membranes permeabilised by a detergent such as Triton X100 or Tween20 to enable antibodies to enter the cells and cellular compartments where the target protein is found. Similar to Western Blot protocol, non-specific binding of antibodies is prevented by incubation with a blocking solution, usually diluted non-fat dry milk, followed by incubation with a primary antibody against the protein of interest. Either primary antibody directly or a secondary antibody against the primary antibody is linked to a fluorescent molecule and can be visualized under a microscope, when the slide is stimulated by light with an antibody specific wave length.

2.6.2 Specific information

Cells were seeded equally in six well plates containing a sterile cover slip. Medium was carefully changed every other day until cells reached about 70% confluence. Then, cells on cover slips were washed very carefully with 1xPBS and subsequently fixed in 100% methanol at -20°C for 15 minutes. Permeabilisation of cell membranes was achieved by incubation with 1% Triton X-100/1xPBS for 5 minutes. Unspecific binding was prevented by incubating the cells in 20% foetal calf serum (FCS/1xPBS) for 60 minutes and cells were incubated overnight with the antibody for PAPSS1 or PAPSS2 (Table 2-4). For negative controls, cover slips were incubated with 1xPBS instead of the primary antibody. After several washing steps with 1xPBS, the appropriate secondary antibody was applied for 45 minutes and the cover slip was mounted on a microscope slide with 10 µL VECTASHIELD mounting medium containing DAPI (Vector laboratories, USA) and fixed with nail varnish.

Antibody	Species raised	Supplier	Dilution
Human PAPSS1	Mouse	Abcam (ab56389)	1:50
Human PAPSS2	Mouse	Abcam (ab56393)	1:50
Alexa Fluor 488 goat anti-mouse IgG (H+L) (green)	Goat	Invitrogen (a11001)	1:250

Table 2-4 Information on antibodies used for ICC

Antibodies against PAPSS1 and PAPSS2 are primary antibodies. Alexa Fluor 488 antibody targets the primary antibodies (mouse) and emits green light when excited by light with a wavelength of 488 nm.

3 Results

3.1 Characterisation of cell lines

3.1.1 Qualitative expression of mRNA

HepG2 cells and human adult liver expressed mRNA of *SULT2A1*, whereas WRL68 cells did not. *STS* mRNA was found in liver, HepG2 and WRL68. Adrenal gland and NCI-h295R1 and NCI-h295R3 expressed more *SULT2A1* than NCI-h295. Expression of *SULT2A1* in SW13 cells was not detected. By qualitative RT-PCR no mRNA expression of *STS* was seen in adrenal and expression in SW13 appeared to be low. NCI-h295, NCI-h295R1 and NCI-h295R3 showed a strong signal for *STS*. All analysed tissues and cell lines showed mRNA expression of *PAPSS1*. The splice variants *PAPSS2a* and *PAPSS2b* exhibited a differentiated pattern of expression in tissues and cell lines. Whilst in liver tissue *PAPSS2b* was predominant, the liver cell lines HepG2 and WRL68 expressed *PAPSS2a*. The adrenal gland was the only tissue with expression of *PAPSS2a* and *PAPSSb*. In SW13, NCI-h295, NCI-h295R1 and NCI-h295R3 only *PAPSS2a* was detected.

In Cos-7 cells, a commonly used cell line for transfection experiments, neither *SULT2A1* nor *STS* expression was detectable by qualitative RT-PCR. *PAPSS1* and *PAPSS2a* were expressed, but not *PAPSS2b*. Results from Cos-7 cells are only preliminary, as experiments were not validated in triplicates or by qualitative RT-PCR. (Figure 3-1)

mRNA expression of several inward and outward transport proteins for DHEA were investigated by qualitative RT-PCR to confirm the presence of transport capacity of DHEAS needed for later functional assays. Whilst in human adult liver *OATP-A, OATP-B, OATP-C, OATP-D* and *OATP-8* were expressed, in HepG2 cells only *OATP-B* and in WRL68 *OATP-D, OATP-E* and *OATP-F* were found. In human adult adrenal tissue, RT-PCR showed expression of *OATP-B* and *OATP-D*.

MRP4 epression of the isoforms of the main efflux transporter for DHEAS was detected in all cell lines, adult liver and adult adrenal tissue without differentiation of isoforms (Figure 3-2).

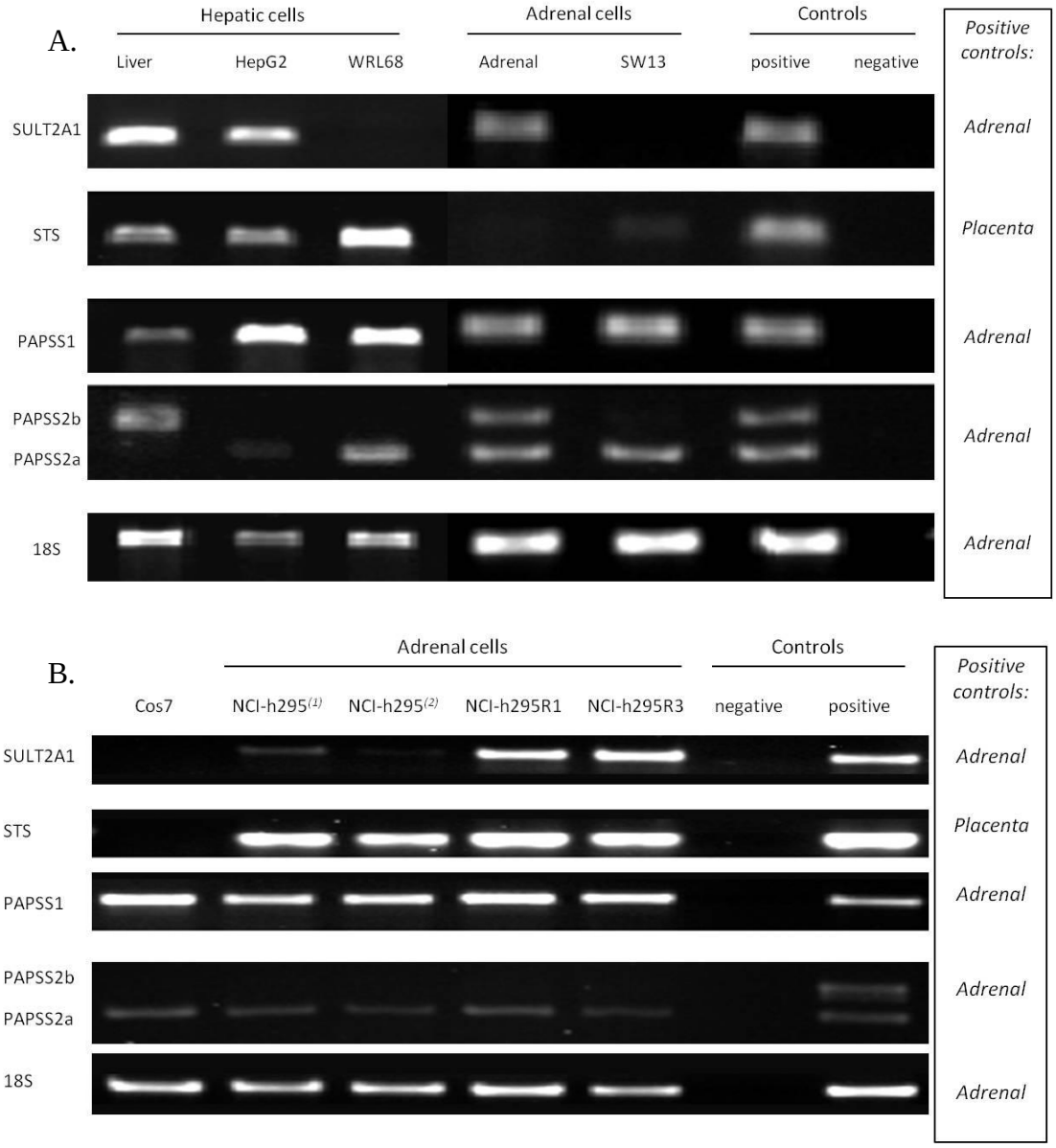


Figure 3-1 mRNA expression of *SULT2A1, STS, PAPSS1 and PAPSS2a/b* in liver, adrenal and various cell lines as detected by qualitative PCR

Interestingly, human adult liver and adrenal expressed *PAPSS2b* while all cell lines expressed *PAPSS2a*. Adrenal tissue expressed both splice variants of PAPSS2 whilst all adrenal cell lines that were investigated only expressed PAPSS2a. SULT 2A1 was not expressed in WRL68, SW13 and surprisingly only a very thin band was found in NCI-h295. Due to problems with cell culturing of NCI-h295 cells this result has to be evaluated critically. (1) and (2) indicate two batches of this cell line and represent two attempts to overcome cell culturing problems. COS7, which is a common cell line for transfection experiments, showed no expression of SULT2A1 but expression of PAPSS1 and PAPSS2a.

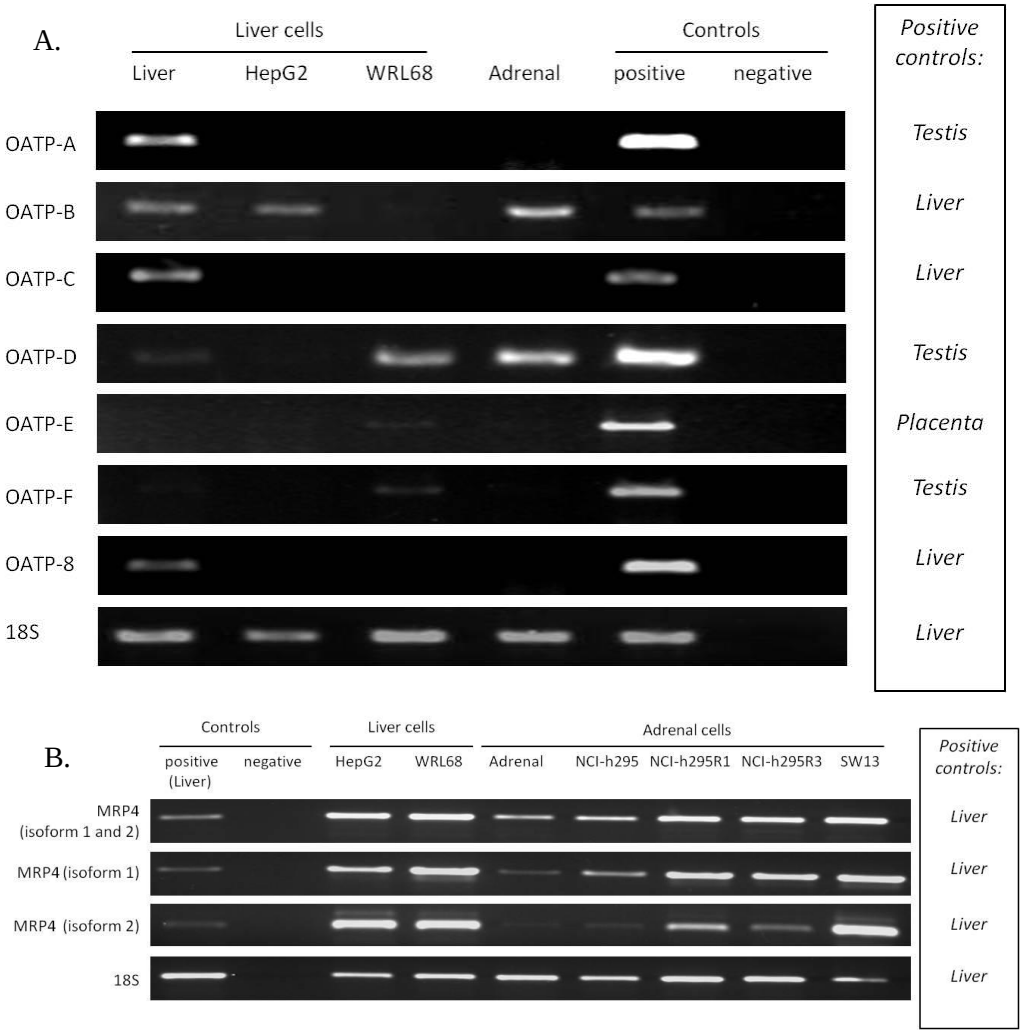


Figure 3-2 OATP and MRP4 expression profile of liver, adrenal and hepatic cell lines determined by RT-PCR mRNA expression of several *OATP*s that are important for DHEAS transport across the plasma membrane were investigated. These proteins are important because DHEAS needs active transport and cannot diffuse freely into the cell due to its hydrophilic properties. In adult liver, expression of several *OATP*s were found. HepG2 seems to express only *OATP-B*. Also, *OATP* expression seems to be very tissue specific since adrenal tissue differed from liver tissue. WRL68 also exhibited another expression profile of *OATP*s, however it has been shown that WRL68 is actually a HeLa contaminant and not a hepatic cell line (Panel A). Two isoforms of *MRP4* have been described before. However, differences in function, expression patterns or activity have not been investigated so far. Panel B shows bands for both isoforms in all examined cells and tissues. Isoform 1 seems to be predominant in adrenal tissue, however the method of RT-PCR is not quantitative.

Adrenal tissue and all analysed adrenal cell lines were also checked for mRNA expression of the enzymes *SRD5A1, SRD5A2, HSD3B1, HSD3B2* and *HSD17B3*, which are involved in steroid metabolism (Figure 1-8). *SRD5A1* and *SRD5A2* were detected in all cells. Whilst HSD3B1 was also expressed in all investigated samples, the cell line SW13 lacked mRNA expression of *HSD3B2*. *HSD17B3* exhibited a strongly different expression profile, only found in the NCI-h295 cell line. (Figure 3-3)

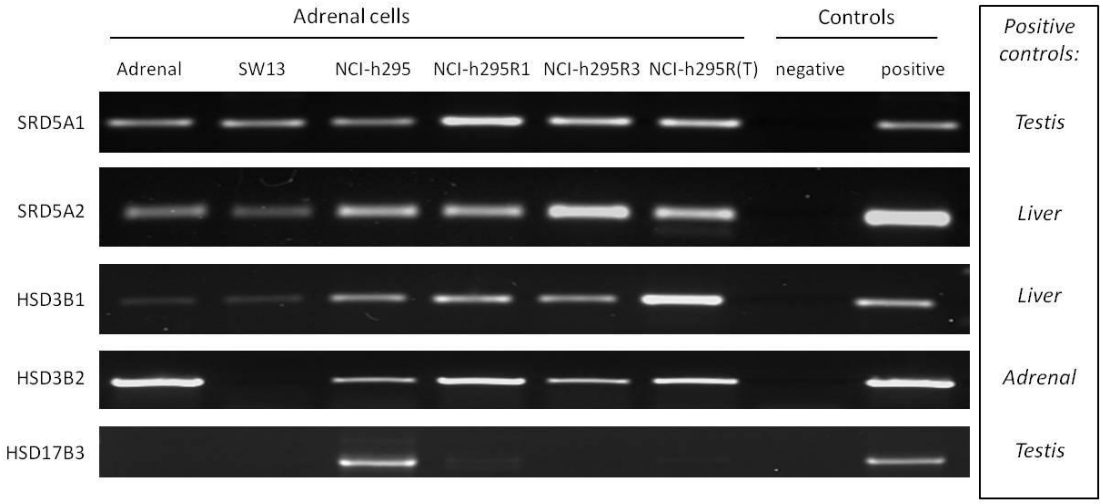
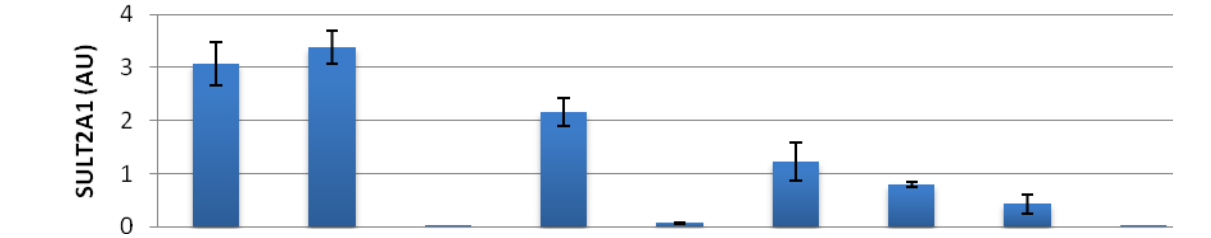


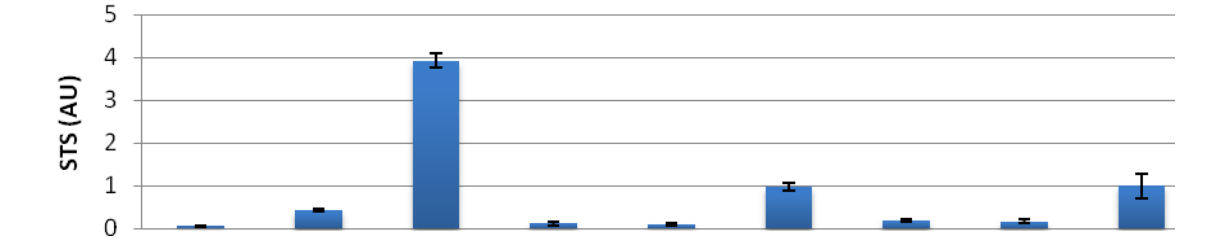
Figure 3-3 Expression of steroid metabolising enzymes in human adrenal and adrenal cell lines
Adrenal and all adrenal cell lines showed mRNA expression of *SRD5A1, SRD5A2* and *HSD3B1* by RT-PCR. By contrast, *HSD3B2* could not be detected in SW13. *HSD17B3* (conversion of androstenedione to testosterone) was detected only in NCI-h295.

3.1.2 **Quantitative expression of mRNA**

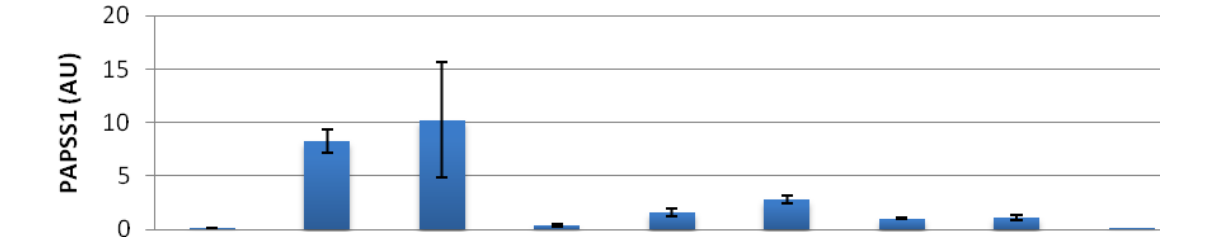
Using real-time RT-PCR, mRNA expression of SULT2A1, STS, PAPSS1 and PAPSS2 in several adrenal and hepatic cell lines were compared with each other as well as adrenal and liver tissue. The liver cell line HepG2 showed a high expression SULT2A1 (ΔC_t=11.5) comparable to adult liver (ΔC_t=11.7) and a low expression of STS (ΔC_t=13.5 versus ΔC_t=18.5). However, expression of STS in placenta, a location of major sulphatase activity, showed very similar mRNA expression of STS (ΔC_t=13.4). In contrast to HepG2, the other liver cell line, WRL68 exhibited very low expression of SULT2A1 (ΔC_t=21.4) but showed abundant expression of STS (ΔC_t=11.3). (Figure 3-4)



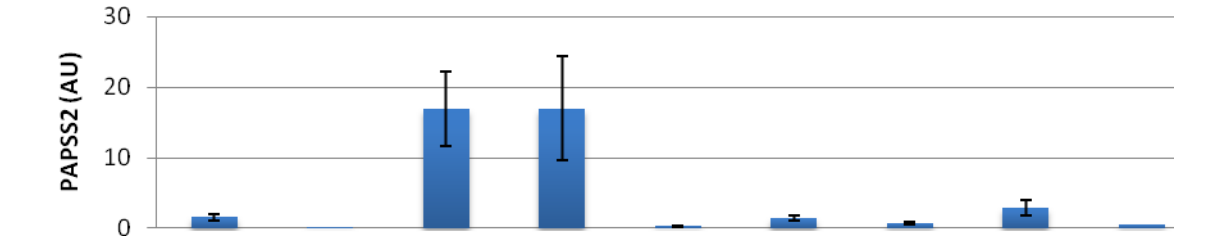
ΔC_t ±S.E.M.	liver	HepG2	WRL68	adrenal gland	h295	h295R1	h295R3	h295R3 (forskolin)	placenta
SULT2A1	11.7±0.2	11.5±0.1	21.4±0.7	12.2±0.2	17.3±0.4	13.1±0.5	13.6±0.1	14.8±0.6	23.8±0.3



ΔC_t ±S.E.M.	liver	HepG2	WRL68	adrenal gland	h295	h295R1	h295R3	h295R3 (forskolin)	placenta
STS	18.2±0.9	13.5±0.1	11.3±0.1	16.7±0.5	16.7±0.3	13.4±0.1	15.8±0.3	16.1±0.5	13.4±0.5



ΔC_t ±S.E.M.	liver	HepG2	WRL68	adrenal gland	h295	h295R1	h295R3	h295R3 (forskolin)	placenta
PAPSS1	20.2±0.9	12.5±0.2	13.7±0.9	18.6±0.8	16.0±0.3	15.2±0.2	16.6±0.1	16.5±0.3	20.0±0.3



ΔC_t ±S.E.M.	liver	HepG2	WRL68	adrenal gland	h295	h295R1	h295R3	h295R3 (forskolin)	placenta
PAPSS2	16.2±0.6	21.9±0.1	12.7±0.5	12.9±0.7	18.7±0.5	16.2±0.5	17.0±0.2	15.4±0.7	17.5±0.1

Figure 3-4 quantitative mRNA expression of *SULT2A1, STS, PAPSS1* and *PAPSS2* (by real-time PCR) mRNA expression is presented as Arbitrary Units (AU) for each enzyme as a conversion of ΔC_t values shown underneath each graph. Experiments were performed in triplicates and ΔC_t values (presented with S.E.M.)

Real-time PCR of adrenal tissue and cell lines confirmed findings by conventional PCR, with high expression of *SULT2A1* in adult adrenal (ΔC_t=12.2), NCI-h295R1 (ΔC_t=13.1) and NCI-h295R3 (ΔC_t=13.6), and low expression in NCI-h295 (ΔC_t=17.3). Interestingly, expression of *SULT2A1* mRNA tends to decrease after 24 hours of treatment with 1 µM forskolin in NCI-h295R3 (no treatment: ΔC_t=13.6, forskolin treatment: ΔC_t=14.8, P=0.12). However, this effect was not significant. *STS* mRNA was found in high abundance in NCI-h295R1 (ΔC_t=13.4), whilst it was low in adrenal tissue (ΔC_t=16.7), NCI-h295 (ΔC_t=16.7) and h295R3 (ΔC_t=15.8). (Figure 3-4)

The expression profile of the *PAPS synthase* isoforms 1 and 2 differed between hepatic cell lines and liver tissue. Adult liver showed low expression of *PAPSS1* (ΔC_t=20.2), whereas it showed high expression of *PAPSS2* (ΔC_t=16.2). In contrast, in HepG2 cells *PAPSS1* was the predominant isoform (*PAPSS1* ΔC_t=12.5 versus *PAPSS2* ΔC_t=21.9) and in WRL68 both isoforms of *PAPSS* were highly expressed (*PAPSS1* ΔC_t=13.7, *PAPSS2* ΔC_t=12.7).

Similarly, the expression profile for adrenal tissue and cell lines differs. Adult adrenal showed low expression of *PAPSS1* (ΔC_t=18.6) and high expression of *PAPSS2* (ΔC_t=12.9). Interestingly, all adrenal cell lines showed only a moderate expression level of *PAPSS1* (NCI-h295 ΔC_t=16.0, NCI-h295R1 ΔC_t=15.2, NCI-h295R3 ΔC_t=16.6) as well as *PAPSS2* (NCI-h295 ΔC_t=18.7, NCI-h295R1 ΔC_t=16.2, NCI-h295R3 ΔC_t=17.0). (Figure 3-4)

3.1.3 **Protein expression analysis**

Protein expression of SULT2A1, PAPSS1 and PAPSS2 was analysed applying western blot technique. In this case, expression was analysed qualitatively and not quantitatively.

Protein expression of SULT2A1 resembled the results of mRNA expression. SULT2A1 was expressed in human adult liver, HepG2, NCI-h295R1 and NCI-h295R3. As shown by real-time PCR, expression in NCI-h295 could be detected, but only on a low level. Human liver tissue acted as a positive control for SULT2A1 expression and purified SULT2A1 protein as a positive control for the antibody. To enable purification, SULT2A1 was tagged with Gluthation-S transferase (GST) with a size of around 25

kDa, which explains the band of a different size in figure Figure 3-5. No protein expression of SULT2A1 was found in WRL68 and SW13.

PAPSS1 protein (70 kDa) was found in all cell lines (HepG2, WRL68, SW13, NCI-h295, NCI-h295R1 and NCI-h295R3). Confirming real-time PCR results, expression in human liver tissue was very low (Figure Figure 3-5). PAPSS2 was detected in all cell lines and human liver tissue. In NCI-h295 cells, expression seemed to be low. Purified PAPSS2 protein with a GST tag was used as a positive control. (Figure 3-5)

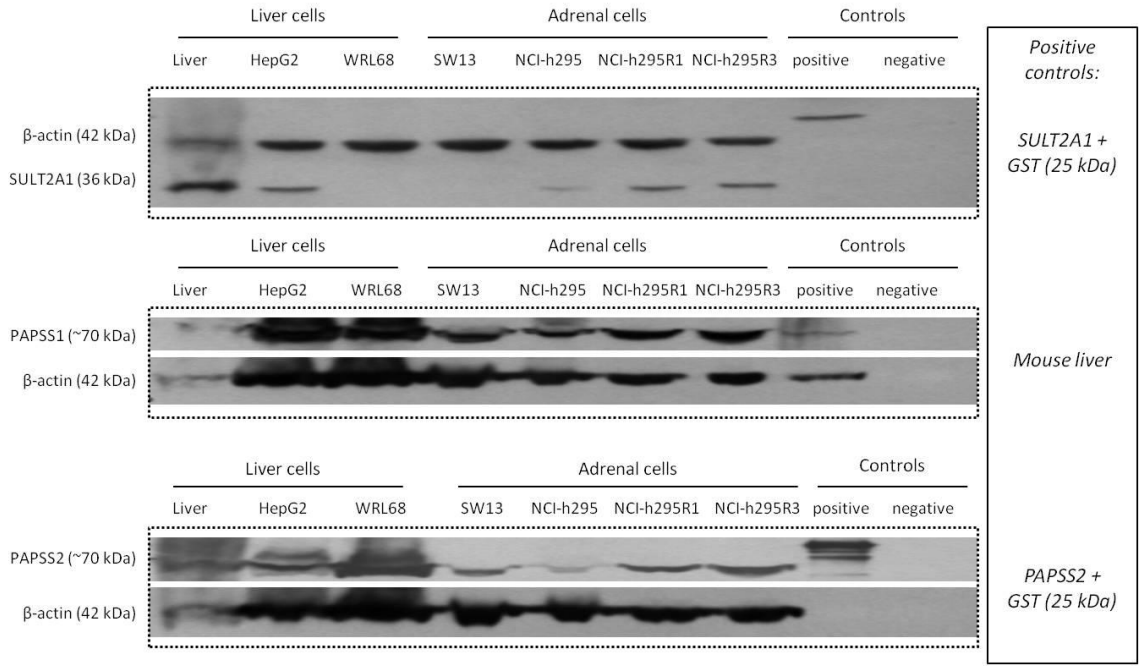


Figure 3-5 Protein expression in hepatic and adrenal cell lines and liver tissue

Western Blot was performed to analyze expression of SULT2A1, PAPSS1 and PAPSS2 in HepG2, WRL68, SW13, NCI-h295, NCI-h295R strain 1 and 3. Human and mouse liver served as a positive control for expression as well as purified recombinant SULT2A1 and PAPSS2 as a positive control for success of primary antibody binding. SULT2A1 and PAPSS2 were tagged with GST, which explains the band in positions of an additional 25 kDa.

3.1.4 **Functional enzyme activity**

Sulphotransferase activity was assessed for different time points after inhibition of HSD3B activity by trilostane for 60 minutes incubation before start of assay. Preceding experiments without trilostane treatment pointed towards relevant activity of HSD3B2 with conversion of DHEA to androstenedione (36% to 65% at 30 and 120 minutes, respectively). When cells had been preincubated with trilostane, DHEA was converted to DHEAS in a time-dependent manner: after 30 minutes, 9.8% of the DHEA was recovered as DHEAS and 17.3% after 60 minutes, respectively; within 90 minutes, 26.9% of DHEA was converted to DHEAS and 37.6% within 120 minutes, respectively. Conversion to androstenedione remained below 10% at all time points when preincubated with trilostane. In addition, further conversion to androstenetriol and 7α-hydroxy-DHEA was below 10% at all time points. (Figure 3-6) Sulphatase activity (conversion of DHEAS to DHEA) was not detected, even at a 24-hours time point (data not shown).

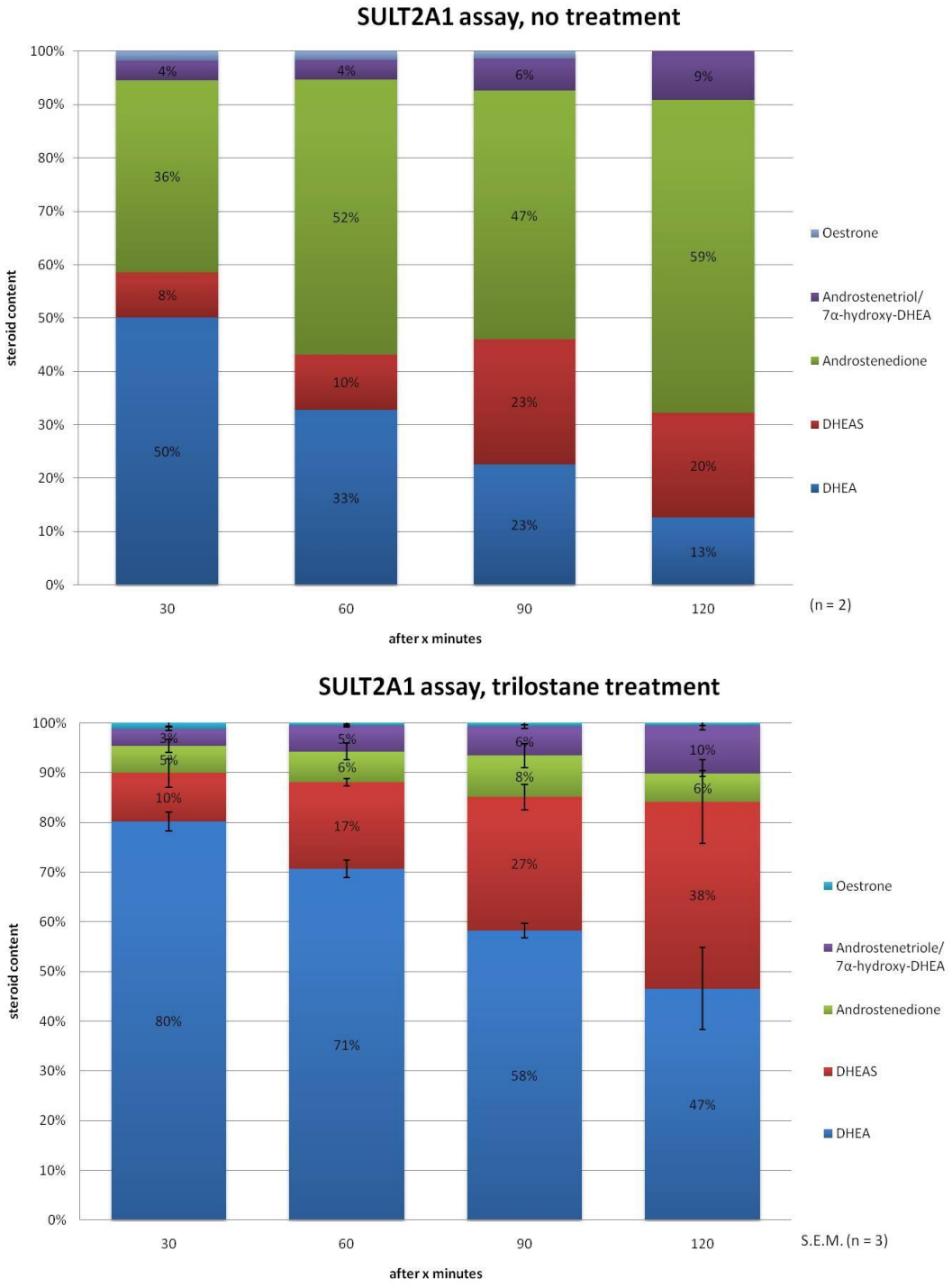


Figure 3-6 Conversion of DHEA to its metabolite in NCI-h295R3

SULT2A1 enzyme assay was performed with NCI-h295R3, steroids separated by TLC and conversion calculated by counts of radio-labeled products of ^{3}H-DHEA. Interestingly, cells showed high conversion to other DHEA metabolites like Androstenedione, Androstenetriole / 7α-hydroxy-DHEA (not separated clearly by TLC) suggesting a high activity of HSD3B2. By applying the HSD3B inhibitor trilostane, conversion to androstenedione was effectively reduced and a time-dependent conversion to DHEAS was seen.

3.1.5 **Immunocytochemistry**

Another interesting aspect of the enzyme PAPS synthase is its subcellular localisation, which can be investigated by immunochemistry. Again, the adrenal cell line NCI-h295R was used. RNA expression of the relevant enzymes was confirmed by PCR before the experiments. Figure 3-7 shows sections of slides with immunostained cells. One can see that PAPSS1 and PAPSS2 are expressed in the cytoplasm and in the nucleus. However, merging the picture of the PAPSS stain with the nucleus representing DAPI stain makes the higher accumulation of PAPSS in the nucleus evident.

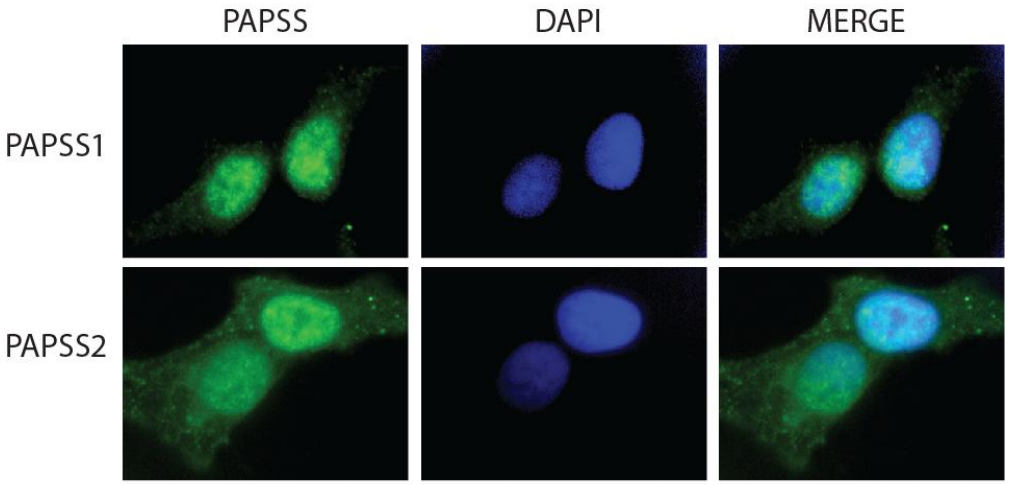


Figure 3-7 Subcellular localisation of PAPSS1 and PAPSS2 in NCI-h295R (T) cells by ICC
PAPSS1 and PAPSS2 are expressed in the cytoplasm and the nucleus with predominance in nuclear expression. For these experiments a vector transfected strain of NCI-h295R was used.

3.2 The DHEA/DHEAS-Shuttle in human foetal tissues

In a second project, mRNA expression in foetal tissues, like adrenal, liver and cartilage from femur, rib and digits were analysed and compared to adult tissue.

3.2.1 **Qualitative analysis of mRNA expression**

As described before, adult liver expressed *SULT2A1*, *PAPSS1* and *PAPSS2b*. Foetal and adult adrenal tissue had the same expression profile of *SULT2A1*, *PAPSS1* and *PAPSS2a* as well as *PAPSS2b*. In contrast, foetal chondrocytes of femur, rib and digit did not exhibit expression *of SULT2A1*. *PAPSS1* and *PAPSS2a* were found, but not *PAPSS2b*. (Figure 3-8)

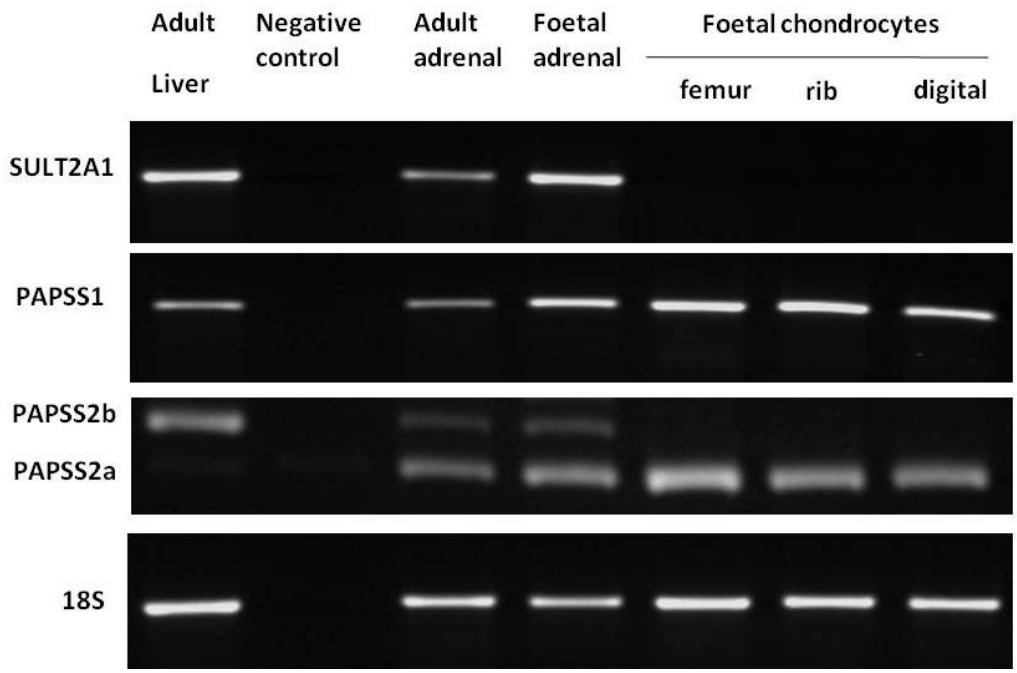
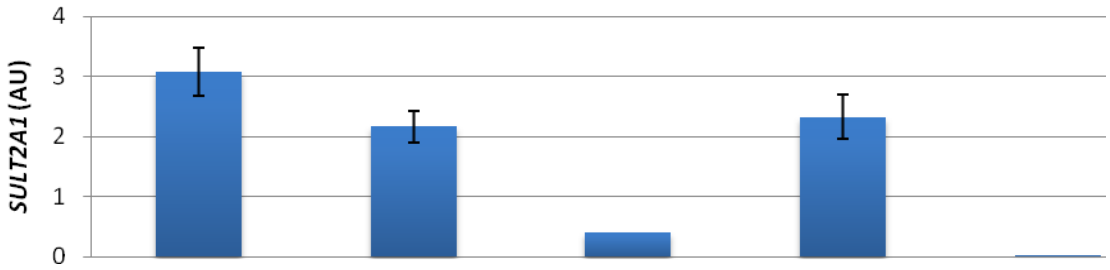


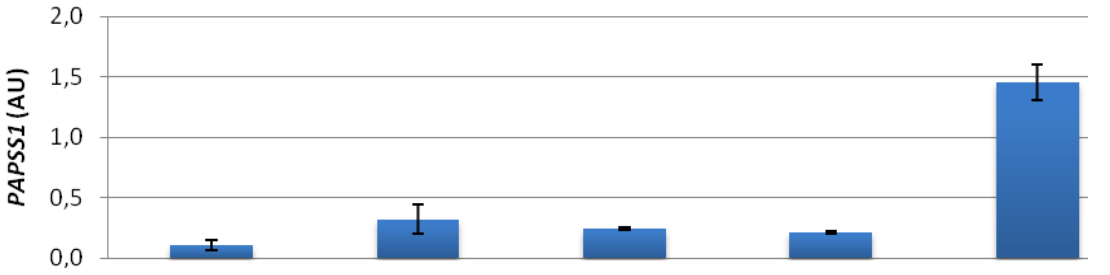
Figure 3-8 mRNA expression of the DHEA-DHEAS-Shuttle in human foetal tissues
mRNA expression of *SULT2A1, PAPSS1* and *PAPSS2a/b* was analysed in human adult liver, adult and foetal adrenal and foetal chondrocytes by qualitative RT-PCR . Adult liver, adult adrenal and foetal adrenal as major sites of sulphonation expressed *SULT2A1* and *PAPSS2b*. Adult liver did not express *PAPSS2a* whereas foetal chondrocytes did not express *PAPSS2b*. Adult and foetal adrenal expressed *PAPSS2a* and *PAPSS2b*. In chondrocytes, *SULT2A1* mRNA could not be detected. *PAPSS1* was expressed ubiquitously.

3.2.2 **Quantitative analysis of mRNA expression (real-time PCR)**

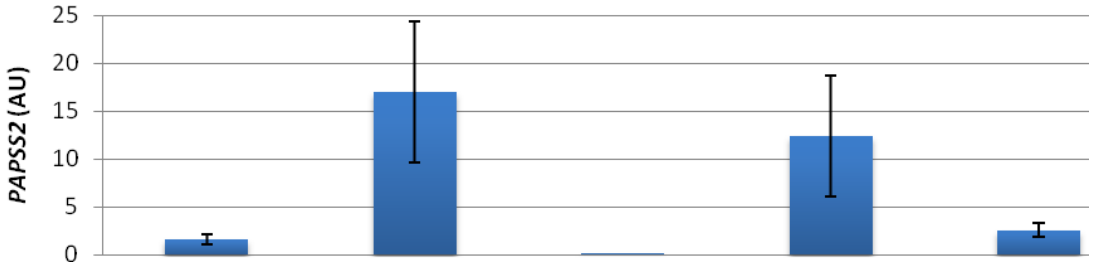
Foetal tissues were available to study mRNA expression of *SULT2A1*, *PAPSS1* and *PAPSS2* by real-time PCR. Confirming conventional RT-PCR, *SULT2A1* was very low in foetal cartilage (ΔC_t=27.5). However, *PAPSS1* (ΔC_t=16.1) and *PAPSS2* (ΔC_t=15.4) were relatively highly expressed. The expression profile of foetal adrenal copied that of adult adrenal for *SULT2A1* (ΔC_t=12.1 versus ΔC_t =12.2), *PAPSS1* (ΔC_t=18.8 versus ΔC_t=18.6) and *PAPSS2* (ΔC_t=13.3 versus ΔC_t=12.9). In contrast, foetal liver exhibits a different expression profile compared to adult liver. *SULT2A1* was lower in foetal liver (ΔC_t=14.6) than in adult tissue (ΔC_t=11.7), *PAPSS1* expression was higher in foetal liver (ΔC_t=18.7) than in adult liver (ΔC_t=20.2), and conversely, *PAPSS2* expression was lower in foetal (ΔC_t=19.2) than in adult liver (ΔC_t=16.2). (Figure 3-9)



ΔC_t ±S.E.M.	adult liver	adult adrenal	foetal liver	foetal adrenal	foetal cartilage
SULT2A1	11.7 ±0.2	12.2 ±0.2	14.6 ±0.1	12.1 ±0.2	27.5 ±0.2



ΔC_t ±S.E.M.	adult liver	adult adrenal	foetal liver	foetal adrenal	foetal cartilage
PAPSS1	20.2 ±0.8	18.6 ±0.8	18.7 ±0.1	18.8 ±0.1	16.1 ±0.1



ΔC_t ±S.E.M.	adult liver	adult adrenal	foetal liver	foetal adrenal	foetal cartilage
PAPSS2	16.2 ±0.6	12.9 ±0.7	19.2 ±0.1	13.3 ±0.7	15.4 ±0.5

Figure 3-9 quantitative mRNA expression of *SULT2A1*, *PAPSS1* and *PAPSS2* in foetal tissue

Quantitative mRNA expression of *SULT2A1*, *PAPSS1* and *PAPSS2* in foetal tissues was analysed by real-time PCR. Results are presented in Arbitrary Units (AU), transformed from ΔC_t values that are shown together with the S.E.M. underneath each graph. Real-time PCR confirmed adult and foetal adrenal as a site of high sulphonation activity. The lack of *SULT2A1* was confirmed by this more sensitive method. *PAPSS2* was predominant in adrenal but also showed high expression in adult liver and foetal chondrocytes.

4 Discussion

4.1 Enzyme expression and function in hepatic and adrenal cell lines

Sulphonation is a major factor, which determines the availability of DHEA. The regulation of the necessary enzymes SULT2A1 and PAPS synthase is not fully understood. Due to lack of CYP17, rodents have no significant adrenal C19 steroid production and are considered poor models for human C19 steroidogenesis. Some primates species appear to be suitable animal models as they undergo adrenarche and develop a zona reticularis [211]. However, ethical considerations limit the use of primates on a larger scale.

Cell lines are an easily available option, but limitations arise from transferring results to actual human physiology. To date, only scarce information is available about gene expression of PAPSS1 and PAPSS2 in cell lines. Since liver and adrenal tissues major sites of sulphonation activity, cell lines from these tissues are good candidates for further experiments. To evaluate the differential regulation of PAPSS1 and PAPSS2, a cell line with similar expression levels of these enzymes was desirable.

Numerous experiments regarding SULT2A1 activity have been conducted in the human hepatoma cell line HepG2 before [36, 212-213]. In my experiments, conventional PCR, real-time PCR and western blot confirmed mRNA and protein expression of SULT2A1 and PAPS synthase isoforms. However, differential expression of PAPSS2a and 2b was investigated for the first time. Interestingly, whilst in human adult liver only PAPSS2b is expressed, only PAPSS2a was found in HepG2 cells. It remains unclear what drives this change. Since PAPSS2a exhibits a lower activity [194] it could simply be an adaption to the fact that in cell culture environment there is less sulphonation in detoxification reactions compared to liver cells *in vivo*. On the other hand, one can speculate that this change in enzyme expression is linked to carcinogenesis since Huang et al also found a significant downregulation of SULT2A1 in hepatocellular carcinoma compared to normal liver tissue [214].

In my experiments, real-time PCR revealed a different expression profile of *PAPSS1* and *PAPSS2* in HepG2 cells compared to adult liver. In contrast to liver tissue, HepG2

abundantly expressed *PAPSS1* and less *PAPSS2*. *SULT2A1* was expressed in a similar range. These findings make HepG2 cells suitable for studying sulphotransferase activity but not to study the regulation of PAPS synthase 2 in particular.

Experiments by Hammer et al had revealed no conversion of DHEAS to DHEA in HepG2 cells despite expression of *STS* [36]. PCR and real-time PCR experiments confirmed the expression in my experiments. A possible explanation for the absent conversion of DHEAS could have been that DHEAS had not been transported across the cell membrane due to lack of the required transport proteins. However, this study shows mRNA expression of OATP-B and MRP4 confirming the ability of HepG2 cells to transport DHEAS across the cell membrane. The OATP expression pattern of HepG2 and liver tissue is somewhat different to the results found earlier by real-time PCR [215]. Hilgendorf et al found expression of all here analysed OATPs in liver tissue with highest levels in OATP-C and OATP-8. By RT-PCR, I confirmed expression of OATP-A, OATP-B, OATP-C, OATP-D and OATP-8. A possible explanation for the absent expression of OATP-E and OATP-F in my experiments could be the more sensitive method of Hilgendorf (real-time PCR). This could also be a reason for the different findings in HepG2. Hilgendorf found low expression of OATP-B, OATP-C, OATP-E and OATP-8 by real-time PCR. In contrast, my RT-PCR experiments detected only OATP-B in HepG2 cells. Summing up, this means that HepG2 cells express a possibly changed expression profile of OATPs compared to liver, however, influx of DHEAS should be sufficiently possible.

WRL68 has long been considered a human embryonic liver cell line and has been used as a hepatocyte model. However, the European Collection of Cell Cultures (EACC) recently identified this cell line as a HeLa contaminant and therefore as a human cervix carcinoma cell line [201]. In addition, this cell line turned out to be not a suitable model for further investigations because of its expression profile of SULT2A1 and PAPSS. Neither by conventional PCR nor by real-time PCR *SULT2A1* was detected. Western blot confirmed the absence of protein expression of SULT2A1. On the contrary, WRL68 cells exhibited mRNA expression of *STS* on a high level in my real-time PCR experiments suggesting sulphatase activity as reported in HeLa cells [216]. Even though

it expressed both *PAPSS1* and *PAPSS2* mRNA abundantly on a similar level, WRL68 cannot be used model due to its lack of SULT2A1 expression unless the cell line is transfected. Regarding influx and efflux proteins of DHEAS, OATP-D, OATP-E, OATP-F and MRP4 were detected by PCR. Therefore, transport of DHEAS across the cell membrane seems to be possible.

In addition to HepG2 and WRL68, the adrenocortical cell lines SW13, NCI-h295, NCI-h295R substrain 1 and substrain 3 were analysed. The SW13 cell line derived from a primary small cell carcinoma of the adrenal cortex. This cell line has been used before to investigate the transcriptional regulation of PAPSS2 [217]. Although a study in 1988 showed secretion of DHEAS by SW13 cells [218], conventional PCR and western blot could not detect expression of SULT2A1 in this cell line. The ubiquitously expressed PAPSS1 and in addition PAPSS2a were found. Nevertheless, the lack of SULT2A1 expression made SW13 cells not suitable for further experiments, so that investigations by real-time PCR were not carried out.

The NCI-h295 and NCI-h295R cell lines have proved to be useful to investigate regulation of adrenal steroidogenesis and adrenal disease [202, 219-223]. However, the NCI-h295 cells showed only relatively low expression of SULT2A1 in my experiments by conventional PCR, real-time PCR and western blot. PCR showed expression of *PAPSS1* and *PAPSS2a* but not *PAPSS2b*. Real-time PCR showed a lower level of *PAPSS2* and an increased level of *PAPSS1* expression compared to healthy adrenal tissue. Although the protein expression was not quantified, western blot showed a less intense band with for PAPSS2 expression in NCI-h295 cells than in other cell lines. Again, the lower expression of SULT2A1 and PAPSS2 argued against the further use of this cell line. Importantly, the results of experiments with NCI-h295 need to be evaluated critically. Even though cells were propagated under appropriate conditions, they repeatedly exhibited a reduced growth rate. This could have led to changes in cell metabolism and therefore also changes in expression of SULT2A1 and PAPSS. A second batch of cells showed the same problems. I suggest a problem with growth medium and its components since this had not been prepared freshly.

Additionally, two substrains of the NCI-h295R cell line were available that derived from the NCI-h295 cell line and that partly exhibit steroid profiles similar to ZG and ZR [203]. Further advantages of the NCI-h295R cell line are that its growth rate is fast, growth media is comparatively inexpensive and cells are easy to handle. Both substrains expressed similar levels of SULT2A1 mRNA protein. However, they differed in the mRNA expression of *STS*, *PAPSS1* and *PAPSS2*.

The higher expression of *STS* mRNA in strain 1 could mean antagonising the effect of *SULT2A1* in functional assays. However, experience from other studies suggest that STS activity is negligible [36]. In contrast to NCI-h295R1, STS levels in strain 3 were lower. More importantly, western blot confirmed protein expression of SULT2A1, PAPSS1 and PAPSS2 so that the NCI-h295R3 cell line was chosen to further analyse SULT2A1 activity.

NCI-h295R3 cells showed SULT2A1 activity at an initial DHEA concentration of 500 nM in the media. However, due to 3β-hydroxysteroid dehydrogenase activity DHEA was also converted to androstenedione. Trilostane is a competitive inhibitor of HSD3B activity that has proven to be effective and specific [210] and has been used for treatment of hypercortisolim [224] and advanced hormone-dependant breast cancer [225]. By incubation with 1 µM trilostane starting 1 hour prior to the experiments, conversion to androstenedione was successfully reduced so that the majority of conversion products was DHEAS.

For future investigations of SULT2A1 activity and its regulation in this cell line, it is important to use the appropriate assay parameters. The applied time point should be within the linear range of the catalytic activity because this can decline with time due to depletion of the substrate, accumulation of the product and inactivation of the enzyme. The time course experiment suggests that for experiments with 500 nM unlabelled DHEA and 1 µM trilostane a time point at 90 minutes would be suitable to detect significant changes in SULT2A1 activity.

For investigations towards relevance of subcellular localisation of PAPSS2, I used the immunocytochemistry (ICC) technique to visualise PAPSS1 and PAPSS2 in NCI-h295 substrain 3. As Besset et al reported in transfected cells, PAPSS1 was also located in the

nucleus of this cell line. In Besset's experiments PAPSS2 localised to the cytoplasm when transfected alone but re-localised to the nucleus when co-expressed with PAPSS1 [195]. By applying ICC, I confirmed this phenomenon. PAPSS 1 and PAPSS2 were both found in the cytoplasm and in the nucleus with a predominance in the nucleus. Since PAPSS1 is expressed ubiquitously, physiological relevance of cytoplasmatic localisation of PAPSS2 without PAPSS1 expression seems to be low. However, these findings are intriguing anyway and raise the question if subcellular localisation plays a role in the function and regulation of PAPSS2 activity. After having established this method, more experiments are being conducted to investigate the relevance of these findings.

4.2 PAPSS2 in chondrocytes and inactivating PAPSS2 mutations in as a cause for hyperandrogenism and skeletal malformation

4.2.1 Case report

Here, I would like to present a case of a girl with androgen excess, premature pubarche, hyperandrogenic anovulation and serum DHEAS levels below the limit of detection where my findings of differential PAPSS2 expression contributed to work out the underlying cause. This case was also presented in the New England Journal of Medicine [192].

The daughter of nonconsanguineous was referred to the Department of Paediatrics – Metabolic and Endocrine disorders at the Radbout University Nijmengen Medical Centre (the Netherlands) at the age of 8 years for endocrine evaluation of pubic and axillary hair growth that had started 2 years earlier. Dr. Noordam performed the clinical and biochemical workup in Nijmengen. At the age of 8 years, pubic hair development was classified as Tanner stage 4 and breast development as Tanner stage 2. Blood pressure was normal at 115/85 mmHg. Bone age was accelerated (12 years according to the classification of Greulich and Pyle), her height was 125 cm and the ratio of her sitting height to her standing height was 0.54.

The patient's plasma DHEAS level was below the detection limit, whilst DHEA was in the upper normal range. Androstenedione and testosterone level were twice as high as expected for girls at her age. Dexamethasone suppression test (0.5 mg administered every 6 hours for 48 hours) decreased cortisol, androstenedione and DHEA levels sufficiently to rule out steroid production by an adrenal and gonadal tumour. In addition, congenital adrenal hyperplasia was ruled out. Gonadotropin levels were within the normal range before and after gonadotropin releasing hormone infusion. 17β-oestradiol was in the normal range.

Menarche occurred at 11 years of age. The girl was followed up without treatment until the age of 12. Her bone age was 16.5 years, her height was 139 cm and her weight was 58.3 kg. X-ray studies revealed no epiphyseal or metaphyseal changes but mild lumbar scoliosis and flattened vertebrae. Pubertal hair development was at Tanner stadium 5 and breast development at Tanner stadium 4. Additionally, she suffered from significant hirsutism and acne. At 13 years of age, secondary amenorrhea developed. Until 14.5 years of age, patent's height remained at 139 cm.

Endocrine assessment at 12 years of age mirrored the findings at 8 years of age. DHEAS levels were still below limit of detection, even with a more sensitive assay. Although it was possible to stimulate DHEA levels, DHEAS levels remained below the limit of detection throughout a cosyntropin (synthetic ACTH) stimulation test. Analysis of urinary steroid metabolites over a 24-hour period showed increased excretion of the major androgen metabolite androsterone.

These findings were explained with a lack of DHEA sulphonation leading to androgen excess. However, genetic analysis of *SULT2A1* showed no mutations whereas the girl had compound heterozygous mutations in *PAPSS2*. Direct sequencing of the *PAPSS2* gene detected a heterozygous substitution of cytosine for guanine at nucleotide position 143, which leads to substitution of threonine by arginine at position 48 in the PAPSS2 protein (T48R). This mutation is situated in the APS-kinase domain of the protein. *In vitro* experiments revealed an activity of approximately 6%. In addition, another mutation in this patient was found in the ATP sulphurylase domain of the protein. Sutbstitution of cytosine for thymidine in cDNA position 985 introduces a stop codon, which predicts to truncate the PAPSS2 protein at position 329 (R329X). Here, *in vitro* experiments showed no residual activity.

Before, a homozygous *PAPSS2* mutation (S475X) had been found to lead to spondyloepimetaphyseal dysplasia, Pakistani type [197], whilst a mutation in chondroitin 6-O-sulfotransfrease (*CHST-3*) results in spondyloepiphyseal dysplasia, Omani type [226]. The acceleration of bone age in our patient was probably due to androgen excess. The absence of a pubertal growth spurt, the reduced height and the increased ratio of sitting to standing height might reflect the bone dysplasia, although no longbone epiphyseal or metaphyseal changes were observed. This is possibly explained by the finding of a residual PAPSS2 activity of approximately 6% for the T28R mutant, which was present in the patient.

4.2.2 *PAPSS2* expression in foetal tissue

Mutations in the *PAPSS2* gene result in skeletal malformations in human and mice [197-198]. Fuda et al demonstrated that in adult human and guinea pig cartilage PAPSS1 is the predominant isoform whilst *PAPSS2* is expressed sparsely. In contrast, guinea pig cartilage from the growth plate exhibited high expression of *PAPSS2* and low expression of *PAPSS1* [194].

For the first time, differential expression of *PAPSS2* splice variants were investigated in human foetal tissue (Figure 3-8). Real-time PCR showed higher expression of *PAPSS2* than *PAPSS1* in human foetal cartilage from the rib, femur and digit. Interestingly, only *PAPSS2a* was found in foetal chondrocytes whilst at major sites of DHEA sulphonation like adult liver, adult adrenal and foetal adrenal *PAPSS2b* was expressed. The reason why PAPSS1 cannot compensate for the loss of PAPSS2 activity might be explained by the lower catalytic efficiency of PAPSS1. *In vitro* studies showed a higher specific activity of PAPSS2b than of PAPSS2a which were still 10 to 15 fold higher than the activity of PAPSS1 [194].

In addition, my real-time PCR results confirmed earlier data [227-228] that *SULT2A1* is most abundantly expressed in foetal adrenal as a site of vast DHEAS biosynthesis. When compared to adult liver, foetal tissue showed a much lower expression of *SULT2A1*.

Given the finding that foetal chondrocytes expressed only the *PAPSS2a* variant (Figure 3-8), wild type and mutant PAPSS2a protein was used for the functional analysis mentioned in the case report above (chapter 4.2.1).

5 Conclusion

DHEA is a precursor for the male and female sex hormones testosterone and estradiol, which are mainly secreted from the testes and the ovary, respectively. In addition, epidemiological studies showed that low serum levels of DHEA and DHEAS correlate with the incidence of autoimmune disease, cancer and cardiovascular disease. *In vitro*, DHEA and DHEAS influenced glucose metabolism in a favourable manner. However, positive effects of DHEA substitution were only significant adrenal insufficiency in women.

Steroid sulphotransferase 2A1 (SULT2A1) is the responsible enzyme for sulphonation of DHEA to DHEAS which is thought to be the inactive form of DHEA. In this role, SULT2A1 acts as a central regulator of steroid synthesis because sulphonation of DHEA withdraws the substrate for further downstream conversion. Another essential cofactor for sulphonation is PAPS, which is produced by the enzyme PAPS synthase (PAPSS) from ATP and anorganic sulphate. PAPSS exists in the different isoforms PAPSS1 and PAPSS2 and splice variants PAPSS2a and PAPSS2b. Changes in PAPSS activity are thought to influence sulphonation of DHEA significantly. However, neither regulation of PAPSS nor its influence on SULT2A1 have been investigated in human cell lines or humans.

The main goal of this thesis was to analyze the enzyme expression of the DHEA/DHEA shuttle, i.e. mRNA and protein of SULT2A1, PAPSS1 and PAPSS2, in various human cell lines. Furthermore, I investigated which cell line could serve as a suitable model for further research regarding regulation of SULT2A1, PAPSS1 and PAPSS2.

Here, I could show that the enzymes of the DHEA/DHEAS shuttle were expressed in the human adrenal cell line NCI-h295R as both mRNA and protein. In enzyme assays, I was able to prove conversion of DHEA to DHEAS as well as to different other steroids. However, applying Trilostane, a potent inhibitor of CYP3B, effectively directed conversion of DHEA to DHEAS. Using these findings, future experiments can investigate for example the influence of certain cytokines or endocrine disruptors on expression and activity of PAPSS1/2 and on sulphonation of DHEA. In particular, the relatively equal expression of PAPSS1 and PAPSS2 will enable us to do knock down

experiments with siRNA to elucidate how the activity of one enzyme changes when the other one fails.

Sulphonation of DHEA by SULT2A1 is thought to happen in the cytoplasm or more precisely in the Golgi apparatus. However, experiments in transfected cells have shown both a cytoplasmatic and a nuclear localisation when both enzymes were expressed at the same time. Immunocytochemistry revealed the same results in the adrenal cell line NCI-h295R, where both enzymes were expressed strongly in the nucleus. The physiological role is not clear and requires further research. Presumably, sulphate is activated in the nucleus. However, one could also speculate that a shift of PAPSS to the nucleus could generate a reservoir, which can be activated by re-localisation to the cytoplasm when more PAPS is needed.

Expression of *SULT2A1* in some foetal tissues has been investigated earlier. Whilst in adult human cartilage *PAPSS1* is predominant, in newly born hamsters *PAPSS2* is more abundantly expressed. The expression of *PAPSS* isoforms in highly sulphonating tissue has not been investigated in humans, so far. This work demonstrated a differential expression of *SULT2A1*, *PAPSS1* and *PAPSS2* in adult and foetal liver, adrenal and foetal cartilage tissue. In adult and foetal adrenal expression was similar. However, foetal and adult liver differed in the expression of *SULT2A1*, which was expressed much more in adult tissue. Most importantly, in foetal cartilage there was only a low expression of *SULT2A1* and PAPS seems to mostly provided by PAPSS1, which was considerably higher expressed in cartilage than in other tissues. In contrast, *PAPSS2* was mainly expressed in adult and foetal adrenal.

Additionally, we reported a case of a female patient who had been investigated for hyperandrogenism. Two mutations in the *PAPSS2* gene had led to massively reduced serum levels of DHEAS. One heterozygous mutation in the domain of the APS kinase of the PAPSS2 protein leads to substitution of one amino acid at position 48 (T48R). *In vitro* experiments showed a residual activity of 6% for this mutation. A second mutation in the ATP sulphurylase domain of *PAPSS2* was found. The introduction of thymidine instead of cytidine leads to a stop codon, which is presumed to truncate the protein at position 329 (R329X). *In vitro,* no residual activity was seen for this mutation. The lack of PAPS reduces sulphonation of DHEA but also sulphonation of proteoglycanes, which leads to skeletal abnormalities. The abundance of DHEA enables massive

downstream conversion to androgens leading to clinical features of hyperandrogenism. Regarding the bone abnormalities, it is interesting and surprising that activity of PAPSS1 compensated to a great extent in cartilage but was not able to keep up a more considerable sulphonation of DHEA. Possibly, the subcellular localisation might play a role in this scenario.

6 Zusammenfassung

DHEA ist eine Vorstufe der männlichen und weiblichen Sexualhormone Testosteron bzw. Oestradiol, welche hauptsächlich in den Testes bzw. Ovarien gebildet werden, aber auch in der Körperperipherie aus DHEA gebildet werden können. Desweiteren konnte in epidemiologischen Studien gezeigt werden, dass niedrige Spiegel von DHEA und DHEAS mit dem Auftreten von Autoimmunerkrankungen, Tumorerkrankungen und Herz-Kreislauf-Erkrankungen korrelieren. *In vitro* konnten beispielsweise günstige Effekte auf den Glukose-Stoffwechsel nachgewiesen werden. Allerdings konnte eine klinisch sinnvolle Gabe von DHEA nur im Rahmen einer Substitution bei Nebenniereninsuffizienz bei Frauen nachgewiesen werden.

Verantwortlich für die Sulfonierung von DHEA ist vor allem die Steroid Sulfotransferase 2A1 (SULT2A1). DHEAS wird als inaktivierte Form von DHEA angesehen. SULT2A1 fungiert als zentraler Regulator der Steroid-Synthese, da durch Sulfonierung von DHEA zu DHEAS der weiteren Konversion das Substrat entzogen wird. Für diese Sulfonierung ist PAPS ein essentieller Kofaktor. Das Enzym PAPS-Synthase, von welchem unterschiedliche Splice-Varianten und Isoformen (PAPSS1 und PAPSS2a/b) vorliegen, stellen PAPS aus ATP und anorganischem Sulfat her. Eine Änderung der Aktivität der PAPS-Synthase kann vermutlich die Aktivität der DHEA Sulfotransferase maßgeblich beeinflussen. Weder die Regulation der PAPS Synthase noch deren Wirkung auf SULT2A1 wurden bisher in menschlichen Zelllinien oder beim Menschen untersucht.

Hauptziel dieser Arbeit war die Analyse der Enzymexpression des DHEA/DHEAS Shuttles (mRNA und Protein von SULT2A1, PAPSS1, PAPSS2) in verschiedenen humanen Zelllinien. Ferner wurde untersucht, ob eine der Zelllinien als Modell geeignet ist, die Regulation von SULT2A1 sowie insbesondere PAPSS1 und PAPSS2 in bestimmten pathophysiologischen Situationen zu untersuchen.

Hier konnte gezeigt werden, dass insbesondere die adrenale Zelllinie NCI-h295R die Enzyme des DHEA/DHEAS Shuttles sowohl als mRNA als auch als Protein exprimiert. Mittels Enzym-Assay konnte eine Konversion von DHEA zu DHEAS und verschiedenen weiteren Steroiden nachgewiesen werden. Eine Hemmung der CYP3B-abhängigen Konversion mittels Trilostane unterdrückt die Bildung von weiteren

Androgenen in NCI-h295R Zellen allerdings effektiv, sodass DHEA größtenteils zu DHEAS konvertiert wurde. Hieraus ergeben sich vielfältige Möglichkeiten, z.B. den Einfluss von Zytokinen oder von endokrinen Disruptoren auf die Sulfonierung von DHEA und auf die Expression von PAPSS1/2 zu untersuchen. Insbesondere kann aufgrund der ähnlichen Expression von PAPSS1 und PAPSS2 in dieser Zelllinie untersucht werden, welche Auswirkung ein Ausschalten eines Enzyms mittels siRNA auf das jeweils andere hat.

Die Sulfonierung von DHEA durch SULT2A1 geschieht im Zytoplasma bzw. im Golgi Apparat. Allerdings haben Untersuchungen an transfizierten Zelllinien gezeigt, dass PAPSS1 bzw. PAPSS2 sowohl im Plasma als auch nukleär vorliegen können, wenn beide gleichzeitig exprimiert waren. Mittels Immunzytochemie konnten diese Ergebnisse auch in der Zelllinie NCI-h295R nachgewiesen werden. Beide Enzyme sind auch hier vor allem nukleär exprimiert. Der physiologische Hintergrund dieser Lokalisierung ist nicht geklärt und erfordert weitere Erforschung. Vermutlich erfolgt die Sulfat-Aktivierung also im Nukleus. Möglicherweise stellt die Verlagerung der Enzyme in den Nukleus aber auch eine Reserve der PAPS Synthese dar, die durch Rückverlagerung ins Zytoplasma dort rasch zusätzliches PAPS zur Verfügung stellen kann.

Die Expression der DHEA Sulfotransferase wurde bereits in einigen fötalen Geweben untersucht. Während in adultem Knorpel beim Menschen die Expression von PAPSS1 dominiert, wird z.B. im Knorpel von neugeborenen Hamstern vor allem PAPSS2 gebildet. Welche Isoform von PAPSS in welchen fötalen Geweben beim Menschen dominiert, wurde bislang nicht untersucht. In dieser Arbeit konnte mittels Realtime PCR eine differenzierte Expression von *SULT2A1*, *PAPSS1* und *PAPSS2* in fötalen Geweben nachgewiesen werden. In adultem und fötalem Gewebe der Nebennieren zeigte sich ein ähnliches Expressionsmuster. Während allerdings in der adulten Leber viel SULT2A1 vorhanden ist, konnte nur eine deutlich niedrigere Expression in fötalem Gewebe gezeigt werden. In fötalem Knorpel findet sich kaum SULT2A1. Dagegen wird in fötalem Knorpel deutlich mehr PAPSS1 gebildet als in adultem und fötalem Leber- bzw. Nebennieren-Gewebe. PAPSS2 ist sowohl beim Erwachsenen als auch beim Fötus hauptsächlich in der Nebenniere exprimiert. Auffällig ist eine relativ geringe Expression in der fötalen Leber.

Ergänzend wird in dieser Arbeit eine Patientin mit Hyperandrogenismus vorgestellt, bei der zwei Mutationen im PAPSS2 Gen zu einem massiv erniedrigten DHEAS Spiegel geführt hatten. Eine heterozygote Mutation liegt im Bereich der APS-Kinase von PAPSS2 und führt zum Austausch einer Aminosäure an Position 48 im PAPSS2a Protein (T48R). *In vitro* konnte für diese Mutation eine Reduktion der Aktivität auf 6% nachgewiesen werden. Eine zweite Mutation fand sich in der ATP Sulfurylase Domäne von PAPSS2. Durch einen Nukleosid-Austausch (Thymidin statt Cytidin) entsteht ein Stop-Codon, was vermutlich an Position 329 zum Abbruch des Proteins führt (R329X). *In vitro* konnte für diese Mutation (R329X) keine Aktivität nachgewiesen werden. Durch das Fehlen von PAPS ist die Sulfonierung von Proteoglykanen im Knorpel gestört, was zu Skelettveränderungen führt. Vor allem aber kommt es durch das Fehlen der Inaktivierung von DHEA zu DHEAS zu einem Überangebot an DHEA. Dieses wird zu aktiven Androgenen konvertiert und verursacht klinisch einen Hyperandrogenismus. Interessant und überraschend ist, dass die PAPSS1-Aktivität im Knorpel eine gewisse Sulfonierung der Proteoglykane ermöglicht. Im Gegensatz dazu trägt PAPSS1 offensichtlich kaum zur Sulfonierung von DHEA bei, da der DHEAS Spiegel extrem niedrig ist. Möglicherweise spielt hier auch die subzelluläre Lokalisation der PAPS Synthase eine entscheidende Rolle.

7 Appendix

7.1 References

1. McNicol, A.M., *Lesions of the adrenal cortex.* Arch Pathol Lab Med, 2008. **132**(8): p. 1263-71.
2. Orth, D.N. and W.J. Kovacs, *The adrenal cortex*, in *Williams textbook of endocrinology*, R.H. Williams and J.D. Wilson, Editors. 1998, Saunders: Philadelphia. p. 517-547.
3. Laragh, J.H., *Atrial natriuretic hormone, the renin-aldosterone axis, and blood pressure-electrolyte homeostasis.* N Engl J Med, 1985. **313**(21): p. 1330-40.
4. Ehrhart-Bornstein, M. and S.R. Bornstein, *Cross-talk between adrenal medulla and adrenal cortex in stress.* Ann N Y Acad Sci, 2008. **1148**: p. 112-7.
5. Miller, W.L., *Androgen synthesis in adrenarche.* Rev Endocr Metab Disord, 2009. **10**(1): p. 3-17.
6. Nakamura, Y., et al., *Type 5 17beta-hydroxysteroid dehydrogenase (AKR1C3) contributes to testosterone production in the adrenal reticularis.* J Clin Endocrinol Metab, 2009. **94**(6): p. 2192-8.
7. Sucheston, M.E. and M.S. Cannon, *Development of zonular patterns in the human adrenal gland.* J Morphol, 1968. **126**(4): p. 477-91.
8. Mesiano, S. and R.B. Jaffe, *Developmental and functional biology of the primate fetal adrenal cortex.* Endocr Rev, 1997. **18**(3): p. 378-403.
9. Narasaka, T., et al., *Temporal and spatial distribution of corticosteroidogenic enzymes immunoreactivity in developing human adrenal.* Mol Cell Endocrinol, 2001. **174**(1-2): p. 111-20.
10. Kempna, P. and C.E. Flück, *Adrenal gland development and defects.* Best Pract Res Clin Endocrinol Metab, 2008. **22**(1): p. 77-93.
11. Allolio, B. and W. Arlt, *DHEA treatment: myth or reality?* Trends Endocrinol Metab, 2002. **13**(7): p. 288-94.
12. Parker, C.R., et al., *Aging alters zonation in the adrenal cortex of men.* The Journal of clinical endocrinology and metabolism, 1997. **82**(11): p. 3898-3901.
13. Nakamura, Y., et al., *Adrenal changes associated with adrenarche.* Reviews in endocrine & metabolic disorders, 2008.
14. Rainey, W.E., et al., *Dissecting human adrenal androgen production.* Trends in endocrinology and metabolism: TEM, 2002. **13**(6): p. 234-239.
15. Remer, T., et al., *Urinary markers of adrenarche: reference values in healthy subjects, aged 3-18 years.* J Clin Endocrinol Metab, 2005. **90**(4): p. 2015-21.
16. Idkowiak, J., et al., *Premature adrenarche - novel lessons from early onset androgen excess.* Eur J Endocrinol, 2011.
17. Hochberg, Z., *Evo-Devo of child growth III: premature juvenility as an evolutionary trade-off.* Horm Res Paediatr, 2010. **73**(6): p. 430-7.
18. Campbell, B., *Adrenarche in comparative perspective.* Am J Hum Biol, 2011. **23**(1): p. 44-52.
19. Hechter, O., et al., *Transformation of cholesterol and acetate to adrenal cortical hormones.* Arch Biochem Biophys, 1953. **46**(1): p. 201-14.

20. Brown, M.S., P.T. Kovanen, and J.L. Goldstein, *Receptor-mediated uptake of lipoprotein-cholesterol and its utilization for steroid synthesis in the adrenal cortex.* Recent Prog Horm Res, 1979. **35**: p. 215-57.
21. Miller, W.L., *Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter.* Biochim Biophys Acta, 2007. **1771**(6): p. 663-76.
22. Miller, W.L., *Androgen biosynthesis from cholesterol to DHEA.* Molecular and cellular endocrinology, 2002. **198**(1-2): p. 7-14.
23. Miller, W.L., *Molecular biology of steroid hormone synthesis.* Endocr Rev, 1988. **9**(3): p. 295-318.
24. Orentreich, N., et al., *Age changes and sex differences in serum dehydroepiandrosterone sulfate concentrations throughout adulthood.* J Clin Endocrinol Metab, 1984. **59**(3): p. 551-5.
25. Labrie, F., et al., *DHEA and the intracrine formation of androgens and estrogens in peripheral target tissues: its role during aging.* Steroids. **63**(5-6): p. 322-328.
26. Dharia, S. and C.R. Parker, *Adrenal androgens and aging.* Seminars in reproductive medicine, 2004. **22**(4): p. 361-368.
27. Arlt, W., *Dehydroepiandrosterone replacement therapy.* Current Opinion in Endocrinology, Diabetes and Obesity, 2006. **13**(3): p. 291-305 10.1097/01.med.0000224811.39318.73.
28. Labrie, F., *Intracrinology.* Mol Cell Endocrinol, 1991. **78**(3): p. C113-8.
29. Labrie, F., *Adrenal androgens and intracrinology.* Seminars in reproductive medicine, 2005. **22**(4): p. 299-309.
30. Fitzpatrick, J.L., et al., *Metabolism of DHEA by cytochromes P450 in rat and human liver microsomal fractions.* Arch Biochem Biophys, 2001. **389**(2): p. 278-87.
31. Selcer, K.W., et al., *Immunohistochemical analysis of steroid sulfatase in human tissues.* J Steroid Biochem Mol Biol, 2007. **105**(1-5): p. 115-23.
32. Gniot-Szulzycka, J. and B. Januszewska, *Purification of steroid sulphohydrolase from human placenta microsomes.* Acta Biochim Pol, 1986. **33**(3): p. 203-15.
33. Farnsworth, W.E., *Human prostatic dehydroepiandrosterone sulfate sulfatase.* Steroids, 1973. **21**(5): p. 647-64.
34. Steckelbroeck, S., et al., *Steroid sulfatase (STS) expression in the human temporal lobe: enzyme activity, mRNA expression and immunohistochemistry study.* Journal of neurochemistry, 2004. **89**(2): p. 403-417.
35. Reed, M.J., et al., *Steroid sulfatase: molecular biology, regulation, and inhibition.* Endocrine reviews, 2005. **26**(2): p. 171-202.
36. Hammer, F., et al., *No evidence for hepatic conversion of dehydroepiandrosterone (DHEA) sulfate to DHEA: in vivo and in vitro studies.* The Journal of clinical endocrinology and metabolism, 2005. **90**(6): p. 3600-3605.
37. Arlt, W., *Androgen therapy in women.* Eur J Endocrinol, 2006. **154**(1): p. 1-11.
38. Arlt, W., *Dehydroepiandrosterone and ageing.* Best practice & research. Clinical endocrinology & metabolism, 2004. **18**(3): p. 363-380.
39. Deslypere, J.P., et al., *Testosterone and 5 alpha-dihydrotestosterone interact differently with the androgen receptor to enhance transcription of the MMTV-CAT reporter gene.* Mol Cell Endocrinol, 1992. **88**(1-3): p. 15-22.

40. Labrie, F., et al., *Physiological changes in dehydroepiandrosterone are not reflected by serum levels of active androgens and estrogens but of their metabolites: intracrinology.* J Clin Endocrinol Metab, 1997. **82**(8): p. 2403-9.
41. Labrie, F., et al., *Changes in serum DHEA and eleven of its metabolites during 12-month percutaneous administration of DHEA.* The Journal of steroid biochemistry and molecular biology, 2008. **110**(1-2): p. 1-9.
42. Labrie, F., et al., *Structure, regulation and role of 3 beta-hydroxysteroid dehydrogenase, 17 beta-hydroxysteroid dehydrogenase and aromatase enzymes in the formation of sex steroids in classical and peripheral intracrine tissues.* Baillieres Clin Endocrinol Metab, 1994. **8**(2): p. 451-74.
43. Arlt, W., et al., *Oral dehydroepiandrosterone for adrenal androgen replacement: pharmacokinetics and peripheral conversion to androgens and estrogens in young healthy females after dexamethasone suppression.* J Clin Endocrinol Metab, 1998. **83**(6): p. 1928-34.
44. Arlt, W., et al., *Biotransformation of oral dehydroepiandrosterone in elderly men: significant increase in circulating estrogens.* J Clin Endocrinol Metab, 1999. **84**(6): p. 2170-6.
45. Arlt, W. and M. Hewison, *Hormones and immune function: implications of aging.* Aging cell, 2004. **3**(4): p. 209-216.
46. Loria, R.M., *Immune up-regulation and tumor apoptosis by androstene steroids.* Steroids, 2002. **67**(12): p. 953-66.
47. Rose, K.A., et al., *Cyp7b, a novel brain cytochrome P450, catalyzes the synthesis of neurosteroids 7alpha-hydroxy dehydroepiandrosterone and 7alpha-hydroxy pregnenolone.* Proc Natl Acad Sci U S A, 1997. **94**(10): p. 4925-30.
48. Muller, C., et al., *Inter-conversion of 7alpha- and 7beta-hydroxy-dehydroepiandrosterone by the human 11beta-hydroxysteroid dehydrogenase type 1.* J Steroid Biochem Mol Biol, 2006. **99**(4-5): p. 215-22.
49. Goodarzi, M.O., N. Xu, and R. Azziz, *Association of CYP3A7*1C and serum dehydroepiandrosterone sulfate levels in women with polycystic ovary syndrome.* J Clin Endocrinol Metab, 2008. **93**(7): p. 2909-12.
50. Miller, K.K., et al., *Stereo- and regioselectivity account for the diversity of dehydroepiandrosterone (DHEA) metabolites produced by liver microsomal cytochromes P450.* Drug Metab Dispos, 2004. **32**(3): p. 305-13.
51. Widstrom, R.L. and J.S. Dillon, *Is there a receptor for dehydroepiandrosterone or dehydroepiandrosterone sulfate?* Semin Reprod Med, 2004. **22**(4): p. 289-98.
52. Webb, S.J., et al., *The biological actions of dehydroepiandrosterone involves multiple receptors.* Drug Metab Rev, 2006. **38**(1-2): p. 89-116.
53. Morissette, M., et al., *Effect of dehydroepiandrosterone and its sulfate and fatty acid ester derivatives on rat brain membranes.* Steroids, 1999. **64**(11): p. 796-803.
54. Peng, W., J.R. Hoidal, and I.S. Farrukh, *Role of a novel KCa opener in regulating K+ channels of hypoxic human pulmonary vascular cells.* Am J Respir Cell Mol Biol, 1999. **20**(4): p. 737-45.
55. Gordon, G., M.C. Mackow, and H.R. Levy, *On the mechanism of interaction of steroids with human glucose 6-phosphate dehydrogenase.* Arch Biochem Biophys, 1995. **318**(1): p. 25-9.

56. Meikle, A.W., et al., *The presence of a dehydroepiandrosterone-specific receptor binding complex in murine T cells.* J Steroid Biochem Mol Biol, 1992. **42**(3-4): p. 293-304.
57. Okabe, T., et al., *Up-regulation of high-affinity dehydroepiandrosterone binding activity by dehydroepiandrosterone in activated human T lymphocytes.* J Clin Endocrinol Metab, 1995. **80**(10): p. 2993-6.
58. Liu, D., et al., *Dehydroepiandrosterone inhibits intracellular calcium release in beta-cells by a plasma membrane-dependent mechanism.* Steroids, 2006. **71**(8): p. 691-9.
59. Charalampopoulos, I., et al., *G protein-associated, specific membrane binding sites mediate the neuroprotective effect of dehydroepiandrosterone.* FASEB J, 2006. **20**(3): p. 577-9.
60. Liu, D. and J.S. Dillon, *Dehydroepiandrosterone activates endothelial cell nitric-oxide synthase by a specific plasma membrane receptor coupled to Galpha(i2,3).* J Biol Chem, 2002. **277**(24): p. 21379-88.
61. Alexaki, V.I., et al., *Dehydroepiandrosterone protects human keratinocytes against apoptosis through membrane binding sites.* Exp Cell Res, 2009. **315**(13): p. 2275-83.
62. Ueda, H., et al., *Neurosteroids stimulate G protein-coupled sigma receptors in mouse brain synaptic membrane.* Neurosci Res, 2001. **41**(1): p. 33-40.
63. Liu, D., et al., *Dehydroepiandrosterone stimulates endothelial proliferation and angiogenesis through extracellular signal-regulated kinase 1/2-mediated mechanisms.* Endocrinology, 2008. **149**(3): p. 889-898.
64. Simoncini, T., et al., *Dehydroepiandrosterone modulates endothelial nitric oxide synthesis via direct genomic and nongenomic mechanisms.* Endocrinology, 2003. **144**(8): p. 3449-55.
65. Baulieu, E.E. and P. Robel, *Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) as neuroactive neurosteroids.* Proc Natl Acad Sci U S A, 1998. **95**(8): p. 4089-91.
66. Corpechot, C., et al., *Characterization and measurement of dehydroepiandrosterone sulfate in rat brain.* Proc Natl Acad Sci U S A, 1981. **78**(8): p. 4704-7.
67. Zwain, I.H. and S.S. Yen, *Dehydroepiandrosterone: biosynthesis and metabolism in the brain.* Endocrinology, 1999. **140**(2): p. 880-7.
68. Stromstedt, M. and M.R. Waterman, *Messenger RNAs encoding steroidogenic enzymes are expressed in rodent brain.* Brain Res Mol Brain Res, 1995. **34**(1): p. 75-88.
69. MacKenzie, S.M., et al., *The transcription of steroidogenic genes in the human cerebellum and hippocampus: a comparative survey of normal and Alzheimer's tissue.* J Endocrinol, 2008. **196**(1): p. 123-30.
70. Cascio, C., et al., *Detection of P450c17-independent pathways for dehydroepiandrosterone (DHEA) biosynthesis in brain glial tumor cells.* Proc Natl Acad Sci U S A, 1998. **95**(6): p. 2862-7.
71. Maninger, N., et al., *Neurobiological and neuropsychiatric effects of dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS).* Front Neuroendocrinol, 2009. **30**(1): p. 65-91.

72. Imamura, M. and C. Prasad, *Modulation of GABA-gated chloride ion influx in the brain by dehydroepiandrosterone and its metabolites.* Biochem Biophys Res Commun, 1998. **243**(3): p. 771-5.
73. Zinder, O. and D.E. Dar, *Neuroactive steroids: their mechanism of action and their function in the stress response.* Acta Physiol Scand, 1999. **167**(3): p. 181-8.
74. Stephenson, F.A., *Structure and trafficking of NMDA and GABAA receptors.* Biochem Soc Trans, 2006. **34**(Pt 5): p. 877-81.
75. Mayer, M.L. and G.L. Westbrook, *Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones.* J Physiol, 1987. **394**: p. 501-27.
76. Monnet, F.P., et al., *Neurosteroids, via sigma receptors, modulate the [3H]norepinephrine release evoked by N-methyl-D-aspartate in the rat hippocampus.* Proc Natl Acad Sci U S A, 1995. **92**(9): p. 3774-8.
77. Bergeron, R., C. de Montigny, and G. Debonnel, *Potentiation of neuronal NMDA response induced by dehydroepiandrosterone and its suppression by progesterone: effects mediated via sigma receptors.* J Neurosci, 1996. **16**(3): p. 1193-202.
78. Kurata, K., et al., *beta-estradiol, dehydroepiandrosterone, and dehydroepiandrosterone sulfate protect against N-methyl-D-aspartate-induced neurotoxicity in rat hippocampal neurons by different mechanisms.* J Pharmacol Exp Ther, 2004. **311**(1): p. 237-45.
79. Hayashi, T. and T.P. Su, *Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival.* Cell, 2007. **131**(3): p. 596-610.
80. Maurice, T. and T.P. Su, *The pharmacology of sigma-1 receptors.* Pharmacol Ther, 2009. **124**(2): p. 195-206.
81. Dong, L., et al., *Neuroactive steroid dehydroepiandrosterone sulfate inhibits 5-hydroxytryptamine (5-HT)-evoked glutamate release via activation of sigma-1 receptors and then inhibition of 5-HT3 receptors in rat prelimbic cortex.* J Pharmacol Exp Ther, 2009. **330**(2): p. 494-501.
82. Rhodes, M.E., et al., *Enhancement of hippocampal acetylcholine release by the neurosteroid dehydroepiandrosterone sulfate: an in vivo microdialysis study.* Brain Res, 1996. **733**(2): p. 284-6.
83. Zheng, P., *Neuroactive steroid regulation of neurotransmitter release in the CNS: action, mechanism and possible significance.* Prog Neurobiol, 2009. **89**(2): p. 134-52.
84. Enomoto, M., et al., *Serum dehydroepiandrosterone sulfate levels predict longevity in men: 27-year follow-up study in a community-based cohort (Tanushimaru study).* J Am Geriatr Soc, 2008. **56**(6): p. 994-8.
85. Baulieu, E.E., et al., *Dehydroepiandrosterone (DHEA), DHEA sulfate, and aging: contribution of the DHEAge Study to a sociobiomedical issue.* Proc Natl Acad Sci U S A, 2000. **97**(8): p. 4279-84.
86. Nair, K.S., et al., *DHEA in elderly women and DHEA or testosterone in elderly men.* The New England journal of medicine, 2006. **355**(16): p. 1647-1659.
87. von Mühlen, D., et al., *Effect of dehydroepiandrosterone supplementation on bone mineral density, bone markers, and body composition in older adults: the DAWN trial.* Osteoporosis international : a journal established as result of

cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA, 2008. **19**(5): p. 699-707.
88. Kritz-Silverstein, D., et al., *Effects of dehydroepiandrosterone supplementation on cognitive function and quality of life: the DHEA and Well-Ness (DAWN) Trial.* J Am Geriatr Soc, 2008. **56**(7): p. 1292-8.
89. Cleary, M.P., A. Shepherd, and B. Jenks, *Effect of dehydroepiandrosterone on growth in lean and obese Zucker rats.* J Nutr, 1984. **114**(7): p. 1242-51.
90. Cleary, M.P., T. Zabel, and J.L. Sartin, *Effects of short-term dehydroepiandrosterone treatment on serum and pancreatic insulin in Zucker rats.* J Nutr, 1988. **118**(3): p. 382-7.
91. Lea-Currie, Y.R., P. Wen, and M.K. McIntosh, *Dehydroepiandrosterone-sulfate (DHEAS) reduces adipocyte hyperplasia associated with feeding rats a high-fat diet.* Int J Obes Relat Metab Disord, 1997. **21**(11): p. 1058-64.
92. Mohan, P.F. and M.P. Cleary, *Effect of short-term DHEA administration on liver metabolism of lean and obese rats.* Am J Physiol, 1988. **255**(1 Pt 1): p. E1-8.
93. Tagliaferro, A.R., et al., *Effects of dehydroepiandrosterone acetate on metabolism, body weight and composition of male and female rats.* J Nutr, 1986. **116**(10): p. 1977-83.
94. Lea-Currie, Y.R., P. Wen, and M.K. McIntosh, *Dehydroepiandrosterone reduces proliferation and differentiation of 3T3-L1 preadipocytes.* Biochem Biophys Res Commun, 1998. **248**(3): p. 497-504.
95. Gordon, G.B., et al., *Inhibition of the conversion of 3T3 fibroblast clones to adipocytes by dehydroepiandrosterone and related anticarcinogenic steroids.* Cancer Res, 1986. **46**(7): p. 3389-95.
96. Shantz, L.M., P. Talalay, and G.B. Gordon, *Mechanism of inhibition of growth of 3T3-L1 fibroblasts and their differentiation to adipocytes by dehydroepiandrosterone and related steroids: role of glucose-6-phosphate dehydrogenase.* Proc Natl Acad Sci U S A, 1989. **86**(10): p. 3852-6.
97. Ishizuka, T., et al., *DHEA improves glucose uptake via activations of protein kinase C and phosphatidylinositol 3-kinase.* Am J Physiol, 1999. **276**(1 Pt 1): p. E196-204.
98. Coleman, D.L., E.H. Leiter, and R.W. Schwizer, *Therapeutic effects of dehydroepiandrosterone (DHEA) in diabetic mice.* Diabetes, 1982. **31**(9): p. 830-833.
99. Sato, K., et al., *Testosterone and DHEA activate the glucose metabolism-related signaling pathway in skeletal muscle.* American journal of physiology. Endocrinology and metabolism, 2008. **294**(5): p. E961-8.
100. Yamashita, R., et al., *Effects of dehydroepiandrosterone on gluconeogenic enzymes and glucose uptake in human hepatoma cell line, HepG2.* Endocrine journal, 2005. **52**(6): p. 727-733.
101. Aoki, K., et al., *Dehydroepiandrosterone suppresses elevated hepatic glucose-6-phosphatase mRNA level in C57BL/KsJ-db/db mice: comparison with troglitazone.* Endocr J, 2000. **47**(6): p. 799-804.
102. Brennan, K., A. Huang, and R. Azziz, *Dehydroepiandrosterone sulfate and insulin resistance in patients with polycystic ovary syndrome.* Fertility and sterility, 2008.

103. Villareal, D.T. and J.O. Holloszy, *Effect of DHEA on abdominal fat and insulin action in elderly women and men: a randomized controlled trial.* JAMA, 2004. **292**(18): p. 2243-8.
104. Morales, A.J., et al., *The effect of six months treatment with a 100 mg daily dose of dehydroepiandrosterone (DHEA) on circulating sex steroids, body composition and muscle strength in age-advanced men and women.* Clin Endocrinol (Oxf), 1998. **49**(4): p. 421-32.
105. Yen, S.S., A.J. Morales, and O. Khorram, *Replacement of DHEA in aging men and women. Potential remedial effects.* Ann N Y Acad Sci, 1995. **774**: p. 128-42.
106. Arlt, W., et al., *Dehydroepiandrosterone supplementation in healthy men with an age-related decline of dehydroepiandrosterone secretion.* J Clin Endocrinol Metab, 2001. **86**(10): p. 4686-92.
107. Basu, R., et al., *Two years of treatment with dehydroepiandrosterone does not improve insulin secretion, insulin action, or postprandial glucose turnover in elderly men or women.* Diabetes, 2007. **56**(3): p. 753-766.
108. Butcher, S.K., et al., *Raised cortisol:DHEAS ratios in the elderly after injury: potential impact upon neutrophil function and immunity.* Aging Cell, 2005. **4**(6): p. 319-24.
109. Hazeldine, J., W. Arlt, and J.M. Lord, *Dehydroepiandrosterone as a regulator of immune cell function.* J Steroid Biochem Mol Biol, 2010.
110. Altman, R., et al., *Inhibition of vascular inflammation by dehydroepiandrosterone sulfate in human aortic endothelial cells: roles of PPARalpha and NF-kappaB.* Vascul Pharmacol, 2008. **48**(2-3): p. 76-84.
111. Apostolova, G., et al., *Dehydroepiandrosterone inhibits the amplification of glucocorticoid action in adipose tissue.* Am J Physiol Endocrinol Metab, 2005. **288**(5): p. E957-64.
112. Mysliwska, J., et al., *Increase of interleukin 6 and decrease of interleukin 2 production during the ageing process are influenced by the health status.* Mech Ageing Dev, 1998. **100**(3): p. 313-28.
113. Jones, S.A., *Directing transition from innate to acquired immunity: defining a role for IL-6.* J Immunol, 2005. **175**(6): p. 3463-8.
114. Straub, R.H., et al., *Serum dehydroepiandrosterone (DHEA) and DHEA sulfate are negatively correlated with serum interleukin-6 (IL-6), and DHEA inhibits IL-6 secretion from mononuclear cells in man in vitro: possible link between endocrinosenescence and immunosenescence.* J Clin Endocrinol Metab, 1998. **83**(6): p. 2012-7.
115. Waage, A., G. Slupphaug, and R. Shalaby, *Glucocorticoids inhibit the production of IL6 from monocytes, endothelial cells and fibroblasts.* Eur J Immunol, 1990. **20**(11): p. 2439-43.
116. Radford, D.J., et al., *Dehdyroepiandrosterone sulfate directly activates protein kinase C-beta to increase human neutrophil superoxide generation.* Mol Endocrinol, 2010. **24**(4): p. 813-21.
117. Oberbeck, R., et al., *Dehydroepiandrosterone decreases mortality rate and improves cellular immune function during polymicrobial sepsis.* Crit Care Med, 2001. **29**(2): p. 380-4.
118. Ben-Nathan, D., et al., *Protection by dehydroepiandrosterone in mice infected with viral encephalitis.* Arch Virol, 1991. **120**(3-4): p. 263-71.

119. Ben-Nathan, D., D.A. Padgett, and R.M. Loria, *Androstenediol and dehydroepiandrosterone protect mice against lethal bacterial infections and lipopolysaccharide toxicity.* J Med Microbiol, 1999. **48**(5): p. 425-31.
120. Chang, C.C., et al., *Antiviral effect of dehydroepiandrosterone on Japanese encephalitis virus infection.* J Gen Virol, 2005. **86**(Pt 9): p. 2513-23.
121. Arlt, W., et al., *Dissociation of serum dehydroepiandrosterone and dehydroepiandrosterone sulfate in septic shock.* The Journal of clinical endocrinology and metabolism, 2006. **91**(7): p. 2548-2554.
122. Kim, M.S., et al., *Suppression of DHEA sulfotransferase (Sult2A1) during the acute-phase response.* American journal of physiology. Endocrinology and metabolism, 2004. **287**(4): p. E731-8.
123. Narvaez, J., et al., *Low serum levels of DHEAS in untreated polymyalgia rheumatica/giant cell arteritis.* J Rheumatol, 2006. **33**(7): p. 1293-8.
124. Sullivan, D.A., et al., *Are women with Sjögren's syndrome androgen-deficient?* The Journal of rheumatology, 2003. **30**(11): p. 2413-2419.
125. Schmidt, M., et al., *Inflammation and sex hormone metabolism.* Annals of the New York Academy of Sciences, 2006. **1069**: p. 236-246.
126. Chang, D.M., et al., *Dehydroepiandrosterone suppresses interleukin 10 synthesis in women with systemic lupus erythematosus.* Annals of the rheumatic diseases, 2004. **63**(12): p. 1623-1626.
127. Petri, M.A., et al., *Effects of prasterone on disease activity and symptoms in women with active systemic lupus erythematosus.* Arthritis Rheum, 2004. **50**(9): p. 2858-68.
128. Chang, D.-M., et al., *Dehydroepiandrosterone treatment of women with mild-to-moderate systemic lupus erythematosus: a multicenter randomized, double-blind, placebo-controlled trial.* Arthritis and rheumatism, 2002. **46**(11): p. 2924-2927.
129. Sawalha, A.H. and S. Kovats, *Dehydroepiandrosterone in systemic lupus erythematosus.* Current rheumatology reports, 2008. **10**(4): p. 286-291.
130. Kasperska-Zajac, A., Z. Brzoza, and B. Rogala, *Dehydroepiandrosterone and dehydroepiandrosterone sulphate in atopic allergy and chronic urticaria.* Inflammation, 2008. **31**(3): p. 141-145.
131. Daynes, R.A., D.J. Dudley, and B.A. Araneo, *Regulation of murine lymphokine production in vivo. II. Dehydroepiandrosterone is a natural enhancer of interleukin 2 synthesis by helper T cells.* Eur J Immunol, 1990. **20**(4): p. 793-802.
132. Tabata, N., H. Tagami, and T. Terui, *Dehydroepiandrosterone may be one of the regulators of cytokine production in atopic dermatitis.* Arch Dermatol Res, 1997. **289**(7): p. 410-4.
133. Yu, C.K., et al., *Attenuation of house dust mite Dermatophagoides farinae-induced airway allergic responses in mice by dehydroepiandrosterone is correlated with down-regulation of TH2 response.* Clin Exp Allergy, 1999. **29**(3): p. 414-22.
134. Yu, C.K., Y.H. Liu, and C.L. Chen, *Dehydroepiandrosterone attenuates allergic airway inflammation in Dermatophagoides farinae-sensitized mice.* J Microbiol Immunol Infect, 2002. **35**(3): p. 199-202.
135. Kasperska-Zajac, A., Z. Brzoza, and B. Rogala, *Serum concentration of dehydroepiandrosterone sulfate and testosterone in women with severe atopic*

eczema/dermatitis syndrome. Journal of investigational allergology & clinical immunology : official organ of the International Association of Asthmology (INTERASMA) and Sociedad Latinoamericana de Alergia e Inmunología, 2007. **17**(3): p. 160-163.
136. Schulz, S., et al., *Mechanisms of cell growth inhibition and cell cycle arrest in human colonic adenocarcinoma cells by dehydroepiandrosterone: role of isoprenoid biosynthesis.* Cancer Res, 1992. **52**(5): p. 1372-6.
137. Liu, S., et al., *Dehydroepiandrosterone can inhibit the proliferation of myeloma cells and the interleukin-6 production of bone marrow mononuclear cells from patients with myeloma.* Cancer research, 2005. **65**(6): p. 2269-2276.
138. Solano, M.E., et al., *Dehydroepiandrosterone and metformin regulate proliferation of murine T lymphocytes.* Clin Exp Immunol, 2008. **153**(2): p. 289-96.
139. Hinson, J.P. and M. Khan, *Dehydroepiandrosterone sulphate (DHEAS) inhibits growth of human vascular endothelial cells.* Endocr Res, 2004. **30**(4): p. 667-71.
140. Sicard, F., et al., *Age-dependent regulation of chromaffin cell proliferation by growth factors, dehydroepiandrosterone (DHEA), and DHEA sulfate.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(6): p. 2007-2012.
141. Gayosso, V., L.F. Montano, and R. Lopez-Marure, *DHEA-induced antiproliferative effect in MCF-7 cells is androgen- and estrogen receptor-independent.* Cancer J, 2006. **12**(2): p. 160-5.
142. Flood, J.F. and E. Roberts, *Dehydroepiandrosterone sulfate improves memory in aging mice.* Brain Res, 1988. **448**(1): p. 178-81.
143. Melchior, C.L. and R.F. Ritzmann, *Neurosteroids block the memory-impairing effects of ethanol in mice.* Pharmacol Biochem Behav, 1996. **53**(1): p. 51-6.
144. Frye, C.A. and J.D. Sturgis, *Neurosteroids affect spatial/reference, working, and long-term memory of female rats.* Neurobiol Learn Mem, 1995. **64**(1): p. 83-96.
145. Davis, S.R., et al., *Dehydroepiandrosterone sulfate levels are associated with more favorable cognitive function in women.* J Clin Endocrinol Metab, 2008. **93**(3): p. 801-8.
146. Valenti, G., et al., *Dehydroepiandrosterone sulfate and cognitive function in the elderly: The InCHIANTI Study.* J Endocrinol Invest, 2009. **32**(9): p. 766-72.
147. Markianos, M., et al., *Plasma testosterone and dehydroepiandrosterone sulfate in male and female patients with dysthymic disorder.* J Affect Disord, 2007. **101**(1-3): p. 255-8.
148. Ritsner, M., et al., *Alterations in DHEA metabolism in schizophrenia: two-month case-control study.* Eur Neuropsychopharmacol, 2006. **16**(2): p. 137-46.
149. Silver, H., et al., *Blood DHEAS concentrations correlate with cognitive function in chronic schizophrenia patients: a pilot study.* J Psychiatr Res, 2005. **39**(6): p. 569-75.
150. Strous, R.D., et al., *Dehydroepiandrosterone augmentation in the management of negative, depressive, and anxiety symptoms in schizophrenia.* Arch Gen Psychiatry, 2003. **60**(2): p. 133-41.
151. Arlt, W., et al., *Dehydroepiandrosterone replacement in women with adrenal insufficiency.* N Engl J Med, 1999. **341**(14): p. 1013-20.

152. Gurnell, E.M., et al., *Long-term DHEA replacement in primary adrenal insufficiency: a randomized, controlled trial.* J Clin Endocrinol Metab, 2008. **93**(2): p. 400-9.
153. Christensen, J.J., et al., *Long-term dehydroepiandrosterone substitution in female adrenocortical failure, body composition, muscle function and bone metabolism - a randomized trial.* Eur J Endocrinol, 2011.
154. Barrett-Connor, E., K.T. Khaw, and S.S. Yen, *A prospective study of dehydroepiandrosterone sulfate, mortality, and cardiovascular disease.* N Engl J Med, 1986. **315**(24): p. 1519-24.
155. Danforth, K.N., et al., *The association of plasma androgen levels with breast, ovarian and endometrial cancer risk factors among postmenopausal women.* Int J Cancer, 2010. **126**(1): p. 199-207.
156. Barrett-Connor, E. and D. Goodman-Gruen, *Dehydroepiandrosterone Sulfate Does Not Predict Cardiovascular Death in Postmenopausal Women : The Rancho Bernardo Study.* Circulation, 1995. **91**(6): p. 1757-1760.
157. Haffner, S.M., et al., *Sex hormones and DHEA-SO4 in relation to ischemic heart disease mortality in diabetic subjects. The Wisconsin Epidemiologic Study of Diabetic Retinopathy.* Diabetes Care, 1996. **19**(10): p. 1045-50.
158. Hautanen, A., et al., *Adrenal androgens and testosterone as coronary risk factors in the Helsinki Heart Study.* Atherosclerosis, 1994. **105**(2): p. 191-200.
159. LaCroix, A.Z., K. Yano, and D.M. Reed, *Dehydroepiandrosterone sulfate, incidence of myocardial infarction, and extent of atherosclerosis in men.* Circulation, 1992. **86**(5): p. 1529-35.
160. Khaw, K.T., *Dehydroepiandrosterone, dehydroepiandrosterone sulphate and cardiovascular disease.* J Endocrinol, 1996. **150 Suppl**: p. S149-53.
161. Bonnet, S., et al., *Dehydroepiandrosterone (DHEA) prevents and reverses chronic hypoxic pulmonary hypertension.* Proc Natl Acad Sci U S A, 2003. **100**(16): p. 9488-93.
162. Bonnet, S., et al., *Dehydroepiandrosterone reverses systemic vascular remodeling through the inhibition of the Akt/GSK3-beta/NFAT axis.* Circulation, 2009. **120**(13): p. 1231-40.
163. Ii, M., et al., *Adrenal androgen dehydroepiandrosterone sulfate inhibits vascular remodeling following arterial injury.* Atherosclerosis, 2009. **206**(1): p. 77-85.
164. Ng, M.K.C., et al., *Dehydroepiandrosterone, an adrenal androgen, increases human foam cell formation: a potentially pro-atherogenic effect.* Journal of the American College of Cardiology, 2003. **42**(11): p. 1967-1974.
165. Rice, S.P., et al., *Effects of dehydroepiandrosterone replacement on vascular function in primary and secondary adrenal insufficiency: a randomized crossover trial.* J Clin Endocrinol Metab, 2009. **94**(6): p. 1966-72.
166. Wang, L., et al., *Dehydroepiandrosterone improves murine osteoblast growth and bone tissue morphometry via mitogen-activated protein kinase signaling pathway independent of either androgen receptor or estrogen receptor.* J Mol Endocrinol, 2007. **38**(4): p. 467-79.
167. Kasperk, C.H., et al., *Gonadal and adrenal androgens are potent regulators of human bone cell metabolism in vitro.* J Bone Miner Res, 1997. **12**(3): p. 464-71.

168. Jankowski, C.M., et al., *Effects of dehydroepiandrosterone replacement therapy on bone mineral density in older adults: a randomized, controlled trial.* J Clin Endocrinol Metab, 2006. **91**(8): p. 2986-93.
169. Jankowski, C.M., et al., *Increases in bone mineral density in response to oral dehydroepiandrosterone replacement in older adults appear to be mediated by serum estrogens.* The Journal of clinical endocrinology and metabolism, 2008. **93**(12): p. 4767-4773.
170. Kullak-Ublick, G.A., et al., *Dehydroepiandrosterone sulfate (DHEAS): identification of a carrier protein in human liver and brain.* FEBS letters, 1998. **424**(3): p. 173-176.
171. Kullak-Ublick, G.A., et al., *Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver.* Gastroenterology, 2001. **120**(2): p. 525-33.
172. Konig, J., et al., *A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane.* Am J Physiol Gastrointest Liver Physiol, 2000. **278**(1): p. G156-64.
173. Abe, T., et al., *Identification of a novel gene family encoding human liver-specific organic anion transporter LST-1.* J Biol Chem, 1999. **274**(24): p. 17159-63.
174. Tamai, I., et al., *Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family.* Biochem Biophys Res Commun, 2000. **273**(1): p. 251-60.
175. Pizzagalli, F., et al., *Identification of a novel human organic anion transporting polypeptide as a high affinity thyroxine transporter.* Mol Endocrinol, 2002. **16**(10): p. 2283-96.
176. Sampath, J., et al., *Role of MRP4 and MRP5 in biology and chemotherapy.* AAPS PharmSci, 2002. **4**(3): p. E14.
177. Russel, F.G., J.B. Koenderink, and R. Masereeuw, *Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules.* Trends Pharmacol Sci, 2008. **29**(4): p. 200-7.
178. Borst, P., C. de Wolf, and K. van de Wetering, *Multidrug resistance-associated proteins 3, 4, and 5.* Pflugers Arch, 2007. **453**(5): p. 661-73.
179. Zelcer, N., et al., *Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4).* Biochem J, 2003. **371**(Pt 2): p. 361-7.
180. Benson, D.A., et al., *GenBank.* Nucleic Acids Res, 2005. **33**(Database issue): p. D34-8.
181. Reed, M.J., et al., *Steroid sulfatase: molecular biology, regulation, and inhibition.* Endocr Rev, 2005. **26**(2): p. 171-202.
182. Willemsen, R., et al., *Ultrastructural localization of steroid sulphatase in cultured human fibroblasts by immunocytochemistry: a comparative study with lysosomal enzymes and the mannose 6-phosphate receptor.* Histochem J, 1988. **20**(1): p. 41-51.
183. Martel, C., et al., *Widespread tissue distribution of steroid sulfatase, 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD), 17 beta-HSD 5 alpha-reductase and aromatase activities in the rhesus monkey.* Mol Cell Endocrinol, 1994. **104**(1): p. 103-11.

184. Kriz, L., M. Bicikova, and R. Hampl, *Roles of steroid sulfatase in brain and other tissues.* Physiol Res, 2008. **57**(5): p. 657-68.
185. Albrecht, E.D. and G.J. Pepe, *Placental steroid hormone biosynthesis in primate pregnancy.* Endocr Rev, 1990. **11**(1): p. 124-50.
186. Strott, C.A., *Sulfonation and molecular action.* Endocr Rev, 2002. **23**(5): p. 703-32.
187. Lindsay, J., et al., *Structure, function and polymorphism of human cytosolic sulfotransferases.* Current drug metabolism, 2008. **9**(2): p. 99-105.
188. Nowell, S. and C.N. Falany, *Pharmacogenetics of human cytosolic sulfotransferases.* Oncogene, 2006. **25**(11): p. 1673-8.
189. Radominska, A., et al., *Human liver steroid sulphotransferase sulphates bile acids.* Biochem J, 1990. **272**(3): p. 597-604.
190. Strott, C.A., *Steroid sulfotransferases.* Endocr Rev, 1996. **17**(6): p. 670-97.
191. Xu, Z., et al., *Human 3'-phosphoadenosine 5'-phosphosulfate synthetase: radiochemical enzymatic assay, biochemical properties, and hepatic variation.* Drug metabolism and disposition: the biological fate of chemicals, 2001. **29**(2): p. 172-178.
192. Noordam, C., et al., *Inactivating PAPSS2 mutations in a patient with premature pubarche.* N Engl J Med, 2009. **360**(22): p. 2310-8.
193. Xu, Z.H., et al., *Human 3'-phosphoadenosine 5'-phosphosulfate synthetase 1 (PAPSS1) and PAPSS2: gene cloning, characterization and chromosomal localization.* Biochemical and biophysical research communications, 2000. **268**(2): p. 437-444.
194. Fuda, H., et al., *Characterization and expression of human bifunctional 3'-phosphoadenosine 5'-phosphosulphate synthase isoforms.* Biochem J, 2002. **365**(Pt 2): p. 497-504.
195. Besset, S., et al., *Nuclear localization of PAPS synthetase 1: a sulfate activation pathway in the nucleus of eukaryotic cells.* The FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2000. **14**(2): p. 345-354.
196. Cho, Y.R., et al., *Under-sulfation by PAPS synthetase inhibition modulates the expression of ECM molecules during chondrogenesis.* Biochem Biophys Res Commun, 2004. **323**(3): p. 769-75.
197. ul Haque, M.F., et al., *Mutations in orthologous genes in human spondyloepimetaphyseal dysplasia and the brachymorphic mouse.* Nat Genet, 1998. **20**(2): p. 157-62.
198. Kurima, K., et al., *A member of a family of sulfate-activating enzymes causes murine brachymorphism.* Proc Natl Acad Sci U S A, 1998. **95**(15): p. 8681-5.
199. Ford-Hutchinson, A.F., et al., *Degenerative knee joint disease in mice lacking 3'-phosphoadenosine 5'-phosphosulfate synthetase 2 (Papss2) activity: a putative model of human PAPSS2 deficiency-associated arthrosis.* Osteoarthritis Cartilage, 2005. **13**(5): p. 418-25.
200. Stelzer, C., et al., *Expression profile of Papss2 (3'-phosphoadenosine 5'-phosphosulfate synthase 2) during cartilage formation and skeletal development in the mouse embryo.* Developmental dynamics : an official publication of the American Association of Anatomists, 2007. **236**(5): p. 1313-1318.
201. Freshney, R.I. and A. Capes-Davis. *Database of Cross-Contaminated or Misidentified Cell Lines*. [Web page] 2009 20/06/2009 [cited 2010 15/04/2010];

Available from: http://www.hpacultures.org.uk/media/E50/3B/Cell_Line_Cross_Contaminations_v6_0.pdf.
202. Rainey, W.E., et al., *Regulation of human adrenal carcinoma cell (NCI-H295) production of C19 steroids.* J Clin Endocrinol Metab, 1993. **77**(3): p. 731-7.
203. Rainey, W.E., K. Saner, and B.P. Schimmer, *Adrenocortical cell lines.* Molecular and cellular endocrinology, 2004. **228**(1-2): p. 23-38.
204. Temin, H.M. and S. Mizutani, *RNA-dependent DNA polymerase in virions of Rous sarcoma virus.* Nature, 1970. **226**(5252): p. 1211-3.
205. Baltimore, D., *RNA-dependent DNA polymerase in virions of RNA tumour viruses.* Nature, 1970. **226**(5252): p. 1209-11.
206. Chomczynski, P. and N. Sacchi, *Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.* Anal Biochem, 1987. **162**(1): p. 156-9.
207. Chomczynski, P. and N. Sacchi, *The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on.* Nat Protoc, 2006. **1**(2): p. 581-5.
208. Goto, M., et al., *In humans, early cortisol biosynthesis provides a mechanism to safeguard female sexual development.* The Journal of clinical investigation, 2006. **116**(4): p. 953-960.
209. Burnette, W.N., *"Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A.* Anal Biochem, 1981. **112**(2): p. 195-203.
210. Tueni, E., et al., *Endocrine effects of Trilostane: in vitro and in vivo studies.* Eur J Cancer Clin Oncol, 1987. **23**(10): p. 1461-7.
211. Abbott, D.H. and I.M. Bird, *Nonhuman primates as models for human adrenal androgen production: Function and dysfunction.* Reviews in endocrine & metabolic disorders, 2009. **10**(1): p. 33-42.
212. Assem, M., et al., *Interactions between hepatic Mrp4 and Sult2a as revealed by the constitutive androstane receptor and Mrp4 knockout mice.* The Journal of biological chemistry, 2004. **279**(21): p. 22250-22257.
213. Fang, H.L., et al., *Positive and negative regulation of human hepatic hydroxysteroid sulfotransferase (SULT2A1) gene transcription by rifampicin: roles of hepatocyte nuclear factor 4alpha and pregnane X receptor.* J Pharmacol Exp Ther, 2007. **323**(2): p. 586-98.
214. Huang, L.R., M.W. Coughtrie, and H.C. Hsu, *Down-regulation of dehydroepiandrosterone sulfotransferase gene in human hepatocellular carcinoma.* Mol Cell Endocrinol, 2005. **231**(1-2): p. 87-94.
215. Hilgendorf, C., et al., *Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and organotypic cell lines.* Drug metabolism and disposition: the biological fate of chemicals, 2007. **35**(8): p. 1333-1340.
216. Fournier, M.A. and D. Poirier, *Estrogen formation in endometrial and cervix cancer cell lines: involvement of aromatase, steroid sulfatase and 17beta-hydroxysteroid dehydrogenases (types 1, 5, 7 and 12).* Mol Cell Endocrinol, 2009. **301**(1-2): p. 142-5.
217. Shimizu, C., et al., *Transcriptional regulation of human 3'-phosphoadenosine 5'-phosphosulphate synthase 2.* Biochem J, 2002. **363**(Pt 2): p. 263-71.

218. Itoh, S., et al., *Dehydroepiandrosterone sulfate (DHEA-S) and 3', 5'-cyclic adenosine monophosphate (cAMP) production in a cultured human adrenocortical carcinoma cell line (SW-13).* Endocrinol Jpn, 1988. **35**(1): p. 149-58.
219. Bird, I.M., et al., *Human NCI-H295 adrenocortical carcinoma cells: a model for angiotensin-II-responsive aldosterone secretion.* Endocrinology, 1993. **133**(4): p. 1555-61.
220. Holland, O.B., et al., *Angiotensin increases aldosterone synthase mRNA levels in human NCI-H295 cells.* Mol Cell Endocrinol, 1993. **94**(2): p. R9-13.
221. Bird, I.M., et al., *Ca(2+)-regulated expression of steroid hydroxylases in H295R human adrenocortical cells.* Endocrinology, 1995. **136**(12): p. 5677-84.
222. Hahner, S., et al., *Etomidate Unmasks Intraadrenal Regulation of Steroidogenesis and Proliferation in Adrenal Cortical Cell Lines.* Horm Metab Res, 2010.
223. Fassnacht, M., et al., *New mechanisms of adrenostatic compounds in a human adrenocortical cancer cell line.* Eur J Clin Invest, 2000. **30 Suppl 3**: p. 76-82.
224. Igaz, P., et al., *Steroid biosynthesis inhibitors in the therapy of hypercortisolism: theory and practice.* Curr Med Chem, 2008. **15**(26): p. 2734-47.
225. Beardwell, C.G., et al., *Trilostane in the treatment of advanced breast cancer.* Cancer Chemother Pharmacol, 1983. **10**(3): p. 158-60.
226. Thiele, H., et al., *Loss of chondroitin 6-O-sulfotransferase-1 function results in severe human chondrodysplasia with progressive spinal involvement.* Proc Natl Acad Sci U S A, 2004. **101**(27): p. 10155-60.
227. Stanley, E.L., R. Hume, and M.W. Coughtrie, *Expression profiling of human fetal cytosolic sulfotransferases involved in steroid and thyroid hormone metabolism and in detoxification.* Mol Cell Endocrinol, 2005. **240**(1-2): p. 32-42.
228. Duanmu, Z., et al., *Developmental expression of aryl, estrogen, and hydroxysteroid sulfotransferases in pre- and postnatal human liver.* J Pharmacol Exp Ther, 2006. **316**(3): p. 1310-7.

7.2 Abbreviations

ABCC	ATP-binding cassette transporter family (≈MRP)
AD	Androstanediol
ADG	5α-androstanediol glucorunide
AKR1C	3α-hydroxysteroid dehydrogenase
AMV	Avian myeloblastosis virus
ANE-DIONE	Androstanedione
APS	Adenosine 5'-phosphosulphate
AR	Androgen receptor
ATG	Androsterone glucorunide
ATP	Adenosine triphosphate
A-Triol	Androstenetriol
AU	Arbitrary Units
BLAST	Basic Local Alignment Search Tool www.ncbi.nlm.nih.gov/BLAST/
cDNA	Complementary DNA
CNS	Central nervous system
cpm	Counts per minute
CVD	Cardiovascular disease
CYP11A1	Cholesterol side chain cleavage enzyme / P450 $_{scc}$
CYP17A1	17α-hydroxylase/17,20-lyase
CYP19	P450 aromatase
CYP7B1	7α-hydroxylase
DAPI	4',6-diaminidino-2-phenylindole
ddH_2O	Double distilled water
dH_2O	Distilled water
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydro-testosterone
DMSO	Dimethyl sulphoxide

DNA	Desoxyribonucleic acid
E1	Oestrone
E2	Oestradiol
ENE-DIOL	Androstenediol
eNOS	Endothelial nitric oxide synthase
ER	Oestrogen receptor
ERK 1 / 2	Extracellular signal-regulated kinas 1 / 2
FCS	Foetal calf serum
G6Pase	Glucose-6 phosphatase
GABA	γ-amino butyric acid
GLUT-4	Glucose transporter 4
GR	Glucocorticoid receptor
HSD11B1 / HSD11B2	11β-hydroxysteroid dehydrogenase type 1 / 2
HSD17B3	17β-hydroxysteroiddehydrogenase 3
HSD3B1 / HSD3B2	3β-hydroxysteroiddehydrogenase 1 and 2
ICC	Immunocytochemistry
IFN-γ	Interferon γ
IL-2 / IL-6 / IL-10	Interleukin 2 / 6 / 10
IMM	Inner mitochondrial membrane
K_d	Dissociation constant
kDa	Kilo Dalton (atomic mass unit)
MAPK	Mitogen-activated protein kinase
MMSE test	Mini-mental state examination
mRNA	Messenger RNA
MRP4	Multidrug resistan
NMDA	N-methyl-D-aspartate
OATP	Organic anion transport protein
OMM	Outer mitochondrial membrane
PAPS	3'-phosphoadenosine-5'-phosphosuphate
PAPSS	PAPS synthase
PBS	Phosphate buffered saline
PCOS	Polycystic ovary syndrome

PCR	Polymerase Chain Reaction
Real-time PCR	Real-time Polymerase Chain Reaction
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse Transcritpin Polymerase Chain Reaction
S.E.M.	Standard error of the mean
SF-36	Short form 36
SLCO	Soluable carrier family (≈OATP)
SRD5A1 / SRD5A2	5α-reductase
STAR	Steroidogenic acute regulatory protein
STS	Steroid sulphatase
SULT2A1	DHEA sulphotransferase
TAE buffer	Tris-acetate-EDTA buffer
Taq polymerase	Thermus aquaticus (bacterium) polymerase
TLC	Thin layer chromatography
ZF	Zona fasciculata
ZG	Zona glomerulosa
ZR	Zona reticularis
Δ4	Androstenedione

7.3 List of Figures

7.4 List of Tables

Acknowledgments

First and foremost, I would like to thank Prof. Wiebke Arlt for giving me the opportunity to work in her lab in Birmingham for my doctoral thesis. I am sincerely thankful for her support - right from the start - and for her supervision of my work. Her helpful thoughts, advice and have been invaluable to me.

I am also grateful to Prof. B. Allolio for supporting and overseeing this project in Germany.

Many thanks to the whole lab team of the IBR 2nd floor for teaching me with endless patience and for helpful discussions.

Very special thanks to Dr. Joanne McNelis for teaching me all kinds of methods and for her guidance during my first days in the lab. Dr. Vivek Dhir deserves my greatest thanks for his help with western blots and immunocytochemistry experiments. Furthermore, his help was invaluable to overcome problems with steroid extraction and thin layer chromatography.

I am also thankful to Dr. Stuart Morgan and Dr. Rowan Hardy not only for their advice on western blots and real-time PCR but also for making my start in Birmingham so easy.

Finally, I thank my family and all friends who supported me all those years!

Lebenslauf

Berufliche Erfahrung

Seit 05/2010	Assistenzarzt Innere Medizin und wissenschaftlicher Mitarbeiter an der Klinik der Johann-Wolfgang Goethe Universität Frankfurt (Medizinische Klinik I, Gastroenterologie, Hepatologie, Pneumologie und Endokrinologie. Direktor: Prof. Dr. S. Zeuzem)

Studium, Famulaturen, Praktisches Jahr

10/2002-11/2009	Studium Humanmedizin an der Julius-Maximilians-Universität Würzburg

Praktisches Jahr

08/2008-12/2008	Innere Medizin, Universitätsklinik Birmingham (UK) Liver Unit (Prof. J. Neuberger), Medical Admission Unit (Dr. L. Lambert)
12/2008-04/2009	HNO, Universitätsklinik Würzburg
04/2009-07/2009	Chirurgie Universitätsklinik Würzburg Hand- und Plastische Chirurgie, Gefäßchirurgie, Viszeralchirurgie

Famulaturen

03/2005	Allgemeinmedizin, Dr. med. E. Schlereth, Oberthulba
09/2005	Innere Medizin, St.-Elisabeth-Krankenhaus, Bad Kissingen
03/2006	Orthopädie, König-Ludwig-Haus, Würzburg
09/2006	Radiologie, Hôpital Sud Fribourgeois, Schweiz
03/2007	Neurologie, Neurologische Klinik, Bad Neustadt a. d. Saale

Doktorarbeit, Kongresse, Publikationen, Preise

07/2007-12/2008	Experimentelle Doktorarbeit am Institute of Biomedical Research in Birmingham (UK) (Prof. Arlt, Birmingham und Prof. Allolio, Würzburg)
05/2009	Publikation im New England Journal of Medicine: Noordam, C., Dhir, V., McNelis, J. C., **Schlereth, F.**, Hanley, N. A., Krone, N., et al. (2009). Inactivating PAPSS2 mutations in a patient with premature pubarche. The New England journal of medicine, 360(22), 2310–2318
06/2009	Medical Student Achievement Award (Endocrine Society, U.S.)

Schulische Ausbildung und Zivildienst

1988-1992	Grundschule Oberthulba
1992-2001	Frobenius-Gymnasium Hammelburg
06/2001	Abitur und Allgemeine Hochschulreife (Note 1,7)
09/2001-06/2002	Zivildienst St.-Elisabeth-Krankenhaus Bad Kissingen

Besondere Kenntnisse

Fremdsprachen	Englisch (fließend, IELTS 8/9), Grundkenntnisse Französisch

Frankfurt, den 23.08.2014